

# The gut bacterial community affects immunity but not metabolism in a specialist herbivorous butterfly

Anne Duplouy (✉ [anne.duplouy@biol.lu.se](mailto:anne.duplouy@biol.lu.se))

Helsingin Yliopisto <https://orcid.org/0000-0002-7147-5199>

Guillaume Minard

Universite Claude Bernard Lyon 1

Marjo Saastamoinen

Helsingin Yliopisto

---

## Research

**Keywords:** Larval development; Larval survival; Antibiotic treatment; Gut microbial community; Metabolites; Immunity

**Posted Date:** January 22nd, 2020

**DOI:** <https://doi.org/10.21203/rs.2.21629/v1>

**License:**   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

---

**Version of Record:** A version of this preprint was published at Ecology and Evolution on July 16th, 2020. See the published version at <https://doi.org/10.1002/ece3.6573>.

# Abstract

**Background** Many plant tissues are not resources of optimal nutritious value. They often lack essential nutritive elements and may contain a range of secondary toxic compounds. As nutritional imbalance in food intake may affect the performances of herbivores, the latter have evolved a variety of physiological mechanisms to cope with the challenges of digesting their plant-based diet. Some of these strategies involve living in association with symbiotic microbes that promote the digestion and detoxification of plant compounds, or supply their host with essential nutrients missing from the plant diet. In Lepidoptera, a growing body of evidence has, however, recently challenged the idea that herbivores are nutritionally dependent on their gut microbial community. It is suggested that many of the herbivorous species may not host a resident microbial community, but rather a transient one, acquired from their environment and diet.

**Results** By coupling comparative meta-barcoding, immune gene expression and metabolomics analyses with experimental manipulation of the gut microbial community of pre-diapause larvae of the Glanville fritillary butterfly (*Melitaea cinxia*, L.), we tested whether the larvae host a gut microbial community that supports growth and survival, and modulates metabolism and immunity during the early stages of development. We successfully altered this microbiota through antibiotic treatments, and consecutively restored it through fecal transplants from conspecifics. Under laboratory conditions, Firmicutes dominated the bacterial microbiota associated with the gut and frass of non-treated larvae, even though these Gram-positive bacteria were not found in association with the host plant, *Plantago lanceolata*. Furthermore our study suggests that the microbiota is involved in the up-regulation of an antimicrobial peptide, but did not affect the life-history traits or the metabolism of early instars larvae.

**Conclusions** This study confirms the poor impact of the microbiota on diverse life history traits of yet another Lepidoptera species. However, it also suggests that potential eco-evolutionary host-symbiont strategies that take place in the gut of herbivorous butterfly hosts might have been disregarded, particularly how the microbiota may affect the host immune system homeostasis.

## Background

Herbivory results in the extraction and assimilation of nutrients and energy from a plant diet. This adaptation supports the development and survival of many vertebrates and invertebrates on Earth. Plant tissues might, however, be of low nutritious value, and many are rich in toxic defensive compounds. In insects, many herbivores are generalists and feed on a wide range of plants that provide a large diversity of nutrients [1]. Specialist herbivores, on the other hand, have evolved a range of adaptive behavioral, physiological and anatomical strategies to optimize their nutrient intakes, and consequently their fitness, from a small range of host-plant species [2, 3]. Some of these strategies call for dependence upon symbiotic associations with microorganisms colonizing the guts or other specialized organs of the hosts [4-6]. These microorganisms, often but not only, bacteria and fungi, can directly provision their host with nutrients lacking from their restricted plant-based diet, or facilitate the digestion of various plant compounds. For example, in aphids, the endosymbiotic bacteria *Buchnera aphidicola*, provide essential amino acids that are generally absent from the phloem sap diet of their host plant [7-9]. Similarly, lower

termites rely upon protozoans and bacteria colonizing their guts to digest the lignocellulose of wood into nutritious fatty acids [10-12]. This direct nutritional impact on individuals fitness is further translated in the long-term impact on the fitness of the entire colony, including colony longevity, colony growth rate, and queen fertility [13].

Lepidoptera often possess an alkaline gut environment [14, 15], which has previously been shown to facilitate the inhibition and degradation of plant toxins [16]. For example, the hydrolysis of plant cyanogenic glucoside defenses leads to the emission of hydrogen cyanide at low pH, but is strongly inhibited in the highly alkaline (pH of 10.6) midgut lumen of the larvae in the six-spot burnet moth, *Zygaena filipendulae* [17]. In this unique environment, instead of being lethal to the feeding larvae, the plant-toxins are sequestered for anti-predator protection [18, 19]. Studies have also suggested that the alkaline gut environment as well as the sequestered plant-toxins could also function as anti-pathogen protection through interference with the immune responses of the herbivorous insect [20-22]. Consequently, a lower diversity of microorganisms generally colonize the gut of Lepidoptera [23, 24] than other insect species, and different elements of the host environment (e.g. soil, host-plant surface)[25]. More than 60% of the gut microbiota of Lepidoptera species is represented by ten phyla, often dominated by bacterial species from the *Pseudomonas*, *Bacillus*, *Enterococcus* and *Staphylococcus* genera [23, 24, 26]. In the *Spodoptera litura* moth, the larval gut is predominantly colonized by *Enterococcus casseliflora* bacteria, which tolerate the alkaloid produced by the host plant diet [24]. A growing body of evidence is supporting the idea that many Lepidoptera species rarely carry beneficial symbionts within their microbiota [23]. Most part of those microbial communities are acquired from the host environment and diet but poorly contribute to the insect nutritional intake (e.g. in *Lycaenid* butterflies [27], and others [23]).

The Glanville fritillary butterfly (*Melitaea cinxia*, Linnaeus 1758) is a specialist herbivore, that during its larval phase feeds on plant species from only two genera, *Plantago* and *Veronica*, across its entire European species range [28]. Based on previous laboratory experiments, we know that the performance of the larvae from the Finnish metapopulation varies between the host plants, and between plants of different quality: larvae fed with *Veronica spicata* generally show higher performance than those fed with *Plantago lanceolata* [29], and larvae fed with *Plantago* leaves infected by the fungal pathogen *Podosphaera plantaginis* develop more slowly, weigh less at diapause [30], and show a reduced overwinter survival rate in the field [31]. Furthermore, pre-diapause larvae more often abandon fungus-infected host-plants than healthy control host-plants [31], while post-diapause larvae show behavioral preference towards the better quality plants in an experiment exposing the larval host-plant to drought (corresponding with higher performance on these plants; [32]). These studies suggest that larval avoidance of the lower-quality food might be a behavioral adaptation of the insect towards maximizing larval performance. Two recent studies also suggest a correlation between the composition of the larval microbiota of the Glanville fritillary butterfly and different aspects of the species fitness, including larval growth and performances [33, 34]. These former two studies have, however, failed to provide critical controls to the microbiota experiments through not testing for the effect of experimental manipulation of the gut microbial community composition [33, 34].

To thoroughly address whether the gut microbiota matters to the larval development and survival of the Glanville fritillary butterfly, we manipulated the microbiota of early instar larvae by empirically disrupting and restoring the community through antibiotic treatments followed by fecal transplants from conspecifics. As the microbiota may also be environmentally acquired, we additionally evaluated the self-resilience of the microbiota by adding one treatment group during which the antibiotic treatment was also maintained during fecal transplant. The efficiency of this protocol was assessed by metabarcoding of the bacterial and fungal communities associated to the gut of the larvae. The community resilience of re-infected individuals was estimated based on the community structure of antibiotic-treated and non-treated individuals. Finally, we were particularly interested in testing whether the manipulation of the gut microbiota affected larval performances, by analyzing variation in life history traits (i.e. larval development and survival), immunity, and metabolism among the treatment groups.

## Results

### *Larval performance*

Of the 2,160 larvae included in this study, 62.6% developed into second larval instar (L2), and 54.5% survived until L3. Larvae from the control group showed faster development (C:7.3  $\pm$ 1.9 days versus A:8.4  $\pm$ 1.7, AR:8.3  $\pm$ 3, and R:8  $\pm$ 3.3 days,  $df=3$ ,  $P=2.2e-12$ , Fig 2A). The ICC value of the model further suggests that 60% of the overall variance in development time to L2 was explained by performance differences among families (after log-transformation, ICC=0.60), with family #2 showing the longest developmental time (around 10 days), and family #29 showing the shortest (under 7 days) (Figure S1A). After correction for the family effect, developmental time remained lowest for the controls, and equally high between the other treatment groups (Fig 2B).

Control groups also showed the highest survival to third larval instar (L3) (C: 13.7 larvae per family group, 68.7%), while survival was equally low between the other three treatment groups (A: 10.5, 52.4%, AR: 9.9, 49.6%, and R: 9.5, 47.4% larvae per family group, Fig 2C; Multiple Comparison of means: Tukey contrast test  $P$ -value $<7.2e-4$ ). Note that as the larvae grew, some of them did not survive to reach L3, thus the average larval group size also vary among the treatment groups with the control groups being the largest when larvae reach L2 (C:15.2  $\pm$ 7.2 larvae, A:11.7  $\pm$ 8.7, AR:11.6  $\pm$ 8.6 and R:11.6  $\pm$ 11.6,  $P=2.16e-4$ , Fig 2C). For example, all larvae from four family groups died before reaching L3, two of these developed under the R treatment, one under the A treatment, and one under the AR treatment (i.e. none of the controls). Although group size at L2 significantly varied among the treatments, we included only the treatment as an explanatory variable in the model testing for development time to L2. We do also note and discuss the potential confounding effect of group size on larval performances. Finally, the ICC value of the model suggests that 46% of the overall variance in survival until day 13 is explained by differences among families, with families #3, 19 and 20 showing the highest survival rate (>15 larvae per treatment group), and families #8, 24 and 25 showing the lowest (<5 larvae per treatment group; Figure S1). After correction for the family effect, survival to day 13 remained highest for the control group, and was equally low between the other three treatment groups (Fig 2D).

## ***Microbiota***

We independently analyzed the bacterial and fungal communities, at the phylum level only, from 156 larval samples (three L3 larva gut samples from 13/27 families for each of the four treatments). In parallel, we also characterized the bacterial communities of five frass samples from L7 larvae (i.e. those used to re-infect the larvae), and five pieces of leaves from five *P. lanceolata* host-plants used to feed the larvae during the experiment. Unfortunately, our fungal metabarcoding data showed very high rates of plant ribosomal sequences (Figure S2). Because of this, we were not able to characterize the fungal communities in larvae, frass and plant samples, neither were we able to test their effects on the performances of the larvae.

We identified 760 bacterial OTUs across all our samples (larval guts, frass and plants). We found differences in the bacterial community composition among the treatment groups (Fig 3). The bacterial  $\alpha$ -diversity of the antibiotic-treated larvae was higher to that of the other larvae (Shannon index, TukeyHSD.test, A vs AR:  $df=5$ ,  $P<2e-16$ , A vs C:  $P<2e-16$ , A vs R:  $P<2e-16$ , Figure S3A), and that of the frass samples ( $P=5.6e-6$ ), but was similar to that of the plant samples ( $P=0.96$ ; Fig 3). The bacterial  $\alpha$ -diversity of the plant samples also differed from the frass samples ( $P=1.14e-4$ ), and of all other treatments ( $P<6.58e-5$ ), except the antibiotic-treated larval samples ( $P=0.96$ ). On average, 20 bacterial OTUs per sample were characterized from the antibiotic-treated larvae, 31 from the plant samples, 11 from the larvae of the three other treatment groups, and seven from the frass samples. The  $\alpha$ -diversity varied between families ( $df=12$ ,  $P=6.03e-4$ ), with family#12 showing significantly higher  $\alpha$ -diversity than family#1 ( $P=4.79e-3$ ), family #7 ( $P=1.78e-3$ ), family #9 ( $P=1.29e-3$ ), family #10 ( $P=8.77e-3$ ), family #19 ( $P=0.02$ ) and family #29 ( $P=0.014$ ; Figure S4A).

Similarly, the bacterial community associated with antibiotic-treated larvae was more heterogeneous (homogeneity of multivariate dispersion,  $df = 3$ , pseudo- $F=7.74$ ,  $P=0.001$ ) (Figure S3B), and differed from that characterized from the larval gut samples in the other treatment groups (*adonis*-ANOVA,  $df=3$ , pseudo- $F=48.489$ ,  $R^2=0.40$ ,  $P=0.001$ ; Table 2). The OTUs assigned as *Planktosalinus* and unclassified Burkholderiaceae were most common in the antibiotic-treated larvae, while unclassified Firmicutes were dominating the bacterial communities in the gut of the larvae from the other treatments (Fig 3 & 4). The frass samples that were used to re-infect the larvae showed a very similar microbial community composition to that of the larval gut of the three treatment groups (C, AR and R), and harbored a high abundance of unclassified Firmicutes (Fig 4), thus contrasting with the microbial community from antibiotic-treated larvae. The plant samples also did not harbor a microbial community similar to any of the other treatment groups, and were mostly composed of unclassified bacteria, unclassified Alphaproteobacteria, *Pedobacter* and unclassified Rhizobiaceae (Fig 3). Finally, 11% of the  $\beta$ -diversity was influenced by the family factor (*adonis*-ANOVA,  $df=14$ ,  $F=2.95$ ,  $R^2=0.11$ ,  $P=0.001$ ; Table 2; Figure S4). It is also interesting to note that before removing the contaminating OTUs from any given sample, the antibiotic-treated larvae harbored a similar community to that of the negative controls (blank extractions,

PCRs and sequencing; Fig 3). This observation supports the conclusion that antibiotic treatments efficiently cleared out the antibiotic-treated larvae from their bacterial microbiota.

For the subset of 13 families for which both microbiota and life-history data were measured, we tested whether variation of the  $\alpha$ - or  $\beta$ -diversity indexes correlated with variation in developmental time to L2 and survival to L3. Although we previously showed that larval developmental time to L2 varied among the treatment groups ( $df=3$ ,  $P=1.26e-9$ ), it was not correlated with variation in the diversity and composition of the gut bacterial community (Shannon index:  $df=1$ ,  $P=0.69$ ; NMDS1:  $df=1$ ,  $P=0.8$ ; Fig 5A). Similarly, even though the larval survival to L3 varied among the treatment groups ( $df=3$ ,  $P=1.08e-4$ ), it was not associated with variation in the diversity and composition of the gut bacterial community (Shannon index:  $df=1$ ,  $P=0.47$ ; NMDS1:  $df=1$ ,  $P=0.87$ ; Fig 5B). Finally, family explained 55% of the variance of development time to L2, and 14% of the variance of survival to L3 in these models, respectively.

### ***Metabolomics***

We analyzed variation in the metabolite profile of larvae from the four treatment groups (49 larval samples) by  $^1H$ -NMR. The total signals (annotated and un-annotated) were then included in a multivariate analysis, focusing only at the first seven PCs (all PCs with Eigen value > 3.0) of a PCA including all metabolite data from the NMR analysis. When considering the whole metabolomics profiles, there was no effect of the treatment group on the metabolite composition of the samples ( $df=3$ ,  $P>0.096$ , Table 3). We also individually extracted the signals corresponding to  $\alpha$ -glucose,  $\beta$ -glucose, Alanine, Formic acid, Acetic acid, Fumaric acid, Ethanol, Aucubin and Catalpol. Consistently with the entire metabolomic profile, the variation in the amount of these nine compounds within the larvae were not significantly affected by the treatment group (Table 4). Metabolites showed little variability across larval families (ICC < 0.001 for most PCs analyzed), except for Family #1 which showed a different metabolome profile compared to other families (observed on PC6 with an ICC= 0.41, Table 3).

We further tested whether the development time to L2, and survival to L3 were correlated with the metabolite content of larvae in each treatment group. There was no significant correlation of either the larval development time to L2 ( $df=1$ ,  $P>0.24$ ; Figure S5A) or survival to L3 ( $df=1$ ,  $P>0.055$ ; Figure S5B) with any of the seven PCs describing the metabolomic profile.

### ***Immune gene expression***

We analyzed the expression fold change (Log2) of seven immunity genes in 136 larvae from 12 families. Six of the seven genes did not show any consistent changes between the treatment groups (TukeyHSD.tests, *Attacin* vs any of the three control genes:  $P<1e-4$ ; any other comparison for other genes:  $P>0.95$ ; Fig 6). However, for *Attacin* the expression levels (corrected for family effect) were the lowest in

the antibiotic-treated ('A') larvae (TukeyHSD.test, A vs C:  $P=7.7e-3$ ; A vs AR:  $P=3e-3$ ; A vs R:  $P=1.3e-6$ ), while the expression levels of the *Attacin* gene in the 'AR' and 'R' samples were similar to those in the controls (TukeyHSD.test, C vs AR:  $P=0.99$ ; C vs R:  $P=0.11$ ; AR vs R:  $P=0.17$ ). Finally, once corrected for the family effect, the expression fold change of the *Attacin* immune gene was negatively correlated with the larval development time to L2, and positively correlated with the larval survival to L3 (df=1,  $F$ -value=9.46,  $P=2.62e-3$ ; and df=1,  $F$ -value=7.43,  $P=7.39e-3$ , respectively; Table 5, Fig 7). In general, family explained over 50% of the variance in the *Attacin* gene expression level, with family#14 showing the highest expression levels (Data not shown).

## Discussion

In this study, we showed that the microbiota of pre-diapause larvae of the Glanville fritillary butterfly (a specialist herbivorous Lepidopteran species) could efficiently be altered by antibiotic treatment, and later restored to a similar composition through empirical fecal transplant. Larvae under constant antibiotic treatment (A) were successfully cleared from unclassified Firmicutes bacteria that dominate the microbiota of untreated larvae (C). In contrast, when their diet was provided with frass from conspecifics, the larvae (R) successfully recovered a gut bacterial community similar to that of their untreated conspecifics. This resilience could be the result of either a relaxation of the selective pressures induced by the antibiotic treatment or by the reinfection process *per se*.

We specifically tested this by including a second control treatment (AR), during which larvae were fed on a diet treated with frass mixed with antibiotic before the relaxation of the antibiotic treatment. This particular treatment allows for testing any potential variation in the larval life histories and metabolism caused by the provision of frass only to the diet, since the frass microbiota was simultaneously removed by antibiotics. In this treatment group, the AR larvae recovered a similar bacterial community to both the untreated and the transplanted (R) larvae. This suggests that the arrest of the antibiotic treatment, rather than the fecal transplant *per se*, allowed the recovery of the microbiota in these larvae. Additionally, in concordance with a study by Minard *et al.* [37], that found no association between plant and larval microbiota, we show that the bacterial community present in the gut of control larvae was dissimilar to that associated to the host plant they fed on. Specifically, the unclassified Firmicutes we commonly found in the control larvae were undetected in the plant samples analyzed. Although Firmicutes are generally rare in the gut microbiota of other butterfly species [27, 35], Ruokolainen *et al.* [33], had identified a similar community structure in both field caught and lab reared post diapause larvae of the Glanville fritillary butterfly, further supporting the robustness of our results. The microbiota characterized from the host plant leaves did, however, resemble that of the antibiotic-treated larvae (A). One possible explanation to this may be that the treated larvae only harbor a transient microbiota, from the microbiota associated with their plant diet. Our results do not allow us to fully exclude the possibility that the Glanville fritillary larvae acquire their microbiota from the environment rather than through vertical transmission, or coprophagy.

Previous studies have shown that the gut microbiota is critical to the development and survival of various insect host species (reviewed in [36]), and consistent associations have been described in major insect orders ([4, 6, 37]. In Lepidoptera, some studies show evidence of a correlation between the microbiota composition and the host plant species composition [23, 33], or the host plant quality [34]. However, a more recent and growing literature seem to suggest that strong host-microbiota mutualism has not evolved in many Lepidoptera species, and that the gut bacteria are rather transient passengers collected from these hosts environments [23, 25, 38]. In the Glanville fritillary butterfly, Ruokolainen *et al.* [33] found a correlation between post-diapause larval growth and the host gut microbial community. They demonstrated that about 50% of the variation in post-diapause larval growth correlated with shifts in the larval gut microbial community composition and the diet of the post-diapause larvae. The larvae hosting a particular microbiota and feeding on a particular host plant species (*P. lanceolata* or *V. spicata*) were developing faster and growing larger [33]. The authors suggested that diverging gut microbial communities could mediate diet-associated differences in the larval growth of the Glanville fritillary larvae. In our study, the larval gut bacterial community alone is not the causal reason for differences among the performance traits of the treatment groups during their early larval (*i.e.* survival and development time). Compared to the controls, a longer development time to the 2<sup>nd</sup> larval instar, and a low survival rate to the 3<sup>rd</sup> larval instar were observed in all three antibiotic-treated larval groups, regardless of the composition of their gut microbiota (*i.e.* treatments A, AR, and R). These patterns are suggestive of a general cost of the antibiotic treatment. Chaturvedi *et al.* [27] did not find any association between larval performance (using larval weight as proxy) and bacterial community composition between populations of the Melissa blue butterfly, *Lycaeides melissa*. Similarly, in *Manduca sexta*, the removal of bacteria did not affect larval weight, development nor survival [23], and in *Danaus chrysippus* and *Ariadne merione*, the removal of the gut microbiota followed by transplants with the frass of conspecifics also did not affect any of the developmental and survival traits investigated [38]. Hence, our results are consistent with the growing literature suggesting that Lepidoptera may not have a resident gut microbiota beneficial to larval growth or survival, but rather host a microbial community, which function and evolutionary importance for its host remain unclear.

Notably, family explained a large proportion of the observed microbiota variations (11%), and of the variations in both pre-diapause larval development time and larval survival. The vertical transmission of symbionts has been documented in a wide range of insects, including Cockroaches, whiteflies, tsetse flies, stinkbugs, beewolf (reviewed in [39]). Such long term mutualistic relationship yet remains to be characterized in the Glanville fritillary butterfly [40]. The environmental conditions vary considerably across the Åland metapopulation, and various selection pressures might act differently on the hosts and their microbial communities. For example, the spatial distribution of the two host plants of the larvae of the Glanville fritillary in the field is variable [41, 42], and many plant species contain variable quantities of iridoid and phenylpropanoid glycosides: namely aucubin, catalpol and verbascoside [43-45]. These plant metabolites are defensive compounds known to affect the fitness of herbivorous insects [18, 19, 46], including the larvae of the Glanville fritillary butterfly [45, 47]. Generally, the microbial community associated to the herbivorous insects has been thought to play a key role in the processing of such toxic

compounds [48, 49], and is thus of importance for insect digestion and metabolism (reviewed by [36]). However, a study performed by Minard et al. [40] investigating field collected diapausing larvae of the Glanville fritillary butterfly showed a poor correlation between *P. lanceolata* metabolome and the composition of diapausing larval microbiota. This result is in concordance with the lack of changes in the metabolite composition we found between treatment groups. These two studies suggest that the microbiota does not impact the metabolism of larvae in the Glanville fritillary butterfly, and is thus unlikely to have been locally selected to optimize local adaptation of the families for their host plants. Our study was however restricted to polar and highly concentrated metabolites, and other protocols might, in the future, help disentangle subtler changes in pre-diapause larval metabolism. Other environmental conditions, such as thermal microclimate and soil composition, may also be spatially variable. As many symbionts are predicted to be heat-sensitive and can be eliminated or lost under thermal stresses (reviewed in Wernegreen [50]), spatial variation in microclimates may also lead to independent selection of host families and symbiont communities.

Insects molt numerous times during the course of the larval development. In mosquitoes, previous studies have shown that metamorphosis and the shedding of the gut membrane led to the partial or complete renewal of the gut microbiota in the host [51]. The bacterial diversity has been shown to drop by 50% between the larval and the pupal stages, and to only increase again after the first feed as an adult in *Heliconius erato* butterflies [52]. Similarly, the microbial community in *Lycaenid* butterflies is reorganized between each larval stage [27], while that of the moth *Spodoptera littoralis* partially shifts between early and late instars, and even more drastically during metamorphosis, with only *Enterococci* bacteria persisting through [53]. Part of the gut microbiota of these Lepidoptera is thus voiding or cleaned from the gut lumen, suggesting that the microorganisms that may be beneficial at early developmental stages may be different from those beneficial at later developmental stages. The larvae of the Glanville fritillary butterfly go through seven to eight larval instars, a six to nine month-long physiologically inactive period during the 5<sup>th</sup> instar (over-winter diapause), and metamorphoses to pupal and adult stages [28, 54]. The gut environment of this species thus most likely also represents an unstable habitat for the microorganisms colonizing it. The pre-diapause larval stages that we studied here are potentially the most critical for the Glanville fritillary, as they show highest mortality rates in the field and the laboratory [28, 55]. Nonetheless, the comparison on the bacterial communities described in our study (pre-diapause larval stages) and in that of Ruokolainen *et al.* [33] (post-diapause larval stages) showed that the gut environment of the larvae is potentially comparable throughout the different larval stages of the Glanville fritillary butterfly. Only, the inactive diapausing larvae showed a different and more variable microbiota [40]. Furthermore, the frass of 7<sup>th</sup> instar larvae, used in this study to transplant some of the antibiotic-treated larvae, harbored the same communities than the gut of the control 3<sup>rd</sup> instar larvae. Nonetheless, a more comprehensive diversity comparison at the bacterial species level, and across each of the host developmental stages should be done to confirm this observation in the Glanville fritillary butterfly. For example, no study yet informs whether the microbiota is constantly maintained or go through bottlenecks with each molting phase, nor during metamorphosis to pupae or the adult stage in the Glanville fritillary butterfly.

The expression levels of the *Attacin* gene, which codes for an antimicrobial peptide, were down in the antibiotic-treated larvae (A) compared to all other treatment groups. This result suggests that the commensal microbiota is somehow involved in the permanent expression of this gene. Two potentially non-exclusive hypotheses may explain the response observed in the antibiotic-treated larvae (A). First, the down-regulation of the *Attacin* gene would suggest a relaxed stress-response against the decrease of some bacteria from the antibiotic-treated larvae [56], but present in the control and recolonized larvae. Alternatively, the host might constantly regulate the growth of its gut microbiota through the expression of the *Attacin* gene [57], and the expression levels of this gene might be relaxed once all or most bacteria are removed from the gut after antibiotic treatments. The microbiota of these larvae might thus contribute to the immune homeostasis of the gut environment of this butterfly species. The up-regulation of the *Attacin* gene in the Glanville fritillary butterfly was previously shown in larvae exposed to both bacterial and fungal pathogens [30], in adult butterflies exposed to bacterial pathogens [58], and in adult butterflies after flight [58, 59]. In our study, the expression levels of this gene were positively correlated with survival and negatively correlated with development time in the pre-diapause of the Glanville fritillary butterfly. Altogether, these studies suggest that in the Glanville fritillary butterfly the regulation of the *Attacin* gene is part of a response to general stress cues that benefits the fitness of the larvae.

## Conclusion

By highlighting both the self-resilience of the bacterial community in the gut of the larvae of the Glanville fritillary butterfly, and its consequences for the host immune homeostasis, our study contributes to the growing understanding of the complex processes that take place in the digestive tracks of Lepidoptera. The experimental removal of the dominant species of the gut bacterial community of the larvae significantly impacted the host immunity by down-regulating the expression of a gene involved in the response against pathogens. Furthermore, increased expression levels of the *Attacin* immune gene were associated to improved measured life-history traits (i.e. faster growth and higher survival). However, neither the life history traits nor the larval metabolism were affected by variations in the microbiota composition. Altogether, this study strongly suggests a link between the gut environment and the immune system of the Glanville fritillary butterfly. In the future, the targeted removal of microbial taxa shall further reveal the functional role of the microorganisms colonizing the gut of insects, and clarify their roles in the evolution of physiological and morphological features of the host species, including their ability to cope with pathogens and other stresses.

## Methods

### *Larvae and plant material*

The Glanville fritillary butterfly, *Melitaea cinxia*, L. 1758, is a widely distributed species across Eurasia and North Africa. Over the last three decades, many aspects of the ecology, life history, demography and eco-evolutionary dynamics of the Finnish population inhabiting the Åland islands, the Baltic Sea, have been intensively studied [60-62]. Our laboratory stock population was originally assembled by collecting three

individual larvae from 38 (F0) families across the Åland metapopulation in September 2015, as previously described [30]. Although larvae were not genotyped, our sampling strategy insured that we worked on representative individuals from the genetic diversity of the metapopulation [63, 64]. Larvae were reared in family groups under optimum conditions (during periods of growth: Day:Night (D:N), 27°C:10°C, 12h:12h; during diapause: D:N, 5°C:5°C, 12h:12h) over two generations at the Lammi Biological station, University of Helsinki, Finland. Butterflies were mated with non-siblings partners in the laboratory, and mated females were individually isolated in small cages to lay eggs on *Plantago lanceolata* host plants. The host plants were checked daily for egg clutches, which were carefully transferred into individually labeled petri dishes.

On the day of emergence from the eggs, L1 (F2) larvae from each of 27 selected families ( $N_{\text{Total}}=2,160$ ) were divided between four treatments in groups of 20 individuals each in a full factorial design (Family x Treatment), and reared in the laboratory until 3<sup>rd</sup> instar (L3), as described below. In parallel, we also reared post-diapause (F1) larvae from five families under optimum laboratory conditions (above), and collected fresh frass every day once the larvae reached 7<sup>th</sup> instar (L7). Sample sizes for each treatment and experiment are described in Table 1.

*Plantago lanceolata* (N = 120) was used as the larval food throughout the experiment (see below). The plants were collected as seeds across the Åland islands in 2015, and grown in optimum laboratory greenhouse conditions at the Lammi Biological Research station (D:N, 27°C:10°C, 12h:12h). Plants were watered every 3<sup>rd</sup> day. Plant leaves were only harvested for the experiment, thus preserving all natural defensive metabolites and original microbial load of the plants for the experiment. We also harvested and froze in liquid nitrogen some extra leaves to provide controls to the experiments described below.

### ***Treatments***

The 2,160 larvae from 27 families were equally divided between four treatments. Each group of 20 larvae was given daily a freshly harvested 1.7cm<sup>2</sup> piece of randomly collected *P. lanceolata* leaf [40], which was supplemented differentially according to treatment (Fig 1):

- **(Control)**: 200µl of sterile water was left to dry on the leaves before being provided to the larvae, from day1 (L1) until the larvae molt into L3.
- **(Antibiotic)**: 200µl of the antibiotic solution was left to dry on the leaves before being provided to the larvae, from day 1 (L1) the larvae molt into L3.
- **(Reinfection)**: 200µl of antibiotic solution was left to dry on the leaves before being provided to the larvae, from day1 to day3 (L1). On day4, 200µl of the antibiotic solution supplemented with 5% of L7's frass was left to dry on the leaves before being provided to the larvae. From day5 and until the larvae molted into L3, 200µl of sterile water was left to dry on the leaves before being provided to the larvae.

- (**Antibiotic during Reinfection**): 200µl of the antibiotic solution was left to dry on the leaves before being provided to the larvae, from day1 to 3 (L1). On day4, 200µl of the antibiotic solution was supplemented with 5% of L7's frass and left to dry on the leaves before being provided to the larvae. From day5, 200µl of sterile water was left to dry on the leaves before being provided to the larvae until the larvae molt into L3.

The antibiotic solution was prepared by mixing three anti-bacterial agents ( $2 \times 10^{-4} \text{g.ml}^{-1}$  of neomycin sulfate, with  $1 \times 10^{-3} \text{g.ml}^{-1}$  of aureomycin,  $6 \times 10^{-5} \text{g.ml}^{-1}$  of streptomycin) and two antifungal agents ( $8 \times 10^{-4} \text{g.ml}^{-1}$  of methyl paraben, and  $6 \times 10^{-4} \text{g.ml}^{-1}$  of sorbic acid) as described by Chung et al. [65].

### ***Larval performance: Development and Survival***

For each larval group, transition to the 2<sup>nd</sup> larval instar was checked every day over a 13 day long period, while survival until 3<sup>rd</sup> larval instar within each group was estimated every third day. On the day the surviving larvae reached the 3<sup>rd</sup> instar, they were frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until further manipulated. As the larvae were not starved before being killed, the gut content of most larvae may still include material from the diet. Due to the large number of larval families and treatment groups, not all larval groups could be reared during the exact same days, instead, the emergence dates of the larvae from the eggs spread over eight successive days. The larvae from the same family all emerged on the same day. Larvae were reared at  $23^{\circ}\text{C}$  with lights on between 8:00-10:00 am, and between 3:00pm and 5:00pm, at  $28^{\circ}\text{C}$  with lights on between 10:00 am and 3:00 pm, and at  $18^{\circ}\text{C}$  in the dark between 5:00 pm and 8:00 am.

### ***Metabarcoding of the gut microbiota***

We surface sterilized three L3 larvae from each of the four treatments for 13 families before individually dissecting their gut out under a microscope in a sterile laminar flow hood. All larval carcasses were preserved to perform the metabolomics analyses described below (see *Metabolomics* section). We individually extracted the DNA from the gut of the 156 larvae under sterile conditions. The DNA was extracted using a Qiagen DNeasy Blood and Tissue kit (Qiagen, Germany) following an optimized protocol as described by Minard, Tran [66]. Three additional extractions were carried out on sterile water to control for environmental contamination during the procedure. We amplified the hypervariable V5-V6 bacterial region of the *rrs* gene using the primers 784F (5'-AGGATTAGATACCCTGGTA) and 1061R (5'-CRRACGAGCTGACGAC) [67], and the LSU region of the *ITS* gene of Ascomycota fungi using the primers LSU200A-F (5'-AACKGCGAGTGAAGCRG) and LSU476A-R (5'-CSATCACTSTACTTGTKC) [68]. Each sample was amplified in duplicate and using 3µl of the DNA extract for each PCR reaction [66], and the duplicates for each sample were pooled in sterile condition after amplification. Sequencing was performed by the

Institute for Molecular Medicine Finland (FIMM, Finland) on a MiSeq v.3. Sequencing platform (Illumina, USA) using both reverse and forward primers.

We analyzed the libraries using *Mothur* v.1.37.6 ([http://www.mothur.org/wiki/MiSeq\\_SOP](http://www.mothur.org/wiki/MiSeq_SOP)). [69]. We selected all 250-350bp-long sequences, with less than eight homopolymers, no ambiguous position, and which aligned to the *rrs* Silva v.123 database. Chimeric sequences were removed using UCHIME implemented in *Mothur* [70]. Sequences were clustered within Operational Taxonomic Units (OTUs) according to average neighbor method with 3% distance maximum within each OTU. All OTUs showing at least a 10x higher proportion in any given sample than in the negative controls were considered as contaminant and removed from our dataset using an in-house R script [40].

### ***Metabolomic analysis of the larval carcasses***

We used the carcasses of the larvae used in the microbiota assays described above for the metabolomics analyses. This allowed us to provide information of the metabolites from the larvae without contamination from the plant diet. After dissection, the three larval carcasses from each family ( $N_{\text{total}}=13$  families) were pooled, and crushed in liquid nitrogen using a sterile pestle. Similarly, we also crushed two samples of 30mg of *P. lanceolata* leaves each and two samples of 30mg of larval frass in liquid nitrogen using sterile pestle, to use as controls for diet metabolite that might still contaminate our larval samples. All 55 samples (52 pooled larval carcasses, one host-plant and two frass controls) were then freeze-dried for 48 hours in a freeze dryer (MechaTech Systems Ltd, UK). Dry samples were weighted on an analytical balance ( $d=0.1\text{mg}$ , Fisher Scientific, UK). Metabolites were then extracted using the protocol described by Kim et al. [71]. In brief, for each sample, we placed 10mg of dry material in 350 $\mu\text{l}$  of  $\text{CD}_3\text{OD}$  (VWR Chemicals, Belgium) and 350 $\mu\text{l}$  of  $\text{KH}_2\text{PO}_4$  (Sigma-Aldrich, Germany) buffer mixed in  $\text{D}_2\text{O}$  (pH6) containing 0.05% (wt/wt) of TSP (sodium trimethylsilylpropionic acid) (Sigma-Aldrich, USA). Samples were then sonicated for 20min, and centrifuged 10min at 17,000g. We transferred 600 $\mu\text{l}$  of the clear supernatants into individual 5mm diameter NMR tube (Wilmad, USA), and the metabolite content of each sample was analyzed using a Bruker 850MHz Advance III HD NMR spectrometer equipped with a TCI Cryoprobe (Bruker, USA) at the Finnish Biological NMR Center, the University of Helsinki, Finland.

Proton Nuclear Magnetic Resonance ( $^1\text{H-NMR}$ ) spectra were acquired at 298K and recorded using 1D pre-saturation pulse sequence (zgpr). For each  $^1\text{H}$  spectrum, 256 transients were collected into 32K time domain points using a  $60^\circ$  flip angle, spectral width of 10.2kHz, relaxation delay of 5.0s, an acquisition time of 1.6s, and a mixing time of 5ms. Fourier transformation of the free-induction decay was applied with zero filling to give 65K frequency domain data points. The preliminary treatments of the  $^1\text{H-NMR}$  spectra were performed using the software MNOVA v.10.0.2 (Mestrelab research S.L., Spain). Standard solutions containing 1mg of one of the five antibiotics used for the treatments were measured individually in order to enable their identification, quantification, and trimming from the antibiotic-treated larval samples.

## ***Immune gene expression***

We individually sampled up to 12 larvae from each of the 12 families, and flash-froze them in liquid nitrogen once they had reached the third larval instar. For five of the 12 families, we included three L3 larvae for each treatment, while for the remaining seven families, we only had three larvae in at least two of the treatment groups, but only one or two larvae for the other treatments due to mortality during development ( $N_{\text{total}} = 133$ ; Table 1).

The RNA from each larva was individually extracted following a protocol described by Woestmann *et al.* [58] using TRIzol reagent (Life Technologies Corporation, Carlsbad, USA), acid-phenol:chloroform:isoamyl alcohol (25:24:1, pH=5) and Chloroform. The RNA was then precipitated using isopropanol, washed in 75% ethanol, air-dried in a flow hood, and re-suspended in 50 $\mu$ L MQ water. Potential genomic DNA contaminants were removed using DNase I (Thermo Fisher Scientific Inc., UK). The RNA was reverse-transcribed using an iScript<sup>TM</sup> cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, USA) following the manufacturer's protocol.

The qPCR assays were performed with three technical replicates for each sample, and one negative control and plate control (same sample across all plates) for each 384-well plate used, in a 10 $\mu$ L volume, on a C1000<sup>TM</sup> Thermal Cycler (Bio-Rad Laboratories, USA). We amplified each of the seven immune genes (*lysozyme C*, *prophenoloxidase*, *Attacin*, *peptidoglycan recognition protein LC*,  *$\beta$ -1,3-glucan recognition protein*, *serpin 3a*, and *pelle*) and three housekeeping genes (*histone variant H2A.Z*, and mitochondrial *ribosomal protein L37* and *S24*) using primers and appropriated protocols described by Woestmann *et al.* [58]. For each qPCR reaction, we mixed 1 $\mu$ L of the 1/5 diluted cDNA, with 5 $\mu$ L of SYBR<sup>®</sup> Green containing master mix (iQ<sup>TM</sup> SYBR<sup>®</sup> Green Supermix, Bio-Rad Laboratories, USA), 3 $\mu$ L of nuclease-free water, and 0.5 $\mu$ L of the forward and reverse primers (10 $\mu$ M). Non-reverse transcribed samples were used as controls for the lack of genomic DNA contamination.

## ***Statistical analysis***

All the statistical analysis were performed with the software R v3.3.1 [72].

*Larval performance*: We first tested correlations among the variables using linear models (lm), from the package *lmer4* [73]. As larval group size at L2 and treatment are highly correlated, only the treatment variable is used in the following models. The development time to L2 was log-transformed prior to analysis. The development time to L2 was compared among larvae from the 27 families using a linear mixed model including the 'treatment' as an explanatory variable and the 'family' as a random variable. The survival rate at day 13 of the L3 larvae from the 27 families were compared using a general linear mixed model assuming a Gamma distribution of the data, with 'treatment' as an explanatory variable and

the 'family' as a random factor. We used the packages *lme4* [73] and *MASS* [74] for the mixed model analyses. Interclass correlation coefficients (ICC) were calculated based on variance of the random factor and residual to estimate how much of the variance was explained by the random factor 'family' in each model.

*Microbiota*: We used VEGAN [75] in R to compute a Bray-Curtis dissimilarity matrix, and analyze bacterial composition variations among samples using non metric multidimensional scaling (NMDS) or distance based redundancy analysis (dbRDA) [76]. The  $\alpha$ -diversity (diversity of the microbiota within each samples) of the microbiota was estimated through the Shannon index while the  $\beta$ -diversity (dissimilarity among samples) of the microbiota was estimated through the Bray-Curtis index. For the  $\alpha$ -diversity comparisons, a linear model was used after a logarithmic transformation of the index. The impact of the treatment, the larval family and their interaction were considered as explanatory variables. Similarly, for the  $\beta$ -diversity comparisons, we used a Permutational analysis of variance (*adonis*-ANOVA) [77] with the treatment, the larval family and their interaction as explanatory variables.

*Metabolomics*: We extracted entire spectra values from each sample using the program MestReNova 12 (Mestrelab Research, Spain) [78]. For multivariate analysis, the signals were binned to 0.04 ppm, the TSP, H<sub>2</sub>O and CD<sub>3</sub>OD signals were removed, and the integral values were transformed following the formula given below: (See Formula 1 in the Supplemental Files)

with '*m*' as the exact dry mass of the sample ( $\pm 0.1$  mg), ' $\delta$ ' as the <sup>1</sup>H chemical shift and '*g*' as to the number of equivalent <sup>1</sup>H atoms contained within the TSP reference molecule. Characteristic signals corresponding to  $\alpha$ -glucose ( $\delta$  4.59, d, J=7.9Hz),  $\beta$ -glucose ( $\delta$  5.19, d, J=3.7Hz), Alanine ( $\delta$  1.49, d, J=7.2Hz), Formic acid ( $\delta$  8.47, s), Acetic acid ( $\delta$  1.91, s), Fumaric acid ( $\delta$  6.53, s) and Ethanol ( $\delta$  1.19, t, J=7Hz) were annotated based on previously published datasets applying the same protocol [71, 79, 80]. *Plantago lanceolata* also contains variable quantities of iridoid and phenylpropanoid glycosides: namely aucubin, catalpol and verbascoside [43-45]. Two characteristic peaks were identified for aucubin ( $\delta$  6.31, dd, J=1.9Hz) and catalpol ( $\delta$  6.40, dd, J=1.9Hz) based on the <sup>1</sup>H-NMR profiles of standard compounds.

We calculated a first principal component analysis (PCA) including all signal bin values from 49 of the 52 larval samples. Three larval samples (family 1: treatment A and AR, and family 19: treatment R) were removed prior PCA as they clearly appeared as outliers driving most of the variation from the dataset. We considered only seven principal components (PCs) as they showed Eigen-values > 3, and together represented over 65% of the observed variation in the dataset. We analyzed the seven PCs using a linear mixed model (*lmer*) [73] after log transformation. Interclass correlation coefficients (ICC) from each model were calculated based on the proportion of the variance explained by the random family factor. We included 'larval treatment' as an explanatory variable and 'family' as a random factor in each model. Tukey tests, after correction for the family effect using the *glht* function, were used as posthoc tests to explore paired comparison between treatments. The resulting *P*-values were corrected for multiple testing using a Bonferroni correction ( $\alpha=0.025$ ).

We tested the relationship between each of the first seven PCs and the development time to L2 and survival to L3 between the treatment groups using a linear mixed model (*lmer*), with 'treatment' and the 'PC' of interest as fixed factors, and 'family' as a random factor to each model. Tukey tests after correction for the family effect with the *glht* function, were used as posthoc tests to explore paired comparison between treatments. The resulting *P*-values were corrected for multiple testing using a Bonferroni correction ( $\alpha=0.025$ ).

*Immune gene expression:* We calculated the mean immune gene expression from the three technical replicates (with exception of few outliers) considering the geometric mean of the three reference genes, for each family but one. For unknown reason, the control samples for the *S24* house-keeping gene was not expressed for the family16, thus, the immune gene expression for family16 was calculated based on the geometric mean of the two remaining house-keeping genes only. The immune genes expression (Log<sub>2</sub>) were compared among larvae from 12 families using generalized linear models [73, 77] including the 'larval treatment' and 'gene' as fixed factors (including interaction term), and 'family' as a random factor. We performed a post-hoc analysis using the *lsmeans* function with Tukey's HSD adjustment for pairwise comparisons [81], to explore paired comparison between treatments and genes, and corrected resulting *P*-values for multiple testing using a Bonferroni correction ( $\alpha=0.025$ ).

Finally, we tested whether development time to L2 (corrected for family effect) and survival to L3 (corrected for family effect) were differently affected by variation in the expression levels (Log<sub>2</sub>) of the *Attacin* immune gene (an antimicrobial peptide active against Gram-negative bacteria [82]) from the different larval treatment groups, including the 'immune genes expression levels' and 'treatment' as explanatory variables. Tukey tests, after correction for the family effect using the *glht* function, were used as posthoc tests to explore paired comparison between treatments. The resulting *P*-values were corrected for multiple testing using a Bonferroni correction ( $\alpha=0.025$ ).

## List Of Abbreviations

L2: second larval instar

L3: third larval instar

A: Treatment group: Antibiotic treated

AR: Treatment group: Antibiotic treated during Reinfection with frass

C: Treatment group: Control

R: Treatment group: Antibiotic treated followed by Reinfection

## Declarations

### Ethics approval and consent to participate

The study complies with animal ethic approvals.

### **Consent for publication**

Not applicable

### **Availability of data and materials**

Raw data from life history assays, qPCR runs, and NMR spectra are publicly stored in *Dryad* (<https://doi.org/10.5061/dryad.9s4mw6mc1>). The raw microbiota data used during the current study are available from the corresponding author on request, and will be made fully accessible from the European Nucleotide Archive (<http://www.ebi.ac.uk/ena>, European Molecular Biology Library - European Bioinformatics Institute, EMBL-EBI) upon acceptance of the manuscript.

### **Competing interests**

There are no conflicts of interest concerning this article.

### **Funding**

The project was funded by the Academy of Finland (Grant #304041 MS) and the European Research Council (Grant #637412, META-STRESS to MS). AD is funded by a Marie Curie Sklodowska Individual Fellowship (#790531, HostSweetHome).

### **Authors' contributions**

AD & GM collected the data, AD & GM analyzed the data, GM designed the research, AD wrote the manuscript with all authors contributing to the final version.

### **Acknowledgements**

We would like to thank M. Journiac, S. Ikonen and several research assistants at the Lammi Biological Station for their help with rearing the larvae and collecting the data, to T. Nyman and J-M. Pitkänen for their help with conducting molecular work in the laboratory, to E. Rosa and A. Kahilainen for fruitful discussions on the analyses and research. Thanks to T. Niemi-Aro from the NMR-laboratory at the BI, the University of Helsinki, for providing us with the Metabolomic spectra, to T. Hannunen from the FIMM laboratory for supervising the high throughput microbial sequencing.

### **Authors' information: ORC IDs**

Anne DUPLOUY: 0000-0002-7147-5199

Guillaume MINARD: 0000-0002-2079-88444

Marjo SAASTAMOINEN: 0000-0001-7009-25277

## References

1. Hagele BF, Rowell-Rahier M. Dietary mixing in three generalist herbivores: nutrient complementation or toxin dilution? *Oecologia*. 1999;119:521-33. doi: 10.1007/s004420050815.
2. Lampert E. Influences of plant traits on immune responses of specialist and generalist herbivores. *Insects*. 2012;19:573-92. doi: 10.3390/insects3020573.
3. Lampert EC, Bowers MD. Host Plant Influences on Iridoid Glycoside Sequestration of Generalist and Specialist Caterpillars. *J Chem Ecol*. 2010;36:1101-4. doi: 10.1007/s10886-010-9849-4.
4. Brune A. Symbiotic digestion of lignocellulose in termite guts. *Nat Rev Microbiol*. 2014;12:168-80. doi: 10.1038/nrmicro3182.
5. Hosokawa T, Koga R, Kikuchi Y, Meng XY, Fukatsu T. Wolbachia as a bacteriocyte-associated nutritional mutualist. *P Natl Acad Sci USA*. 2010;107:769-74. doi: 10.1073/pnas.0911476107.
6. Douglas AE. Nutritional interactions in insect-microbial symbioses: Aphids and their symbiotic bacteria Buchnera. *Annual Review of Entomology*. 1998;43:17-37. doi: DOI 10.1146/annurev.ento.43.1.17.
7. Hansen AK, Moran NA. Aphid genome expression reveals host-symbiont cooperation in the production of amino acids. *P Natl Acad Sci USA*. 2011;108:2849-54. doi: 10.1073/pnas.1013465108.
8. McCutcheon JP, Moran NA. Extreme genome reduction in symbiotic bacteria. *Nature Reviews Microbiology*. 2012;10:13-26. doi: 10.1038/nrmicro2670.
9. Poliakov A, Russell CW, Ponnala L, Hoops HJ, Sun Q, Douglas AE, et al. Large-Scale Label-Free Quantitative Proteomics of the Pea aphid-Buchnera Symbiosis. *Mol Cell Proteomics*. 2011;10. doi: 10.1074/mcp.M110.007039.
10. Bandi C, Sacchi L. Intracellular symbiosis in termites. In: Abe T, Bignell DE, Higashi M, editors. *Termites: evolution, sociality, symbiosis, ecology*. Dordrecht, Netherlands: Kluwer Academic Publishers; 2000. p. 261-73.
11. Nazarczuk RA, O'Brien RW, Slaytor M. Alteration of the Gut Microbiota and Its Effect on Nitrogen-Metabolism in Termites. *Insect Biochem*. 1981;11:267-75. doi: Doi 10.1016/0020-1790(81)90004-4.
12. Slaytor M, Chappell DJ. Nitrogen-Metabolism in Termites. *Comp Biochem Phys B*. 1994;107:1-10. doi: Doi 10.1016/0305-0491(94)90218-6.
13. Rosengaus RB, Zecher CN, Schultheis KF, Brucker RM, Bordenstein SR. Disruption of the Termite Gut Microbiota and Its Prolonged Consequences for Fitness. *Appl Environ Microb*. 2011;77:4303-12. doi: 10.1128/Aem.01886-10.
14. Berenbaum M. Adaptive significance of midgut pH in larval Lepidoptera. *The American Naturalist*. 1980;115:138-46. <https://www.jstor.org/stable/2460837>.
15. Dow JAT. Ph Gradients in Lepidopteran Midgut. *J Exp Biol*. 1992;172:355-75. <Go to ISI>://WOS:A1992KF72000030.
16. Wouters FC, Blanchette B, Gershenzon J, Vassao DG. Plant defense and herbivore counter-defense: benzoxazinoids and insect herbivores. *Phytochem Rev*. 2016;15:1127-51. doi: 10.1007/s11101-016-

9481-1.

17. Zagrobelny M, Bak S, Ekstrom CT, Olsen CE, Moller BL. The cyanogenic glucoside composition of *Zygaena filipendulae* (Lepidoptera: Zygaenidae) as effected by feeding on wild-type and transgenic lotus populations with variable cyanogenic glucoside profiles. *Insect Biochem Mol Biol*. 2007;37:10-8. doi: 10.1016/j.ibmb.2006.09.008.
18. Zagrobelny M, Moller BL. Cyanogenic glucosides in the biological warfare between plants and insects: the Burnet moth-Birdsfoot trefoil model system. *Phytochemistry*. 2011;72:1585-92. doi: 10.1016/j.phytochem.2011.02.023.
19. Hartmann T, Theuring C, Beuerle T, Bernays EA, Singer MS. Acquisition, transformation and maintenance of plant pyrrolizidine alkaloids by the polyphagous arctiid *Grammia geneura*. *Insect Biochem Molec*. 2005;35:1083-99. doi: 10.1016/j.ibmb.2005.05.011.
20. Ojala K, Julkunen-Tiito R, Lindstrom L, Mappes J. Diet affects the immune defence and life-history traits of an Arctiid moth *Parasemia plantaginis*. *Evol Ecol Res*. 2005;7:1153-70. <Go to ISI>://WOS:000234549900005.
21. Bukovinszky T, Poelman EH, Gols R, Prekatsakis G, Vet LEM, Harvey JA, et al. Consequences of constitutive and induced variation in plant nutritional quality for immune defence of a herbivore against parasitism. *Oecologia*. 2009;160:299-308. doi: 10.1007/s00442-009-1308-y.
22. Vogelweith F, Thiery D, Quaglietti B, Moret Y, Moreau J. Host plant variation plastically impacts different traits of the immune system of a phytophagous insect. *Funct Ecol*. 2011;25:1241-7. doi: 10.1111/j.1365-2435.2011.01911.x.
23. Hammer TJ, Janzen DH, Hallwachs W, Jaffe SP, Fierer N. Caterpillars lack a resident gut microbiome. *Proceedings of the National Academy of Sciences of the United States of America*. 2017;114:9641-6. doi: 10.1073/pnas.1707186114.
24. Vilanova C, Baixeras J, Latorre A, Porcar M. The Generalist Inside the Specialist: Gut Bacterial Communities of Two Insect Species Feeding on Toxic Plants Are Dominated by *Enterococcus* sp. *Front Microbiol*. 2016;7. doi: ARTN 1005  
10.3389/fmicb.2016.01005.
25. Whitaker MRL, Salzman S, Sanders J, Kaltenpothz M, Pierce NE. Microbial Communities of Lycaenid Butterflies Do Not Correlate with Larval Diet. *Frontiers in Microbiology*. 2016;7. doi: ARTN 1920  
10.3389/fmicb.2016.01920.
26. Voirol LR, Frago E, Kaltenpoth M, Hilker M, Fatouros NE. Bacterial symbionts in Lepidoptera: their diversity, transmission, and impact on the host. *Frontiers in Microbiology*. 2018;9:556. doi: 10.3389/fmicb.2018.00556.
27. Chaturvedi S, Rego A, Lucas LK, Gompert Z. Sources of Variation in the Gut Microbial Community of *Lycaeides melissa* Caterpillars. *Sci Rep*. 2017;7:11335. doi: 10.1038/s41598-017-11781-1.

28. Kuussaari M, Singer MC. Group size, and egg and larval survival in the social butterfly *Melitaea cinxia*. *Ann Zool Fenn.* 2017;54:213-23. doi: Doi 10.5735/086.054.0119.
29. Van Nouhuys S, Singer MC, Nieminen M. Spatial and temporal patterns of caterpillar performance and the suitability of two host plant species. *Ecol Entomol.* 2003;28:193-202. doi: DOI 10.1046/j.1365-2311.2003.00501.x.
30. Rosa E, Woestmann L, Biere A, Saastamoinen M. A plant pathogen modulates the effects of secondary metabolites on the performance and immune function of an insect herbivore. *Oikos.* 2018;127:1539-49. doi: 10.1111/oik.05437.
31. Laine AL. Resistance variation within and among host populations in a plant-pathogen metapopulation: implications for regional pathogen dynamics. *J Ecol.* 2004;92:990-1000. doi: DOI 10.1111/j.0022-0477.2004.00925.x.
32. Salgado AL, Saastamoinen M. Developmental stage-dependent response and preference for host plant quality in an insect herbivore. *Anim Behav.* 2019;150:27-38. doi: 10.1016/j.anbehav.2019.01.018.
33. Ruokolainen L, Ikonen S, Makkonen H, Hanski I. Larval growth rate is associated with the composition of the gut microbiota in the Glanville fritillary butterfly. *Oecologia.* 2016;181:895-903. doi: 10.1007/s00442-016-3603-8.
34. Rosa E, Minard G, Lindholm J, Saastamoinen M. Moderate plant water stress improves larval development, and impacts immunity and gut microbiota of a specialist herbivore. *PLoS One.* 2019;14:e0204292. doi: 10.1371/journal.pone.0204292.
35. Phalnikar K, Kunte K, Agashe D. Dietary and developmental shifts in butterfly-associated bacterial communities. *R Soc Open Sci.* 2018;5:171559. doi: 10.1098/rsos.171559.
36. Engel P, Moran NA. The gut microbiota of insects - diversity in structure and function. *Fems Microbiology Reviews.* 2013;37:699-735. doi: 10.1111/1574-6976.12025.
37. Ceja-Navarro JA, Vega FE, Karaoz U, Hao Z, Jenkins S, Lim HC, et al. Gut microbiota mediate caffeine detoxification in the primary insect pest of coffee. *Nat Commun.* 2015;6:7618. doi: 10.1038/ncomms8618.
38. Phalnikar K, Kunte K, Agashe D. Disrupting butterfly caterpillar microbiomes does not impact their survival and development. *Proc Biol Sci.* 2019;286:20192438. doi: 10.1098/rspb.2019.2438.
39. Funkhouser LJ, Bordenstein SR. Mom knows best: the universality of maternal microbial transmission. *PLoS Biol.* 2013;11:e1001631. doi: 10.1371/journal.pbio.1001631.
40. Minard G, Tikhonov G, Ovaskainen O, Saastamoinen M. The microbiome of the *Melitaea cinxia* butterfly shows marked variation but is only little explained by the traits of the butterfly or its host plant. *Environmental Microbiology.* 2019;21:4253-69. doi: 10.1111/1462-2920.14786.
41. Hanski I, Singer MC. Extinction-colonization dynamics and host-plant choice in butterfly metapopulations. *Am Nat.* 2001;158:341-53. doi: 10.1086/321985.
42. Hanski I. Spatially realistic theory of metapopulation ecology. *Naturwissenschaften.* 2001;88:372-81. <https://www.ncbi.nlm.nih.gov/pubmed/11688412>.

43. Marak HB, Biere A, Van Damme JMM. Direct and correlated responses to selection on iridoid glycosides in *Plantago lanceolata* L. *J Evolution Biol.* 2000;13:985-96. doi: DOI 10.1046/j.1420-9101.2000.00233.x.
44. Duff RB, Bacon JSD, Mundie CM, Farmer VC, Russell JD, Forrester AR. Catalpol and Methylcatalpol - Naturally Occurring Glycosides in *Plantago* and *Buddleia* Species. *Biochem J.* 1965;96:1-8. <Go to ISI>://WOS:A19656606600001.
45. Nieminen M, Suomi J, Van Nouhuys S, Sauri P, Riekkola ML. Effect of iridoid glycoside content on oviposition host plant choice and parasitism in a specialist herbivore. *J Chem Ecol.* 2003;29:823-44. <https://www.ncbi.nlm.nih.gov/pubmed/12775146>.
46. Adler LS, Schmitt J, Bowers MD. Genetic-Variation in Defensive Chemistry in *Plantago-Lanceolata* (Plantaginaceae) and Its Effect on the Specialist Herbivore *Junonia-Coenia* (Nymphalidae). *Oecologia.* 1995;101:75-85. doi: Doi 10.1007/Bf00328903.
47. Saastamoinen M, van Nouhuys S, Nieminen M, O'Hara B, Suomi J. Development and survival of a specialist herbivore, *Melitaea cinxia*, on host plants producing high and low concentrations of iridoid glycosides. *Ann Zool Fenn.* 2007;44:70-80. <Go to ISI>://WOS:000245604400007.
48. Berasategui A, Salem H, Paetz C, Santoro M, Gershenson J, Kaltenpoth M, et al. Gut microbiota of the pine weevil degrades conifer diterpenes and increases insect fitness. *Molecular ecology.* 2017; 26:4099-110.
49. Wybouw N, Dermauw W, Tirry L, Stevens C, Grbić M, Feyereisen R, et al. A gene horizontally transferred from bacteria protects arthropods from host plant cyanide poisoning. *Elife.* 2014;3.
50. Wernegreen JJ. Mutualism meltdown in insects: bacteria constrain thermal adaptation. *Curr Opin Microbiol.* 2012;15:255-62. doi: 10.1016/j.mib.2012.02.001.
51. Moll RM, Romoser WS, Modrzakowski MC, Moncayo AC, Lerdthusnee K. Meconial peritrophic membranes and the fate of midgut bacteria during mosquito (Diptera : culicidae) metamorphosis. *J Med Entomol.* 2001;38:29-32. doi: Doi 10.1603/0022-2585-38.1.29.
52. Hammer TJ, McMillan WO, Fierer N. Metamorphosis of a Butterfly-Associated Bacterial Community. *Plos One.* 2014;9. doi: ARTN e86995  
10.1371/journal.pone.0086995.
53. Chen BS, Teh BS, Sun C, Hu SR, Lu XM, Boland W, et al. Biodiversity and Activity of the Gut Microbiota across the Life History of the Insect Herbivore *Spodoptera littoralis*. *Sci Rep-Uk.* 2016;6. doi: ARTN 29505  
10.1038/srep29505.
54. Saastamoinen M, Ikonen S, Wong SC, Lehtonen R, Hanski I. Plastic larval development in a butterfly has complex environmental and genetic causes and consequences for population dynamics. *J Anim Ecol.* 2013;82:529-39. doi: 10.1111/1365-2656.12034.

55. Kahilainen A, van Nouhuys S, Schulz T, Saastamoinen M. Metapopulation dynamics in a changing climate: Increasing spatial synchrony in weather conditions drives metapopulation synchrony of a butterfly inhabiting a fragmented landscape. *Glob Chang Biol*. 2018. doi: 10.1111/gcb.14280.
56. Asling B, Dushay MS, Hultmark D. Identification of early genes in the *Drosophila* immune response by PCR-based differential display: the Attacin A gene and the evolution of attacin-like proteins. *Insect Biochem Mol Biol*. 1995;25:511-8. <https://www.ncbi.nlm.nih.gov/pubmed/7742836>.
57. Login FH, Balmand S, Vallier A, Vincent-Monégat C, Vigneron A, Weiss-Gayet M, et al. Antimicrobial peptides keep insect endosymbionts under control. *Science*. 2011; 334:362-5.
58. Woestmann L, Kvist J, Saastamoinen M. Fight or flight? - Flight increases immune gene expression but does not help to fight an infection. *J Evol Biol*. 2017;30:501-11. doi: 10.1111/jeb.13007.
59. Kvist J, Mattila AL, Somervuo P, Ahola V, Koskinen P, Paulin L, et al. Flight-induced changes in gene expression in the Glanville fritillary butterfly. *Mol Ecol*. 2015;24:4886-900. doi: 10.1111/mec.13359.
60. Nieminen M, Siljander M, Hanski I. Structure and dynamics of *Melitaea cinxia* metapopulations. In: Ehrlich PR, Hanski I, editors. *On the Wings of Checkerspot: A Model System for Population Biology* New York: Oxford University Press; 2004. p. 63-91.
61. Duploux A, Ikonen S, Hanski I. Life history of the Glanville fritillary butterfly in fragmented versus continuous landscapes. *Ecol Evol*. 2013;3:5141-56. doi: 10.1002/ece3.885.
62. Hanski IA. Eco-evolutionary spatial dynamics in the Glanville fritillary butterfly. *Proc Natl Acad Sci U S A*. 2011;108:14397-404. doi: 10.1073/pnas.1110020108.
63. Fountain T, Nieminen M, Siren J, Wong SC, Lehtonen R, Hanski I. Predictable allele frequency changes due to habitat fragmentation in the Glanville fritillary butterfly. *Proc Natl Acad Sci U S A*. 2016;113:2678-83. doi: 10.1073/pnas.1600951113.
64. Nair A, Fountain T, Ikonen S, Ojanen SP, van Nouhuys S. Spatial and temporal genetic structure at the fourth trophic level in a fragmented landscape. *Proc Biol Sci*. 2016;283. doi: 10.1098/rspb.2016.0668.
65. Chung SH, Rosa C, Hoover K, Luthe DS, Felton GW. Colorado potato beetle manipulates plant defenses in local and systemic leaves. *Plant Signaling & Behavior*. 2013;8. doi: 10.4161/psb.27592.
66. Minard G, Tran FH, Van VT, Goubert C, Bellet C, Lambert G, et al. French invasive Asian tiger mosquito populations harbor reduced bacterial microbiota and genetic diversity compared to Vietnamese autochthonous relatives. *Front Microbiol*. 2015;6. doi: 10.3389/fmicb.2015.00970.
67. Andersson AF, Lindberg M, Jakobsson H, Backhed F, Nyren P, Engstrand L. Comparative analysis of human gut microbiota by barcoded pyrosequencing. *PLoS One*. 2008;3:e2836. doi: 10.1371/journal.pone.0002836.
68. Asemaninejad A, Weerasuriya N, Gloor GB, Lindo Z, Thorn RG. New Primers for Discovering Fungal Diversity Using Nuclear Large Ribosomal DNA. *PLoS One*. 2016;11:e0159043. doi: 10.1371/journal.pone.0159043.
69. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, et al. Introducing mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing

- Microbial Communities. *Appl Environ Microb.* 2009;75:7537-41. doi: 10.1128/Aem.01541-09.
70. Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics.* 2011;27:2194-200. doi: 10.1093/bioinformatics/btr381.
71. Kim HK, Choi YH, Verpoorte R. NMR-based metabolomic analysis of plants. *Nat Protoc.* 2010;5:536-49. doi: 10.1038/nprot.2009.237.
72. RCoreTeam. R: A language and environment for statistical computing. R Foundation for Statistical Computing. 2016:Vienna: Austria.
73. Bates D, Machler M, Bolker BM, Walker SC. Fitting Linear Mixed-Effects Models Using lme4. *J Stat Softw.* 2015;67:1-48. <Go to ISI>://WOS:000365981400001.
74. Ripley B, Venables B, Bates DM, Hornik K, Gebhardt A, Firth D. MASS: Support functions and datasets for Venables and Ripley's MASS2016.
75. Oksanen J, Blanchet FG, Kindt R, Legendre P, O'Hara RB, Simpson GL, et al. vegan: Community ecology package. R package version. 2011.
76. Anderson MJ, Willis TJ. Canonical analysis of principal coordinates: A useful method of constrained ordination for ecology. *Ecology.* 2003;84:511-25. doi: Doi 10.1890/0012-9658(2003)084[0511:Caopca]2.0.Co;2.
77. Anderson MJ. A new method for non-parametric multivariate analysis of variance. *Austral Ecol.* 2001;26:32-46. doi: DOI 10.1046/j.1442-9993.2001.01070.x.
78. Willcott MR. MestRe Nova. *J Am Chem Soc.* 2009;131:13180-. doi: 10.1021/ja906709t.
79. Gogna N, Hamid N, Dorai K. Metabolomic profiling of the phytomedicinal constituents of *Carica papaya* L. leaves and seeds by <sup>1</sup>H NMR spectroscopy and multivariate statistical analysis. *Journal of pharmaceutical and biomedical analysis.* 2015;115:74-85.
80. Agudelo-Romero P, Ali K, Choi YH, Sousa L, Verpoorte R, Tiburcio AF, et al. Perturbation of polyamine catabolism affects grape ripening of *Vitis vinifera* cv. *Trincadeira*. *Plant physiology and biochemistry.* 2014;74:141-55.
81. Lenth RV. Least-squares means: the R package lsmeans. *Journal of Statistical Software.* 2016;69:1-33. doi: 10.18637/jss.v069.i01.
82. Imler JL, Bulet P. Antimicrobial peptides in Drosophila: structures, activities and gene regulation. *Chemical Immunology and Allergy.* 2005;86:1-21. doi: 10.1159/000086648.

## Tables

**Table 1: Sample size for each treatment and each experimental assay.** All assays were run individually for each sample but the Metabolomics for which the carcasses of three larvae of the same family were pooled. L3 and L7: third and seventh instar larvae, respectively. Treatments are colored following the color code used in the study figures.

Sample type & Treatment	Development & Survival	Microbiota	Gene expression	Metabolomics
<b>C</b> Control larvae (No treatment)	20 larvae x 27 families	3 guts x13 families	3 larvae x10 families + (2 larvae x1family) + (1 larva x1 family)	3 carcasses pooled x 13 families
<b>A</b> Antibiotics from emergence to L3	20x27	3x13	3x10 + (2x1)+(1x1)	3pooledx13
<b>AR</b> Antibiotics from emergence to L3 + L7 frass on day4	20x27	3x13	3x12	3pooledx13
<b>R</b> Antibiotics until day3 + L7 frass on day4	20x27	3x13	3x9 + (2x1)+(1x2)	3pooledx13
<b>Frass</b> (10mg)	-	5	-	2
<b>Plant leave</b> (10mg)	-	5	-	1
<b>Control</b> (sterile water)	-	5	3	-
<b>Total</b> (N=)	2,160	171	136	55

**Table 2. Permutational analysis (*adonis-ANOVA*) of the gut bacterial community of the larvae of the Glanville fritillary butterfly.**

Covariates	df	pseudoF	R <sup>2</sup>	P
<b>Treatment</b>	3	48.489	0.40	0.001***
<b>Family</b>	14	2.953	0.11	0.001***
<b>Treatment x Family</b>	36	1.898	0.19	0.001***
<b>Residuals</b>	106		0.29	

**Table 3. Analysis on the first seven principal components of a principal component analysis for metabolite composition of 49 samples from four larval treatments ('A', 'AR', 'R' or 'C').** The treatment group does not explain the variation observed between samples, but family effect explains most of the variance between samples for PC6 only.

	PC1	PC2	PC3	PC4	PC5	PC6	PC7
<b>Eigen value</b>	7.97	5.54	5.13	4.09	3.78	3.41	3.18
<b>Cumulative proportion of variance</b>	0.24	0.35	0.45	0.52	0.57	0.62	0.65
<b>Treatment</b>	<i>P</i> =0.498 df=3	<i>P</i> =0.169 df=3	<i>P</i> =0.169 df=3	<i>P</i> =0.169 df=3	<i>P</i> =0.296 df=3	<i>P</i> =0.096 df=3	<i>P</i> =0.25 df=3
<b>Family (ICC)</b>	0.001	0.001	0.001	0.001	0.1e-16	0.408	5.9e-16

**Table 4. Impact of the treatments on the abundance of nine annotated metabolites.**

Compound	df	F	P <sup>1</sup>
α-glucose	3,48	1.11	0.35
β-glucose	3,48	1.16	0.33
Alanine	3,36	2.24	0.10
Formic acid	3,36	0.40	0.75
Acetic acid	3,36	0.83	0.49
Fumaric acid	3,48	0.24	0.87
Ethanol	3,48	0.33	0.80
Aucubin	3,36	2.53	0.07
Catalpol	3,36	0.89	0.46

<sup>1</sup> Corrected for family effect

**Table 5. Impact of the treatments, expression levels of the *Attacin* gene, and interactions, on the development time to L2 and survival to L3 of the larvae of the Glanville fritillary butterfly. *P*-values corrected for multiple testing after Bonferroni correction ( $\alpha=0.025$ ).**

	df	F	<i>P</i> <sup>1</sup>
<b>Development to L2</b>			
<i>Attacin</i> expression level (Log2)	1	9.46	2.62e-3 **
Treatment	3	11.042	1.95e-6 ***
<i>Attacin</i> expression:Treatment	3	0.344	0.794
<b>Survival to L3</b>			
<i>Attacin</i> expression level (Log2)	1	7.43	7.39e-3 **
Treatment	3	13.124	1.91e-7 ***
<i>Attacin</i> expression:Treatment	3	0.518	0.671

<sup>1</sup>Corrected for family effect

## Additional File Legends

**Figure S1: Between family variations of the effect of microbial depletion through antibiotic treatment on the (A.) development rate to L2 and (B.) survival to L3 of pre-diapause larvae.** Data include larvae from 27 families under four different treatments (blue): antibiotic-treated, (salmon): antibiotic-treated even during re-infected, (gray): control, and (yellow): antibiotic-treated followed by re-infection by L7 larval frass.

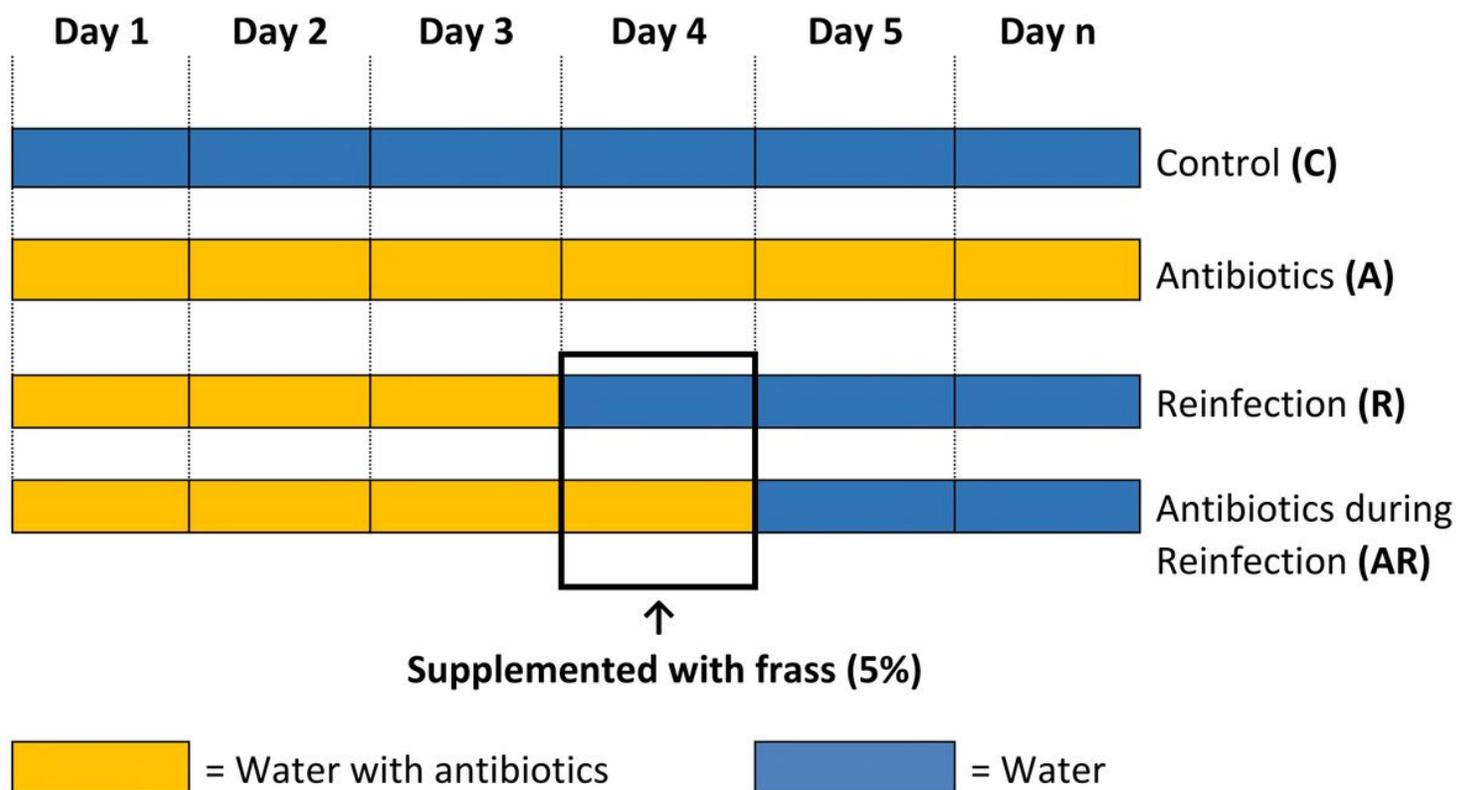
**Figure S2: The plant-contaminated gut fungal community of pre-diapause larvae of the Glanville fritillary butterfly.** Data include three larvae from each of 13 families, and under four different treatments (A): antibiotic-treated, (AR): antibiotic-treated even during re-infected, (C): control larvae, and (R): antibiotic-treated followed by re-infection by frass from L7 larvae of the same families. Note the large contamination of the fungal analysis with DNA from *Streptophyta*.

**Figure S3. Effects of antibiotic treatments on the (A.)  $\alpha$ -diversity (Shannon index), (B.) heterogeneity (Distance to centroid), and (C.)  $\beta$ -diversity of the bacterial gut communities of pre-diapause larvae and frass of the Glanville fritillary butterfly, and of the larval host plant *Plantago lanceolata*.** Data include three larvae from each of 13 families, and under four different treatments: (A-blue- squares): antibiotic-treated, (AR-orange-circles): antibiotic-treated even during re-infected, (C-gray-triangles): control larvae, and (R-yellow+): antibiotic-treated followed by re-infection by frass from L7 larvae of the same families. (turquoise-x): frass, and (green-diamond shapes): plant samples.

**Figure S4: Effects of family on the (A.)  $\alpha$ -diversity (Shannon index) and (B.)  $\beta$ -diversity of the gut bacterial communities of pre-diapause larvae, frass and host plant (*Plantago lanceolata*) of the Glanville fritillary butterfly.** Data include larvae from 13 families. Bacterial communities of five larval frass samples and five host plants (1cm<sup>2</sup> leave piece/plant) were added to the analysis for comparison. There is no difference between families in the bacterial community of the gut.

**Figure S5: PC1 values from the metabolite study according to (A) development time to L2 and (B) survival to L3 of pre-diapause larvae for each treatment group.** Average data from up to three larvae from 13 families reared under four treatment groups: (A-Blue): antibiotic-treated, (AR-Orange): antibiotic-treated even during re-infected, (C-Gray): control, and (R-Yellow): antibiotic-treated followed by re-infection by L7 larval frass. Ellipses give the 2% confidence interval for respective treatment group.

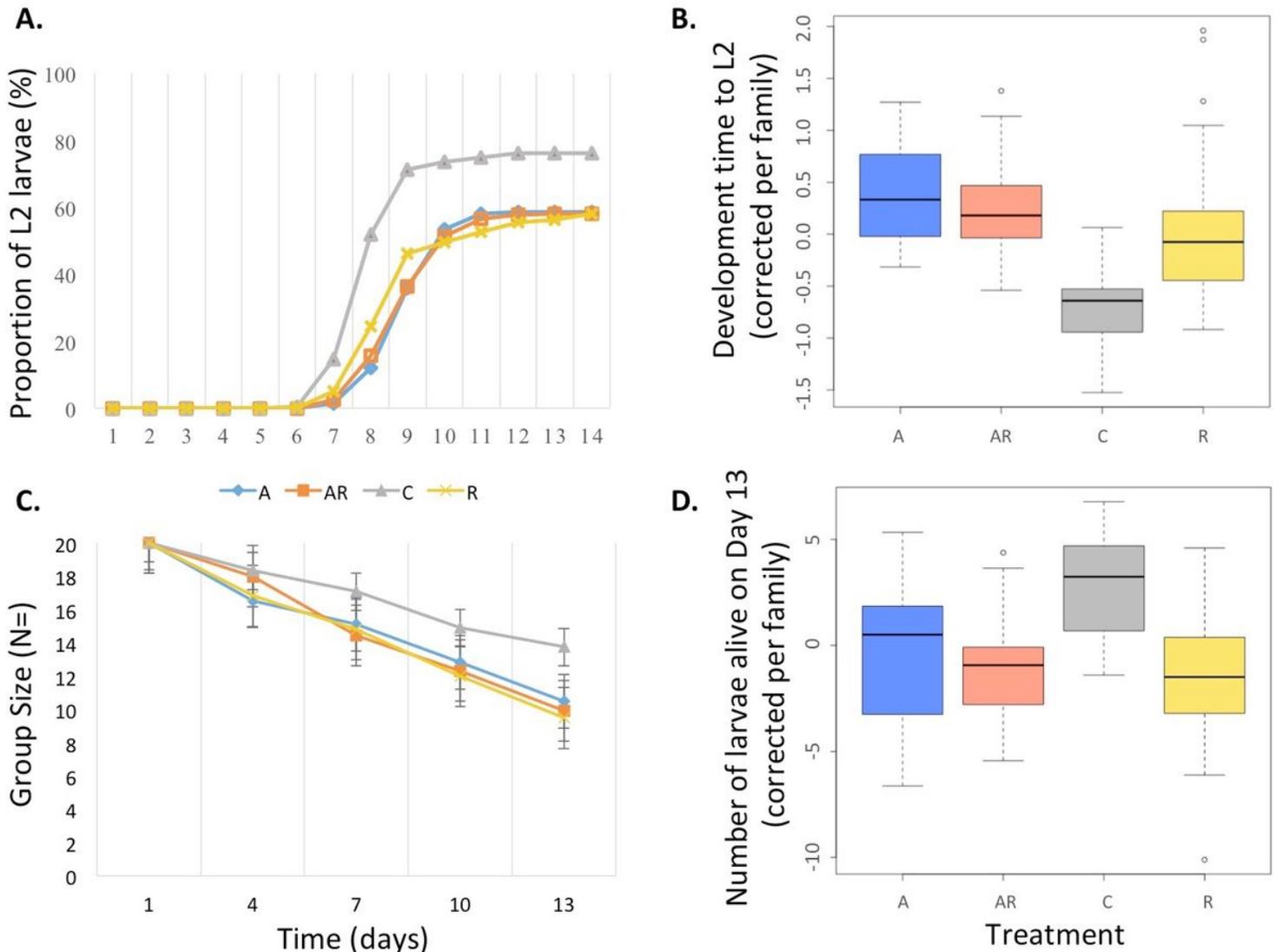
## Figures



**Figure 1**

Daily description of the four larval treatment groups used in the study. (C) A daily amount of 200 $\mu$ l of sterile water was left to dry 2h on the leaves before being provided to the larvae, from day1 (L1) until the larvae molt into L3. (A) 200 $\mu$ l of the antibiotic solution was left to dry 2h on the leaves before being provided to the larvae, from day1 (L1) the larvae molt into L3. (R) 200 $\mu$ l of antibiotic solution was left to dry 2h on the leaves before being provided to the larvae, from day1 to day3 (L1). On day 4, 200 $\mu$ l of the antibiotic solution was supplemented with 5% of L7's frass was left to dry 2h on the leaves before being

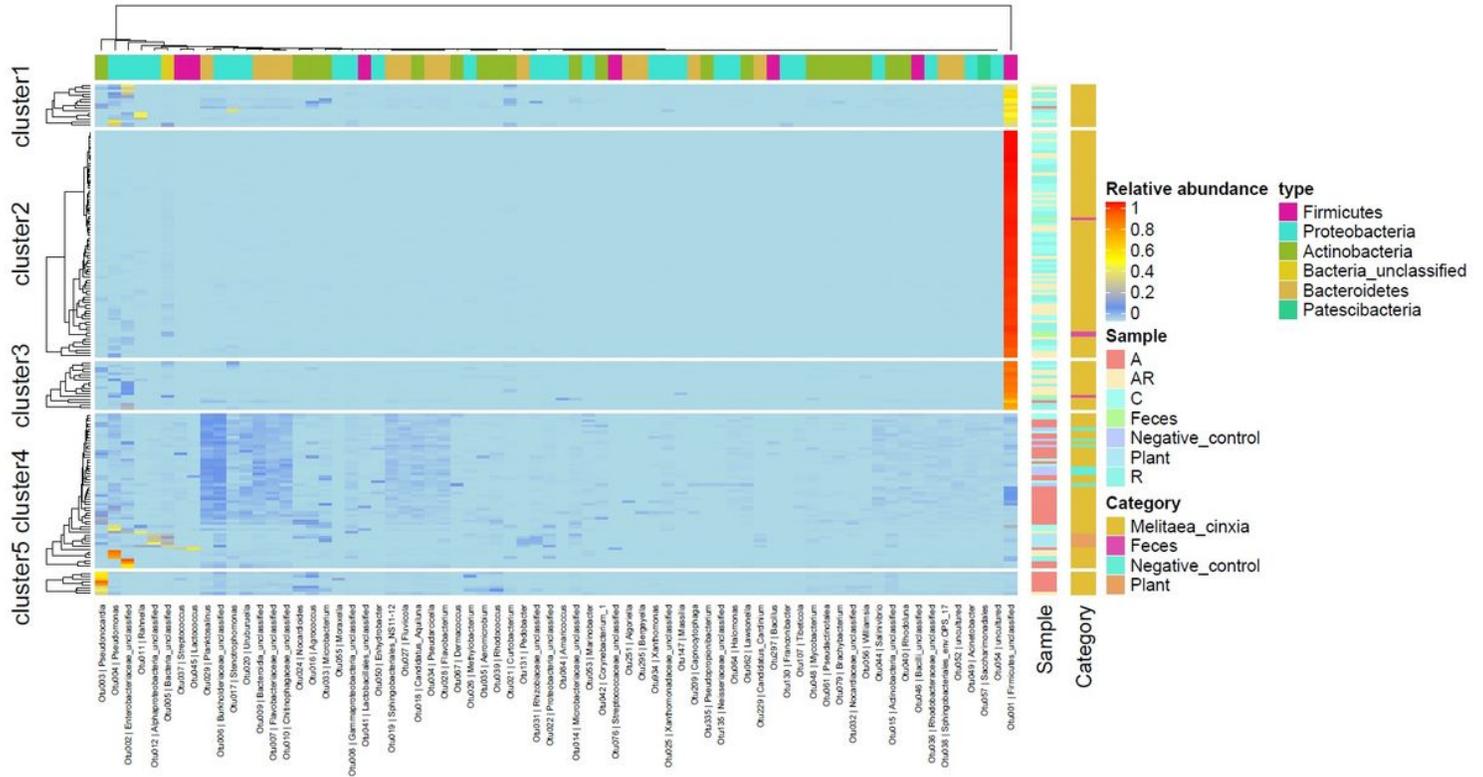
provided to the larvae. From day5 and until the larvae molted into L3, 200µl of sterile water was left to dry 2h on the leaves before being provided to the larvae. (AR) 200µl of the antibiotic solution was left to dry 2h on the leaves before being provided to the larvae, from day1 to 3 (L1). On day4, 200µl of the antibiotic solution was supplemented with 5% of L7's frass and left to dry 2h on the leaves before being provided to the larvae. From day5, 200µl of sterile water was left to dry 2h on the leaves before being provided to the larvae until the larvae molt into L3.



**Figure 2**

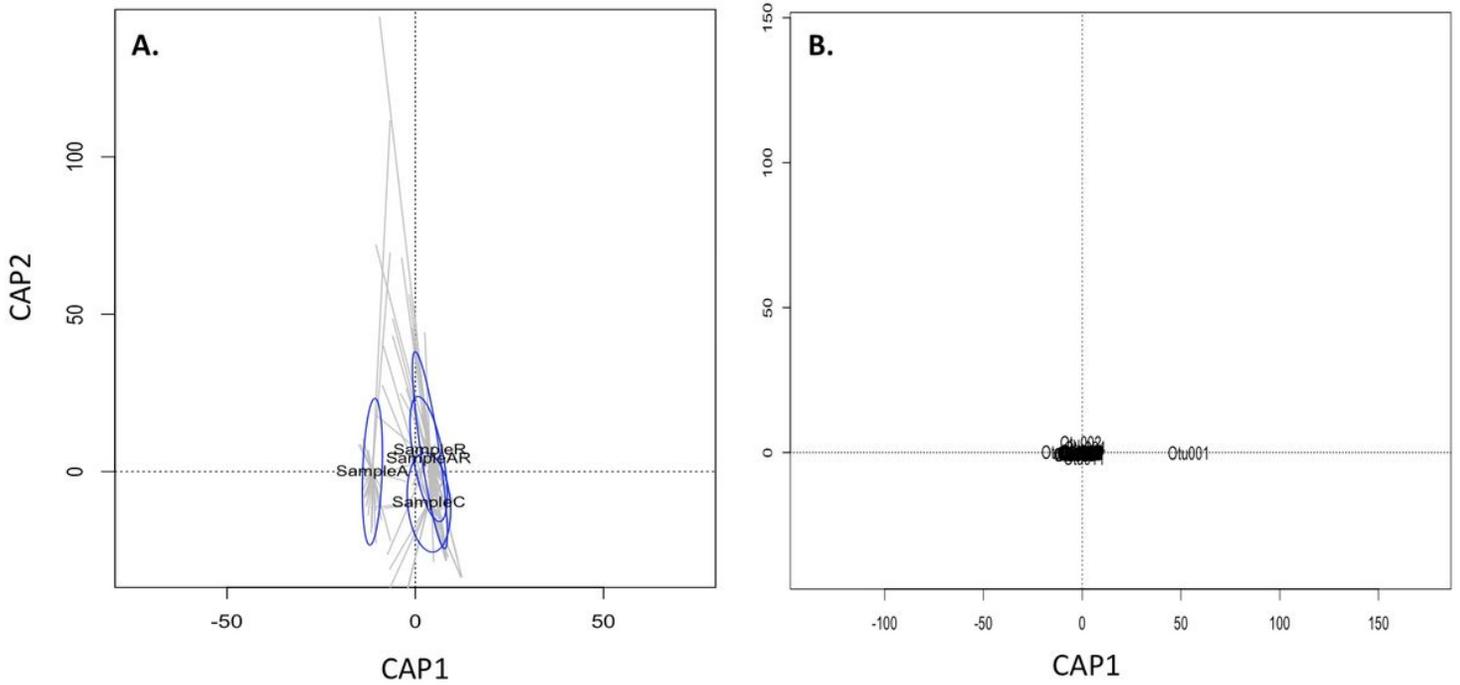
Effects of microbial depletion through antibiotic treatment on the development and survival of pre-diapause larvae of the Glanville fritillary butterfly. (A.) Proportion of larvae from each treatment that reached L2, and (B.) development time of larvae into L2 for each treatment. Data include larvae from 27 families under four different treatments (A-Blue): antibiotic-treated, (AR-Orange): antibiotic-treated even during re-infected, (C-Gray) control, and (R-Yellow): antibiotic-treated followed by re-infection by L7 larval frass. (C.) Survival at day 1, 4, 7, 10 and 13 after the start of the experiment, and (D.) survival at day 13 for each treatment. Data include larvae from 27 families under four different treatments (A): antibiotic-

treated, (AR): antibiotic-treated even during re-infected, (C) control, and (R): antibiotic-treated followed by re-infection by L7 larval frass.



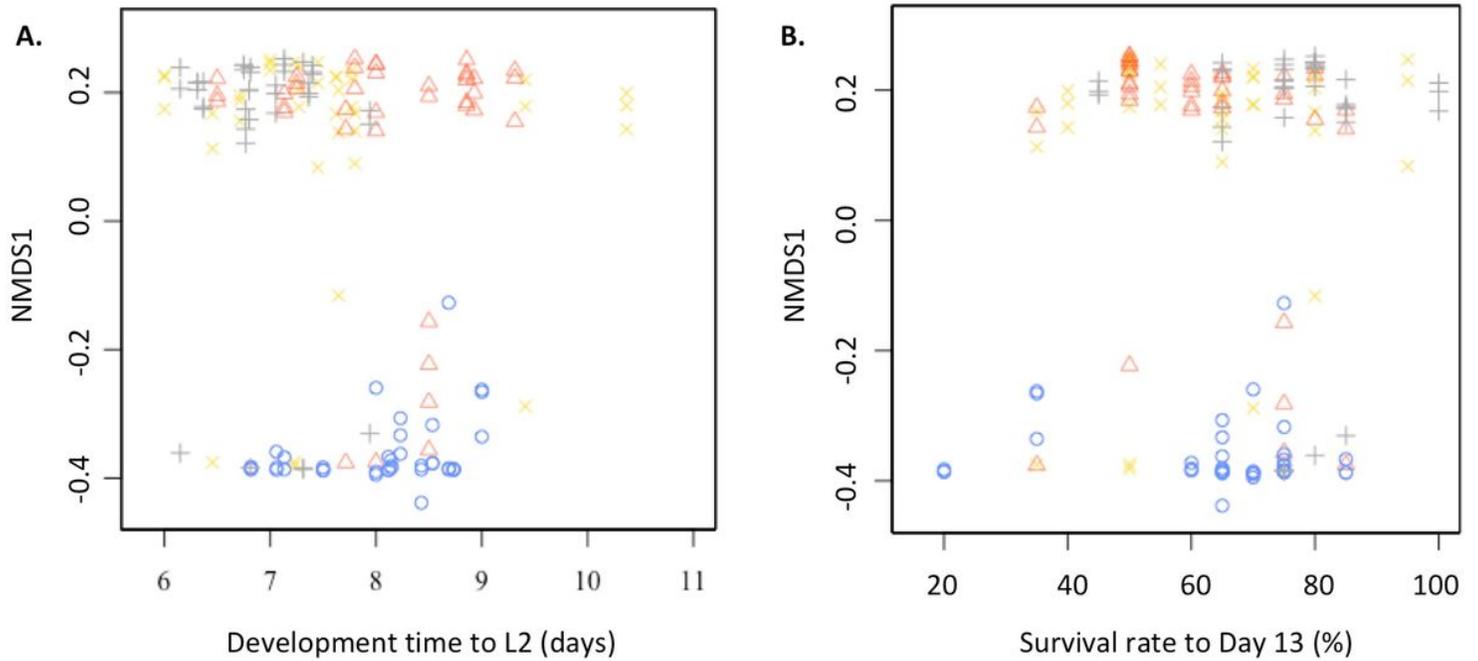
**Figure 3**

Composition of the microbiota of the host plant, the frass, and the gut of the Glanville fritillary larvae. The bacterial OTUs were reported with their taxonomical classification at the Genus and Phylum level (type) within the plants used to feed the larvae (Plant), the frass used to re-infect the larvae (Feces), and within the gut of larvae from four treatment groups. (C): non-treated, (A) fed with antibiotics, (AR) fed with antibiotics while transplanted with frass, or (R) fed with antibiotics before being transplanted with frass. This dataset also includes negative controls, from blank extractions, PCR and sequencing (negative\_control).



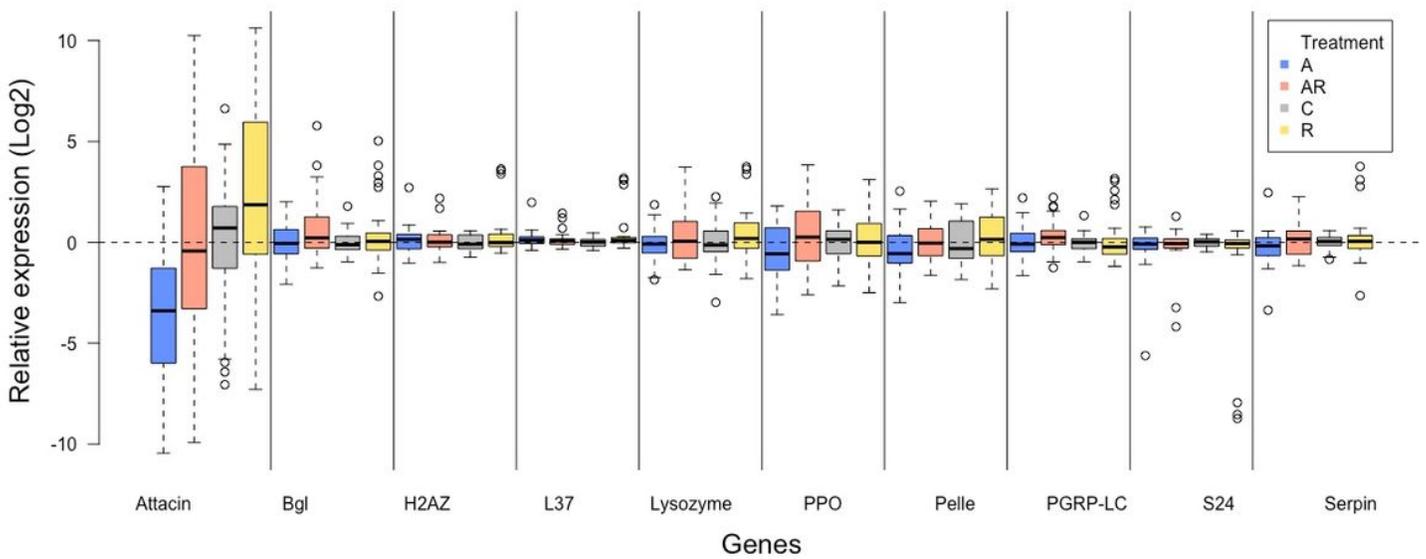
**Figure 4**

Distance based redundancy analysis (dbRDA) highlighting the impact of treatment on the bacterial communities in the larvae of the Glanville fritillary butterfly. The dbRDA was performed with the Bray-Curtis distances reflecting the differences among the bacterial community of the samples. The treatments were used to constrain the analysis, which was also corrected for the family effect. (A.) The samples treatments corresponded to larvae that were either non-treated (SampleC), fed with antibiotics (SampleA), fed with antibiotics while re-infected with frass (SampleAR), or fed with antibiotics before being reinfected with frass (SampleR). (B.) The OTUs that correlated with the separation of the samples are plotted on the same axis. The Otu001 corresponds to sequences that belonged to bacteria from the Firmicutes phylum that could not be classified to any downstream taxonomical level.



**Figure 5**

Effects of manipulation of the gut bacterial community through antibiotic treatments on the (A.) development rate to L2 (days), and (B.) survival rate to L3 in the Glanville fritillary larvae. Average values from three larvae from 13 families reared under four different treatments (Blue  $\square$ ): antibiotic-treated, (Orange  $\Delta$ ): antibiotic-treated even during re-infection, (Gray  $+$ ): control larvae, and (Yellow  $\times$ ): antibiotic-treated followed by re-infection by frass from L7 larvae of the same families.



**Figure 6**

Effects of microbial depletion through antibiotic treatment on the expression levels of seven immune genes in pre-diapause larvae of the Glanville fritillary butterfly. Data include larvae from 12 families under four different treatments (A-Blue): antibiotic-treated, (AR-Orange): antibiotic-treated even during re-infected, (R-Yellow): antibiotic-treated followed by re-infection by L7 larval frass, and (C-Gray): controls. Expression levels of the three house-keeping genes (H2AZ, L37 & S24) are also shown.

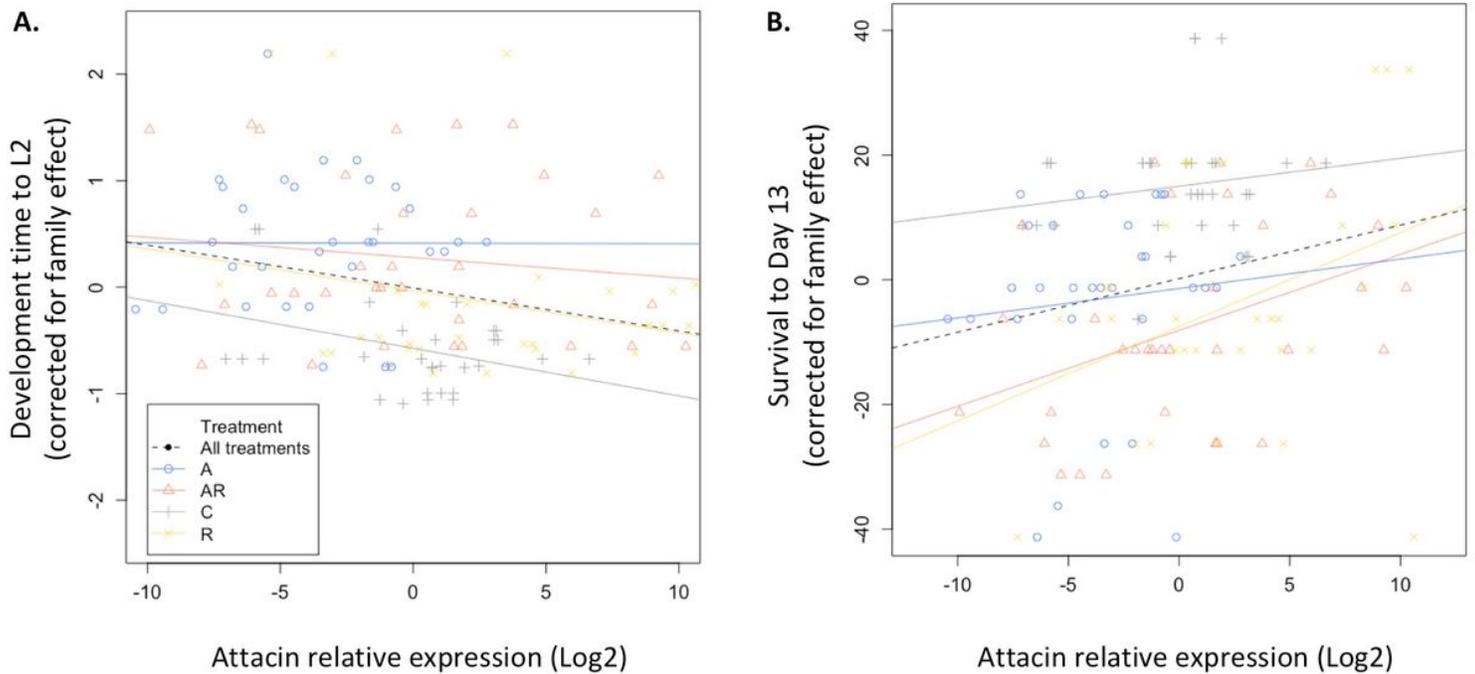


Figure 7

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [AdditionalFile1SupplementaryMaterial.docx](#)