

Dear Konstantinos Kostas Kormas,

Thank you for your decision on our manuscript entitled “Microbiome turnover during offspring development varies with maternal care, but not moult, in a hemimetabolous insect”.

We have carefully considered the reviewers' comments and made all the changes they suggested. In particular, we have edited the manuscript (1) to clarify the experimental design by separating the two experiments and (2) to add supplemental information regarding the behaviour of our model among other and other limits or our methods. We have also addressed all other minor concerns regarding the syntaxes.

Overall, we believe that the new version of the manuscript is now much clearer and stronger. We would like to thank you and the two reviewers, Guillaume Minard and Enric Frago, for the time you have spent on our manuscript and for the insightful comments and suggestions.

A detailed point-by-point response to the comments is provided below. Changes made to the manuscript are shown in yellow.

Sincerely,
Marie-Charlotte Cheutin, on the behalf of all co-authors.

REVIEWER 1 - Guillame Minard

The manuscript synthesizes a study that refers to the effect of development, moult and maternal care on the bacterial microbiota associated with an Hemimetabolous species (*i.e.*/ the European earwigs). All in all, I found the manuscript very well written. The investigations are relevant in the light of the current litterature in ecology. Indeed, the effect of development on the microbiota of hemimetabolous insects has poorly been regarded and the exact same remark goes for maternal care. The experiments and data analyses have been seriously performed. This leads me to recommend only minor edits that are listed in detail in the attached pdf document. Download the review

https://microbiol.peercommunityin.org/download/t_reviews.review_pdf.9d0cee47b5815ddc.417574686f7227732050726f6f665f72657669657765642e706466.pdf

Thank you very much for reviewing our manuscript and for the nice feedback on our study. We appreciated your comments and have edited our manuscript according to your remarks.

Line 35: In some cases, such interactions poorly impact the life history traits of insects. Maybe this can be mentioned as well. <https://doi-org.inee.bib.cnrs.fr/10.1093/femsle/fnz117>

We agree that exceptions occur (such as in caterpillars). As we aimed to understand the potential (even predicted) role of its microbiome, we oriented the introduction toward the beneficial role of the microorganisms associated with insects. However, as we have some evidence that the microbiome associated with *F. auricularia* does not play a major role in this species, we have edited the discussion to develop the exceptions that occur in some insects that don't seem to need a microbiome. (L683-688): “Here again, these predicted functions need to be taken with caution, especially as a recent study demonstrated that the alteration of the microbiome does not affect mother earwigs (Van Meyel *et al.*, 2021) and that its natural variation does not explain its aggregation behaviour (Cheutin *et al.*, 2024), calling into question the need to have a microbiome (Hammer *et al.*, 2017, 2019).”

Line 36: maybe "those" or "such associations" since this is a combination of associations with several microorganisms.

Thank you for the suggestion. We have changed "this association" by "such associations" (L37).

Line 63: Maybe this can be developed a little bit more. The most common vertical paths are indeed intracellular transmission of symbionts within the eggs but also transmission through egg smearing and other funny transmissions path such as capsules that fed larvae have been described in the literature. I forget the name of the review but I think that Takema Fukatsu synthesized it in a paper a decade ago.

Thank you for the reference. As we have already given some examples of vertical transmission occurring after oviposition on line 68, we have edited this section to be more exhaustive and include examples provided by the reviewer. We have also changed the text to clarify the distinction between horizontal and vertical transmission, as suggested by reviewer 2.

(L68-79) "For instance, mothers can deposit microorganisms directly on the eggshell of their future juveniles or produce symbiont capsules that they place next to the eggs for future ingestion by newly hatched juveniles, as reported in stinkbugs of the families Pentomatidae and Scutelleridae (Fukatsu and Hosokawa, 2002; Kikuchi *et al.*, 2008; Hosokawa *et al.*, 2013). After hatching, other mechanisms of vertical and horizontal transmission can also occur by social interactions between family members, such as trophallaxis through mouth-to-mouth or mouth-to-anus contact (Powell *et al.*, 2014; Zhukova *et al.*, 2017) or allo-coprophyagy through consumption of parental feces (vertical) and/or sibling feces (horizontal) (Lombardo, 2008; Onchuru *et al.*, 2018). Access to maternal care and family life can thus ensure the acquisition and reacquisition of beneficial microbes by moulting juveniles, thus possibly strengthening the stability and evolutionary trajectory of symbiotic associations."

Line 117: 16S rRNA metabarcoding will only give you access to the prokaryotic microbiota composition but unfortunately not the whole microbiome (since you will miss eukaryotic microbes). Please, rephrase the sentence.

We apologise for the vagueness of the term we used. By "whole" we meant the entire body (not just the gut). We have edited the text to clarify the meaning. We have also introduced the term "core microbiome" to follow reviewer 2's suggestion and emphasize that we focused our analyses on the filtered sequences that were not randomly distributed.

(L131-136): "We then used 16S rRNA metabarcoding to analyse the prokaryotic fraction of their core microbiome, *i.e.*, the sequences non-randomly distributed across all datasets (see details below). The second experiment tested whether and how the presence of the mother affected the microbiome of her first instar nymphs and resulting adult offspring. To do this, we reared five additional earwig families following the same protocol, but where the mother was removed from the clutch shortly after the eggs hatched."

We have also edited the abstract to clarify: "We reared European earwig juveniles with or without mothers and used 16S rRNA metabarcoding to analyse the prokaryotic

fraction of the core microbiome of eggs, recently and old moulted individuals at four developmental stages and the resulting adults.” (L15-16)

Figure 1 : I understand that the letter O and Y goes for old and young as well as M and F goes for females and males but since those names are used later on in other figures without it being defined in their captions, I would either (i) add its meaning of those names in this figure or (ii) define it in every captions.

Sorry for the omission of the label’s description. As we used to mention these labels throughout the manuscript, we did not edit the figure itself but we clarified the labels Y, O, F and M in the legends as such:

(L203-210) **Figure 1: Overview of the experimental design.** In Experiment 1, we sampled each developmental stage from egg to adult for microbiome analysis. We sampled L1 to L4 nymphs both just after they had moulted to the new stage (grey - Y for young nymphs) and just before they moulted to the next stage (coloured - O for old nymphs). We also sampled female (Adult-F) and male (Adult-M) adults. This gave us 150 samples across all developmental stages in families with mothers during early nymph development. In Experiment 2, we sampled old first instar nymphs and adult males and females. This gave us 58 samples in families without mothers.”

In addition, as the text with the description of the experimental design appeared after the figure, we took the liberty to move the Figure 1 at the end of the section L202 for a better understanding.

We also edited the legends of the Figure 3, to specify L411 “between females (Adult-F) and males (Adult-M)”

Line 182: It was a little bit difficult for me to follow this part (even with the figure). I would recommend the authors to simplify it by adding a table that recapitulates the lifestages, treatments, families and number of samples collected and analysed for each combinations of factor. I can see that the authors mentioned a table in supplementary data that may fit my recommendations but I could not see the table (since PCI only sent me a link to the main document). Anyways, I would add such table to the main document if possible.

As the table represents more than a page, we decided to provide the sampling size in the Figure 1. The supplementary table is now available on Zenodo DOI: 10.5281/zenodo.10776543.

Line 271: This is also a beta diversity measure. I would rephrase this sentence since it might not be clear to someone who is not familiar with such analyses.

Sorry for the shortcut. To be clearer, we have edited the sentence to add “quantitative (weighted) beta diversity distances” at the end L290.

Line 295: For the richness, you may prefer GLMM with a Poisson or Negative Binomial distribution since the explanatory variable is not continuous.

This is a very good suggestion, thank you. We totally agree and edited the methods to perform a GLMM with a Negative Binomial distribution on the observed richness. This does not qualitatively change our results. The results part and the supplemental figure have been modified according to the new statistical approach. Specifically, L315-318: "As observed richness can be considered as counts, we performed a negative binomial mixed model instead of mixed linear models that were used for the three other alpha diversity proxies."

The Tables 1 and 3, with the Figure S4A are edited according to the new results.

Figure 2 : I would probably also add the maternal care effect in this figure.

The text that represent the label of each sample is in small font that cannot be read. I would remove it if not necessary to the understanding of the figure.

We agree on clarifying the figure by added the orphans and by removing the sample labels from the polar plot. As we added the maternal effect on the Figure 2, we also changed the legend by adding L368-374 "**Figure 2: Composition of the European earwig core microbiome. Data from Experiments 1 and 2.** Individual core microbiome at family scale, ordered by relative importance, grouped, and coloured by bacterial family. Specimens are ordered according to their developmental stage with freshly moulted young nymphs (grey) on one side of the circle, and old nymphs (black) on the other side, with adult females (Adult-F) and males (Adult-M) separated. The orphan specimens related with the first instar and adult stages are indicated with dotted circles."

Line 401: Maybe this should be rephrased e.g. "Accordingly to what was reported for...", "In the same way..."

Thank you for the suggestion. We have rephrased the beginning of the sentence as proposed L425-426: "Accordingly to what was reported for alpha diversity, the beta diversity of the earwig gut microbiome changed as the offspring developed."

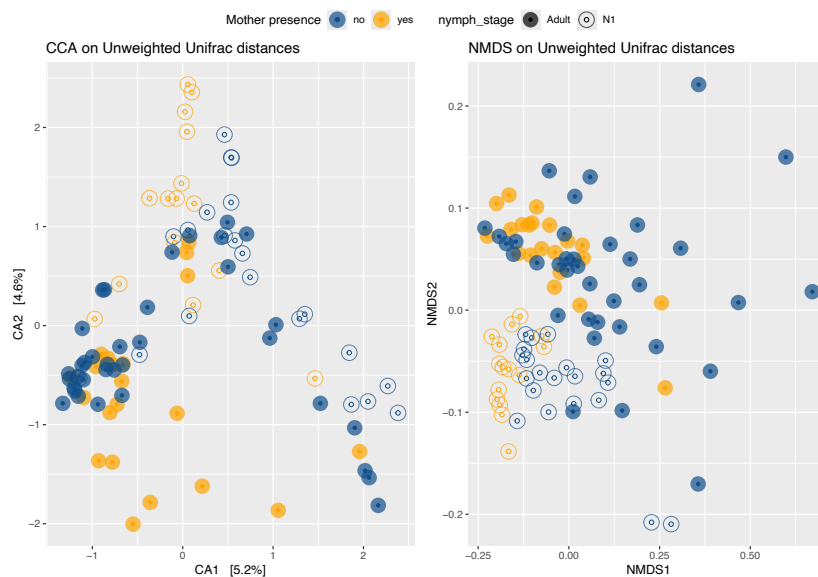
Line 449: In the previous sentence you reported Bray-Curtis distances. Therefore, this was a little bit confusing to me. If you observed differences for several betadiversity measurements I would report them all. Also this may be interesting since you mentioned that there was no differences for alphadiversity metrics that did not depend on the bacterial phylogeny.

We have added the results associated with all the distances. We have also edited the sentence to mention that the effect of maternal care on the total variance explained is limited, which was also supported by the weak clustering effect on the ordination while the effect was still significant. (L471-474): "In terms of beta diversity, maternal presence affected the structure of the bacterial communities (for all metrics, $0.009 \geq P \geq 0.001$) (Figure 5B, C; Figure S7; Table 1), although it only explained a limited proportion of the total variance (for all metrics, $0.074 \geq R^2 \geq 0.023$)."

Line 459 : The difference between blue and yellow dots is not obvious from this graph. Is it possible that separations appear in other PCo axes? Alternatively, does it differ when the

variation is constrained to two dimensions with an NMDS ? If the answer is "yes", then maybe this representation is more appropriate.

Even using CCA or NMDS methods, colors are hardly distinguishable into clusters (see below). This is because data are overall explained by the stage effect (and so points are overall grouped into full versus empty circles). However, it was our choice to imply both stage and mother presence effects in the same plot as it is the visualization of our model. Having said that, the results of the PERMANOVAs are quite persistent with our representation as R^2 associated with the mother presence variable does not exceed 7,4% (whatever the metric) of the total variance that corresponds to a slight effect that can be hardly seen into an ordination while it is still significant. As such, we thought that mentioning these values in the results should be pertinent (see previous comment).



Line 515 : In the discussion I would use the term "bacterial microbiome" or "prokaryotic microbiome" instead of "microbiome". This is totally ok to study the bacterial microbiome (I have been doing that a lot) but some studies include both eukaryotic and prokaryotic metabarcoding so it is better to explicitly mention which community is tested.

We agree with the reviewer. We have changed "microbiome" to "bacterial microbiome" throughout the manuscript (Lines 531,536, 538,541, 542, 550, 559, 584, 597, 598, 622, 653, 657, 660, 666, 692, 702) and added "prokaryotic" L15 and L24

Line 585 : Just a comment that is apart from my reviewer role :

I do not know whether this has ever been studied or not but if those microbes are also involved in cuticle digestion that would be an awesome thing to test from an evolutionary perspective (since cuticle production is costly and digestion of polymers like chitin involves a large diversity of enzyme) :)

New perspectives are always very welcome! Indeed, understanding the evolution of the associated chitin-degrading bacteria and their chitinases could be a good question. It is

well documented in marine systems, as it is one of the most abundant polysaccharides, but we have to admit that it beyond the scope of the current manuscript.

Having said that, we are accumulating evidence to support the idea that the European earwig does not really need a microbiome. The origin and the role of the chitin-degrading bacteria we found (among others) will need to be followed up by functional approaches, even though these bacteria might probably be acquired from the environment.

Line 658: Maybe somewhere in the discussion, I would mention the compositional nature of metabarcoding data that may also bias a little bit some interpretations. This is currently a hot topic with this technic and some studies now involve qPCR to tackle this issue. However, I do not want to ask the authors to perform qPCR since this is rarely done but mentioning it would be enough to inform the reader that some changes in the betadiversity may also be due to variations in absolute abundances of some specific taxa.

Here is an example of a review that refers to that issue in case the authors are not familiar with it : <https://www.frontiersin.org/journals/microbiology/articles/10.3389/fmicb.2017.02224/full>

This is a relevant point, thank you. We developed the section with the metabarcoding biases coupled with the limits of the predicted functions L643-651: “Although these predicted functions may provide insights into our general understanding of the driver of microbiome changes during offspring development, they must be considered with caution. Indeed, metabarcoding, as a qualitative approach might be biased by the compositional nature of the data and must be validated by quantitative approach (Gloor *et al.*, 2017). In addition, the further functional predictions should be confirmed by transcriptomic analyses as the approach is debatable due to the short length of the amplicons and the lack of genome reference concerning insect-associated microbial communities (Djemiel *et al.*, 2022).”

Line 673: I do not think that references should be add in the conclusion. Maybe this is something specific to this journal but if this is not, then I would remove it and send those references to the discussion section.

As these references were already mentioned in the main text, we removed the references from the conclusion section as suggested.

REVIEWER 2 – Enric Frago

In this study Marie-Charlotte Cheutin, Manon Boucicot and Joël Meunier explore the dynamic changes in microbiota during development of an insect that has the peculiarity of having maternal care over young instars. The authors rear a group of earwigs from egg to adulthood and screen bacterial associates in different instars, and in two groups: one with and another without maternal care. Individuals not exposed to maternal care also reach adulthood as care by mothers is not necessary for the insect to reach adulthood. In addition to following the dynamics of the microbiota, the authors also apply an algorithm to detect microbes likely to be the core microbiota in this species, and they also associate the different bacteria found to putative functions. The manuscript is well written and timely, the topic of microbiota development in insects that do not perform a complex metamorphosis, and that show maternal care is important, and has been poorly studied.

Despite these merits, I think there are few elements of this manuscript that could be changed to make it more appealing to readers.

Thank you for your supportive feedback and for the constructive comments. We have made the suggested changes to improve the attractiveness of our study for readers.

My main criticism is on the way results are structured and presented. I think that this study presents two different experiments, but such separation is not clear to me. The first experiment explores how microbiota changes through insect development, the second (and more interesting to me) how maternal care influences such development. These two parts should be clearly separated even if some insects are used in both parts. In the first experiment, microbiota development is assessed by screening bacteria present in all insect stages, whereas in the maternal care experiment only newborn insects and adults are screened. Newborn and adult insects in the first experiment can thus be used as controls in the second one. Parts where this distinction was particularly unclear to me is in the last paragraph of the introduction section and in figure 1 where the experimental design is presented. Maybe the most confusing part was when the authors mention that 20 groups of insects were used and that only 5 were exposed to maternal care. At that point I wondered about such an unbalanced design.

Sorry for the lack of clarity. First, we have edited the last paragraph of the introduction to emphasize that we have done two experiments: (L123-136) “We conducted two experiments in which we analysed the prokaryotic microbiome of 20 families of the European earwig *F. auricularia*. The first experiment tested whether and how their microbial community changes during development. We reared fifteen earwig families to adulthood using a standard protocol in which mothers remained with the clutch until 14 days after hatching, i.e. for the normal duration of family life. During this time, we sampled offspring from each developmental stage (from eggs to adult male and female offspring) at both the beginning and end of each developmental stage. We then used 16S rRNA metabarcoding to analyse the prokaryotic fraction of their core microbiome, i.e., the sequences non-randomly distributed across all datasets (see details below). The second experiment tested whether and how the presence of the mother affected the microbiome of her first instar nymphs and resulting adult offspring. To do this, we reared five additional earwig families following the same protocol, but where the mother was removed from the clutch shortly after the eggs hatched.”

Second, we have edited the legend of Figure 1 to clarify the number of family used and the fact that two experiments have been done: (L203-210) “**Figure 1: Overview of the experimental design.** In Experiment 1, we sampled each developmental stage from egg to adult for microbiome analysis. We sampled L1 to L4 nymphs both just after they had moulted to the new stage (grey - Y for young nymphs) and just before they moulted to the next stage (coloured - O for old nymphs). We also sampled female (Adult-F) and male (Adult-M) adults. This gave us 150 samples across all developmental stages in families with mothers during early nymph development. In Experiment 2, we sampled old first instar nymphs and adult males and females. This gave us 58 samples in families without mothers.”

Finally, we have edited the subtitle of the different parts of the results to clarify to which experiment they were referring to (L357; 368-369;465 and 495). Overall, we hope that these changes will help readers to clarify the different parts of our experimental design and the rationale for the apparent unbalanced design.

There are a few key results and analyses that are not clearly introduced. As a reader we discover these results and analyses once we reach the results section, but a clear statement of why they are important is missing in the introductory section. These include the analysis of core microbiome elements, and the study of the microbiota of young and old individuals within the same instar. Even if I see the value of these approaches, I believe they should be introduced earlier in the manuscript.

We have edited the last paragraph of the introduction to clarify what we did and to what extent it was linked to the different research questions (see previous comment regarding the changes L123-136).

In the introduction section, I found the dichotomy between vertical and horizontal transmission unclear. The authors assume that environmental acquisition is always horizontal, but there are many instances in which parents deliver symbionts externally that are subsequently acquired by their offspring. For example, in L69 the authors state that "...juveniles can acquire these symbionts soon after hatching by ingesting their parents' feces". Wouldn't that be environmental and at the same time vertical transmission?

This is a good point. We agree that coprophagy can be a mixed mode of transmission because it might imply fecal transmission from the parents – in our model, the mother (vertical) but also from other group-members (horizontal thus). In our case, we strictly focus on the transmission from the parents (thus vertical). As suggested by the reviewer 1, we have edited this section by adding more concrete examples with distinction between horizontal and vertical modes L68-79 "For instance, mothers can deposit microorganisms directly on the eggshell of their future juveniles or produce symbiont capsules that they place next to the eggs for future ingestion by newly hatched juveniles, as reported in stinkbugs of the families Pentomatidae and Scutelleridae (Fukatsu & Hosokawa, 2002; Hosokawa et al., 2013; Kikuchi et al., 2008). After hatching, other mechanisms of vertical and horizontal transmission can also occur by social interactions between family members, such as trophallaxis through mouth-to-mouth or mouth-to-anus contact (Powell et al., 2014; Zhukova et al., 2017) or allo-coprophagy through consumption of parental feces (vertical) and/or sibling feces (horizontal) (Lombardo, 2008; Onchuru et al., 2018). Access to maternal care and family life can thus ensure the acquisition and reacquisition of beneficial microbes by moulting juveniles, thus possibly strengthening the stability and evolutionary trajectory of symbiotic associations."

I think that the role of parental care in the species studied should be detailed more precisely. What do mothers do to eggs and young instars? They may protect them against predators, but also clean them from pathogens. I missed a basic assessment of fitness consequences once insects were prevented from maternal care. Also, I think that pathogens should be mentioned at some point in this manuscript. It is quite likely that what maternal care does is to remove pathogenic species.

We have edited the text to add this missing information: (L103-118) "In this species, females oviposit in individual burrows in early winter (Meunier et al., 2012; J. Tourneur & Meunier, 2020) after which they stop their foraging activity and provide extensive forms of care to their eggs. For instance, mothers fiercely protect their eggs from predators (Trumbo, 2012; Wong & Kölliker, 2012), move their clutches when faced with extreme temperature changes (J.-C. Tourneur et al., 2022), and frequently groom their eggs to remove fungal spores and deposit cuticular hydrocarbons to protect them from desiccation (Boos et al., 2014; Diehl & Meunier, 2018). About 50 days later, the eggs

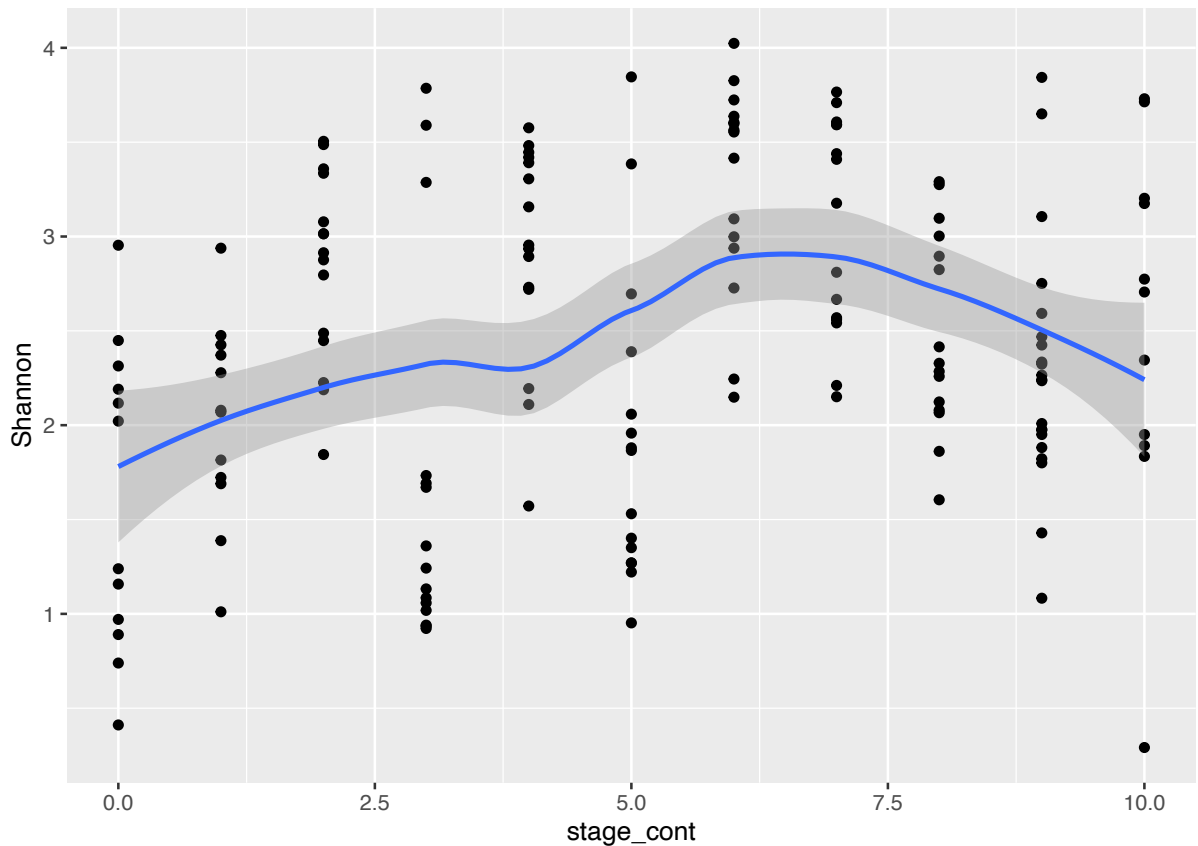
hatch and the mothers stay with their new juveniles for about two more weeks. During this time, they continue to provide care to their nymphs, such as allo-grooming and food provisioning (Kölliker, 2007; Lamb, 1976). Interestingly, maternal presence is not required after hatching, as nymphs can develop and survive without contact with a mother (Kölliker, 2007; Kramer et al., 2015; Thesing et al., 2015). The family naturally splits shortly after the nymphs have moulted for the second time (the first moult occurs at the time of hatching), and the nymphs then moult three more times before reaching adulthood two months later (Thesing et al., 2015; Tourneur et al., 2020)."

Changes in alpha and beta diversity during offspring development. I think there is room for a better exploration of this quite exciting data. Pairwise comparisons are difficult to grasp in this example because there are many groups (particularly in Figure 3A). An alternative solution to analyse this data would be to transform the larval stage into a continuous numeric variable [from 0 (egg) to 5 (adult)] and include as a factor whether the insect was a young or an old individual (pre and post moult). This would allow for a formal test of diversity changes with time and status.

This is an interesting suggestion. We have thought hard about the "best" way to analyse our data. We agree that an intuitive option would have been to run a statistical model with stage, age and the interaction between the two as explanatory variables. However, this would have forced us to omit a large part of our data set: data on eggs (as we do not have them for young and old eggs) and data on adults, as we have males and females and only one age for this developmental stage. Another approach would have been to pool certain parts of the dataset to gain homogeneity, but the choice of what to pool would have been arbitrary and therefore likely to be of little relevance. Therefore, given the non-homogeneity of the sampling with both eggs and adults, and the main question of our study (whether and how the microbiota changes during development), we believe that the pairwise approach we used is the most accurate, meaningful and statistically valid way to analyse our data.

Even if changes through development are not lineal, at least plotting changes with a scatter chart with a smoothed line separating pre and post moult individuals would help visualising trends. To my taste this may help support several statements in the results section that suggest, for example, that microbial diversity increases with development.

We have produced this figure (see below), but we believe that it makes the whole figure more difficult to read and understand than the original figure. For this reason, we would like to keep the original figure in the main text.



I wonder if the information provided in Figure 3B and 4 are not redundant. In the first plot beta diversity differences are represented in a PCA and in the second with a clustered heatmap. I am aware that in the second figure only 62 genera indicators of developmental stages were selected, but it is not clear to me why samples were discriminated using a PCA technique in one section and with a heatmap in the other.

We wanted to understand whether the bacterial (or functional) communities as a whole were different between stages using the PCoA (Figure 3B). As we found significant results, we then asked which bacteria (or predicted functions) explained these results through the heatmap. We agree that the cluster below the heatmap can be redundant in the message, but overall we wanted to show which bacteria were likely to be associated with each developmental stage.

Some minor comments follow:

In L67 the authors consider that "mothers can deposit external secretions containing symbionts on the eggshell" as parental care, but I am not sure I agree.

We gently disagree on this comment. The deposition of external secretions with symbionts on the eggshell fits within the classic definition of parental care: "parental care is any parental trait that enhances the fitness of a parent's offspring, and that is likely to have originated and/or is currently maintained for this function" (Smiseth, Per T, Mathias Kölliker, et Nick J Royle. « What is parental care? » In The evolution of parental care, édité par Nick J Royle, Per T Smiseth, et Mathias Kölliker, 1-17. Oxford: Oxford University Press, 2012.)

L88. Please divide this sentence into 2.

We have followed this suggestion: L93-98 “However, it is not clear whether this stability is universal across species. Importantly, more information is needed to determine whether this stability is due either to the non-purging effect of moulting on the microbial community, to the host microbial niche not changing during development and therefore selecting for the same microbial community, and/or to maternal care ensuring maintenance of the microbial community through vertical transmission.”

L112. Please provide a scientific name.

We have added the scientific name “We conducted two experiments in which we analysed the prokaryotic microbiome of 20 families of the European earwig *F. auricularia*.” (L123-124)

L149. Was moist sand sterilised? It can be a source of microbes

The sand was not sterilized and our laboratory conditions were not sterile (food was not autoclaved, petri dishes were not sterile). We cannot exclude the possibility that some bacteria came from the rearing environment (soil but also food), assuming that they could enter and develop in the tested earwigs. To clarify this point, we have edited the discussion: “Since these bacteria are often generalists, associated with laboratory rearing conditions (Malacrino, 2022), and common in the mothers tested, they are likely to come from the non-sterile rearing environment.” (L671-674)