**JoVE59270**

**Author’s response Letter to the editor and reviewers**

We want to thank you sincerely for your detailed comments and suggestion which we included in our work. We feel that it significantly improved our manuscript.

**Editorial comments:**

**1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.**

**Comment:** Thank you for the advice to thoroughly proofread our manuscript to avoid spelling or grammar problems.

**2. Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”**

**Comment:** Thank you for this suggestion we rephrased the Summary to describe the protocol and the method applications.

**3. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).**

**Comment:** Thanks for the comment. We exchanged centrifuge speeds from rpm to xg.

**4. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).**

**Comment:** Thank you for this suggestion we rephrased the protocol to avoid any personal pronouns.

**5. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.**

**Comment:** Thank you for this advice. We revised the protocol and used the imperative tense throughout the protocol. In rare cases we included a “note” when the imperative tense could not be used. Further we moved discussion into the discussion section.

**6. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Oxoid AnaeroGen, TRI reagent, RNasin, Nanodrop, Phusion, Qubit, QuantiTect, QuantiFast SYBR, etc.**

**Comment:** Thank you for this comment since we have not been aware of the commercial language. We revised the protocol and used generic terms instead. Sometimes we mentioned as advised “see table of materials”.

**7. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.**

**Comment:** We agree to add more details to the protocol steps. (see point 8-13.)

**8. 4.1: Please describe how to homogenize the larvae and specify the incubation temperature.**

**Comment:** We added more details to this essential step of the protocol. We provided more details for how the homogenization needs to be performed and mentioned the incubation time.

**9. 4.3: Is the pellet discarded? Is 500 µL isopropanol added to the new tube containing the upper layer? Please specify.**

**Comment:** Yes, the pellet is discarded and the isopropanol is added to the upper layer. We modified the step to contain more details.

**10. 5.1: Please specify PCR conditions.**

**Comment:** We specified the PCR recipe and the program. Further we changed 5.1 into 5.1.1since there were more details and we still want the protocol to be clearly structured.

**11. 5.2, 5.3, 5.5, 5.7, 6.2, 6.3: Please add more details to these steps. This step does not have enough detail to replicate as currently written. Alternatively, add references to published material specifying how to perform the protocol action.**

**Comment:** We added more details to these steps and changed 5.2 into 5.1.2-5.1.5, 5.3 into 5.1.6-5.1.7, 5.5 into 5.2.1, 5.7 into 5.3 (5.3.1 and 5.3.2), and6.2 into 4.9, 6.3 was left without renaming it. The renaming of the points should help to keep the clearly structure of the protocol.

**12. Please ensure that conditions and primers are listed all PCR procedures.**

**Comment:** We added more details and described the PCR recipe and the program.

**13. 6.1: Please describe how to assess the primer efficiency.**

**Comment:** We added more details to the primer efficiency assessment and devided 6.1 into 6.1 and 6.2. We provided essential information to calculate the primer efficiency.

**14. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.**

**Comment:** After having introduced all the suggested and advised changes to the protocol we highlighted the essential steps of the protocol for the video. The highlights include almost 2 pages.

**15. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.**

**Comment:** We assured to highlight the complete sentences.

**16. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.**

**Comment:** We followed this advice and highlighted the details to perform the highlighted steps.

**17. Figure 2: Please change “100 6” to “106” (i.e., delete 0 and the space between).**

**Comment:** We followed this advice and performed the suggested changes.

**18. Figure 3: Please describe the asterisk symbols in the figure legend.**

**Comment:** We agree to explain the asterix symbol. Therefore we added the descriptions in the figure legends.

**19. Discussion: As we are a methods journal, please also discuss critical steps within the protocol, any modifications and troubleshooting of the technique, and any limitations of the technique.**

**Comment:** Thank you for this comment we discussed critical steps like rearing in thediscussion section. Further we discussed limitation like the lack of gut-specific RNA expression analysis.

**20. References: Please do not abbreviate journal titles.**

**Comment:** We followed this advice and performed the suggested changes

**21. Table of Materials: Please sort the items in alphabetical order according to the name of material/equipment.**

**Comment:** Thanks for the comment. We sorted the items in alphabetical order and included more necessary material/equipment since we noticed that they were missing.

**Reviewer 1:**

**Abstract: Improve and revise. Firstly, I advise to describe the focus of paper and the relation between invertebrate and vertebrate, link the importance of reduce of the number of vertebrate as the authors have already done. Finally, describe briefly the importance of intestinal microbiota and explain the validity of G. mellonella model in this contest and a final consideration about the obtained data**.

**Comment:** Thank you for the advice to revise the abstract.

**Protocol: line 119 : replace rpm with xg**

**Comment:** Thanks for the comment. We changed rpm for xg.

**Why the authors did not extract only the gut of the larvae? the procedure is quite simple.**

**Comment:** We agree that the procedure of extracting the gut from the larvae is not difficult, and we performed this in a previous project but the expression analysis of midgut tissue was not conclusive. Since AMPs can be produced in several tissue types we chose to analyze “global” expression. That is why we use the RNA of whole larval individuals for RNA expression analysis. In our manuscript we mentioned this point already in the discussion section: “During the establishment of the oral administration model and the study of immune response kinetics we found gene expression to be not locally expressed in the midgut.”

**Protocol/Representative results: line 132: Authors described that larvae were incubated between 1-6 hours; in line 229, authors suggested that 16s gene copy of both bacteria decreased within 24 hours. Is not clear if the incubation is between 1-6 hours or 1-24 hours? and if authors incubated larvae within 24 hours, are they sure that the administration of 10⁷ bacteria not killed larvae in this interval?**

**Comment:** We agree that these statements lead to confusion since we missed to mention the 24 h incubation in the protocol. That is why we corrected the incubation times in the protocol into “1-24 h”.

Further, we are sure that the larvae were still alive after 24 h since the RNA was only extracted from living *G. mellonella*. We added this information “living” in the protocol (4.1). After 24 h the oral-administered larvae were still light without any melanization symptoms. The administered bacteria are not pathogenic and lack any virulence factors.

**Fig. 4 and 5: I advice to write one time the legend for these figures, the bacteria are only two. Define only one axis for "y", authors used different scale for the four graphs.**

**Comment:** We followed this advice and included the legend of Figure 4 and 5 only once to each of the figures. Further, we changed the axis to have the same scale.

**Caption of figure : "\*" explain**

**Comment:** We agree to explain the asterix. Therefore we added the explanations in the figure legends.

**Reviewer 2:**

**I suggest to cite more pioneering original papers in which Galleria mellonella has been established as a powerful model for bacterial human pathogens to illustrate its importance as an alternative model host.**

**Comment:** We followed this advice and cited more papers in the introduction to highlight the comparative character of *G. mellonella* and mice to support its alternative host model capabilities.

**I would add more information on the selected antimicrobial peptides of Galleria mellonella (properties, activities). Gallerimycin is an antifungal peptide whereas cecropin has been demonstrated to display activity against human pathogen bacteria.**

**Comment:** We thank for the advice to include more detailed information about the AMPs we selected, since this provide more helpful details for the reader. Therefore we introduced the following section into the introduction: **“**Generally, the AMPs have quite broad host specificity against Gram-positive and Gram-negative bacteria and fungi and have to provide a potent response since insects are lacking any adaptive response. Gloverins is an AMP which is active against bacteria and fungi and inhibits outer membrane formation. Moricins exhibit their antimicrobial function against Gram-positive and Gram-negative bacteria by penetrating the membrane and forming a pore. Cecropins provide activity against bacteria and fungi and permeabilize the membrane similarly like moricins. Gallerimycin is a defensin-like peptide with anti-fungal properties. Interestingly, it was found that the combination of cecropin and gallerimycin had a synergistic activity against E. coli.”

**Lane 27: replace homologous by similar because this term implicates a common ancestral origin.**

**Comment:** We followed this advice and changed “homologous” to “similar”.