A very well performed research on fish microbiomes on a rather understudied subject

This study investigated the gut microbiota of two medaka genetic lines to investigate wehther fishing pressure and/or environmental affect the development of the gut microbiota. Significant differences in the gut microbiota profiles were found between the two lines, and this effect seems to have been mediated by light intensity, but the host's fitness seemed unrelated to these microbiota changes. This part, host fitness in relation to the gut microbiota, could be explored in greater depth by the authors, with more targeted data analysis (no need for extra data or reanalysis of their raw sequencing data). Similar studies are not so common in the field, especially ones that paid extra attention to its experimental design and maticulate data analysis like this one. There are also several comments and suggestions provided by the three reviewers, which I also find accurate and righfully spotted, that could considerable increase the quality and the reading flow of the paper. Overall, I find this paper an important contribution in the field of fish-microbe interactions and co-evolutionary mechanisms which I believe it will be fully appreaciated by the scientists in the microbiome field when published.
*****Our response: We kindly thank Dr. Kormas for his positive comments on our manuscript. We have responded to all comments from the Reviewers and details are provided below.**

Reviews

Reviewed by anonymous reviewer, 11 Apr 2023 14:56

The authors of this study investigated the gut microbiota of two lines of medaka to explore possible fishing pressure and/or environmental effects on the development of the gut microbiota. They detected significant differences in the gut microbiome composition and richness between the two lines, and this effect was mediated by light intensity (represented nutrient availability). However, fitness was not correlated to changes in the gut microbiota.

Understanding the relationship between environmental conditions and fish gut microbiota diversity could reveal important mechanisms influencing the adaptability and resilience of wild fisheries stocks, thus leading to improved management strategies and more sustainable fish stocks.

With that being said, the approach used in this study (e.g., Illumina MiSeq short-amplicon sequencing) is adequate for revealing temporal changes at higher taxonomic levels. However, it lacks the resolution required for species identification, but more importantly, functional services offered by the gut microbiota. This comment has nothing against the methods used; it just prevents understanding how changes within the community structure impact provided functional services, which could impact fitness in a particular life stage. The authors, however, do point this out in their discussion.

Overall, the paper is well-written and nicely structured. There are only a few discussion points:

Introduction

Comment 1: 1) In addition to diet composition and water quality, what about links to deterministic or stochastic differences that might arise between different lines? This has at least been reported in other fish species, such as S. salar.

*****Our response: We agree that stochastic processes have been shown to explain variability in microbiome composition of the medaka, but this is not something we can test with our experiment design. However, we have added a new sentence in the Discussion: "The present study focused on the deterministic processes that influenced the gut microbiome composition of medaka, but it is important to note that stochastic processes may also explained substantial variability in the bacterial composition of medaka (Jones et al. 2022)." (l. 516-519).**

Jones E.W., Carlson J.M. & Ludington W.B. (2022). Stochastic microbiome assembly depends on context. *Proceedings of the National Academy of Science of the United States of America* 119 (7)e2115877119. https://doi.org/10.1073/pnas.211587711

Comment 2: 2) Lines 100-104 – move to methods

*****Our response: We truly think that the two lines should be presented in the Introduction to facilitate readability. This requires providing general information about the selection procedure. Therefore, we have refrained to move this section to the methods. However, as recommended by the Reviewer, we have included a new paragraph (l.115-134) that (1) provides information about the two lines and (2) compares these information to the literature (see our response to the following comment)**

Comment 3: 3) Lines 105-108 – turn this information into a paragraph, linking it to other studies

*****Our response: We have added a new paragraph: "In fisheries science, laboratory size-selection experiments are commonly used to mimic the evolutionary consequences of size-selective fishing, while controlling for phenotypic plasticity (Conover and Munch 2002; Uusi-Heikkilä et al. 2015). Recently, Renneville et al. (2020) performed a size-selection experiment using medaka (*Oryzias latipes*) as a model species. Native to East Asian countries, medaka is a small cyprinodont fish (adult length = 32 mm) that has a short generation time and is easily reared in the laboratory, making it an ideal species for selection experiments (Ruzzante and Doyle 1993, Renneville et al. 2020, Bouffet-Halle et al. 2021). The species is omnivorous with an animal-based diet preference but can also feed on diatoms and filamentous algae (Edeline et al. 2016). The size-selection procedure consisted of mimicking either fishing mortality where only small-bodied fish were allowed to reproduce (small-breeder SB line), or a more natural mortality regime favoring the reproduction of large-bodied fish (large-breeder LB line) (Reneville et al. 2020, Le Rouzic et al. 2020). As expected from the literature (Stearns 1992, Conover and Munch 2002), the LB and SB lines evolved opposite life-history traits and behaviors: small-breeder medaka grew slower, matured earlier and were less efficient foragers than the large-breeder medaka (Diaz Pauli et al.**

2019, Evangelista et al. 2021). The logical next step is to examine to what extent changes in the gut microbiota of LB and SB medaka are driven by the interaction between fisheries-induced changes in both evolution (life-history shift) and environmental conditions (reduced fish density and concomitant increased resource availability).” (l. 115-134)

Methods

***Comment 4:** Line 139-142 – starting with the "On average … " sentence, move to results

****Our response: These results (l. 188-191) refer to size/growth and maturity differences between the two lines under controlled laboratory conditions and were already published by Reneville et al (2020). Thus, these results are not directly related to the present pond experiment, and we have refrained to move them to the Result section of the manuscript.**

***Comment 5:** Line 144-148 – move to results

****Our response: This sentence indicates the number of individuals used in the pond experiment, as well as their initial size, which constitute crucial methodological information. We have decided to keep the sentence in the Method section but we have revprahsed it to improve clarity: “Specifically, for each line, 180 mature fish (initial standard body length: mean ± SD; SL_i in small-breeder = 18.9 mm ± 1.4; SL_i in large-breeder = 19.4 mm ± 1.4; ANOVA: $F_{1, 358} = 13.70$, $P < 0.001$) were selected to generate 24 experimental populations composed of individuals from the same line (48 populations in total), but from distinct families to limit inbreeding (mean kinship coefficient = 0.23 ± 0.1 and 0.17 ± 0.1 SE in LB and SB lines, respectively; further details available in Le Rouzic et al. 2020).” (l. 193-198).**

Comment 6: Line 195 – more information about how the tools were sterilized (e.g., 10% was used, followed by a sterile water wash).

****Our response: Tools were thoroughly washed using 96% laboratory grade ethanol. This information has been added to the revised version of the manuscript (l. 251).**

Comment 7: Line 227-230 – The final dataset ….. move to results

****Our response: While we have decided to keep relevant information for the subsequent statistical analyses (i.e. sampling size, sequence reads before standardization and standardization method; l. 294-298), we have added a new paragraph to the Result section: “After standardization of the data, we identified a total of 3,189,868 sequence reads (mean = 30,969 reads per sample) and 627 ASVs for 103 samples. As expected, dominant phylum of the medaka gut microbiome included Proteobacteria (60.5% and 59.2% of reads in LB and SB medaka, respectively), followed by Bacteroidetes (2.5 and 2.4%, respectively), Verrucomicrobia (2.0 and 3.2%, respectively) and Actinobacteria (2.2 and 3.9%, respectively). Bacterial communities were also characterized by large relative abundance of Cyanobacteria (29.2 and 28.8%, respectively), while Firmicutes represented only 0.7% and 0.5% of reads in LB and SB medaka, respectively (Fig. S2).” (l. 341-348).**

Results

Comment 8: Line 291…if you change the Additionally, to However, it will be easier for the reader to understand a comparison switch.

*****Our response: Done (l. 415)**

Discussion

Comment 9: Line 326: Would this prediction become true given more time or across different life stages?

*****Our response: It could become true if we assess juvenile growth because previous results indicated some differences (even though not significant) between LB and SB juvenile growth (Evangelista et al. 2021). We have slightly rephrased the sentence to: "However, contrary to our prediction, variation in microbiome diversity or composition was not associated with any of the measured growth-related traits of adults." (l. 458-459).**

Comment 10: Line 332-333: Aeromonas is a decent size genus, so it would be nice if you also mentioned beneficial species and expanded on the pathogenic ones.

*****Our response: We agreed, and we also think that the pathogenic or beneficial role of Aeromonas is likely context-dependent, as observed in many bacterial taxa. In addition, as we are not able to provide species-level identifications, we can only speculate about the functional role Aeromonas might play for medaka in this context. Therefore, to avoid speculation we have decided to remove this sentence from the revised version of the manuscript (l. 464-465). The rest of the paragraph has been revised accordingly (l. 466-470, 473).**

Comment 11: Line 335-336: LB medaka produced more offspring and grew faster – isn't this correlated to fitness? Fitness is about success at surviving and reproducing, so it appears that LB could have higher fitness.

*****Our response: True, LB medaka had a higher fitness than SB medaka. However, we did not find evidence of direct association between adult growth rate and microbiome composition (l. 428-431). LB medaka produced more offspring but this is not something we could related to the microbiome composition. Overall, this entire section has been revised to avoid confusion (see response to comment 10 above).**

Comment 12: Line 384: do you mean phenotypic plasticity or genomic plasticity?

*****Our response: We mean phenotypic plasticity, and the sentence has revised rephrased accordingly (l. 535).**

Reviewed by Marco Basili, 06 May 2023 18:59

The manuscript describes the experimental analysis performed in the gut microbiome of two different lineages of medaka, selected based on the size. The authors test the variation that occurred in microbiomes in different environmental conditions.

The topic analyzed is certainly of great interest, even if the species used is not among the most commonly studied in the bibliography. In terms of expository clarity, the title,

abstract, introduction, and final discussion appear sufficient to satisfy the journal parameters.

Comment 1: The authors fail to adequately describe the results obtained: the description of beta diversity is very hasty and lacks the part concerning the different densities of organisms tested. This result, although not significant, should be described in more detail, also associating it with the results obtained from the different exposure to light.
*****Our response: Results related to the light and density treatments on the gut microbiome have been removed from the Appendix and are now reported in the main document (l. 387-393; Fig.3). Please note that, as recommended by the Reviewer 3, we have re-run the PERMANOVA using weighted UniFrac distances. This has slightly changed the results related to the light intensity treatment, which did not affect the gut composition of medaka in the revised version of the manuscript.**

Comment 2: The host fitness part should be well explored in term of values and correlation with other factors.
*****Our response: To explore correlation between adult medaka's traits (i.e. somatic growth rate, body condition and standard length) and gut microbial composition, a new Canonical Correlation Analysis has been performed using the mixOmicx package (Rohart et al. 2017; l. 334-337). Results of the CCA have been added to the revised version of the manuscript (l. 432, Figure 5). We did not test the effect of treatments on medaka's fitness because this (1) was not the main objective of the present manuscript, (2) was already investigated (Evangelista et al. 2021).**

Comment 3: One of the main questions that occur reading the manuscript is in relation to the size of the population, wherein the methods have described the mean and SD of the two different lineages, showing the significative differences. Not having certainty about the actual differences of genetic background, I would suggest to the authors to show more details on the correlations between size and alpha diversity (also in terms of microbial composition of each individual), or even how size is distributed in relation to beta diversity.
*****Our response: We have included correlation tests between medaka body size and gut microbiome diversity, but these were not significant (adjusted P > 0.876; l. 332-333, 429-431, Fig.S3). In addition, a new Canonical Correlation Analysis (CCA) has been performed to explore relationships between medaka's traits and microbial composition but revealed no strong correlations (l. 432, Fig. 5).**

Comment 4: line 272: Desulfovibrionaceae were significantly more abundant in the gut of LB than SB medaka, while in the plot is oriented in the SB direction (even if is colored in orange).
*****Our response: Thank you for pointing out this issue on the figure. We contacted Dr. Chi Liu the maintainer of the microeco R Package and he explained that the odd visualization was due to the negative LDA scores for *Desulfovibrionaceae*. Usually, the bar plot is only used to show the features with high LDA scores. As recommended by Dr. Liu, we have re-run the analyses using alpha = 0.05 and LDA threshold = 2 (Fig. 2c).**

Comment 5: Figure 2c: In the barplot, the family labeled as "Family II" and "Family XI" should be better characterized with the name of the higher taxa level.
*****Our response: Done (Fig. 2b)**

Comment 6: line 700: Figure S4 caption, change the letter "d" with "c"
*****Our response: The typo has been corrected and the figure has been updated according to the new analyses (see comment about weighted and unweighted UniFrac distances) and moved to the main document (Fig. 3).**

Reviewed by Laetitia Wilkins, 21 May 2023 13:33

GENERAL

The manuscript entitled “Within-species variation in the gut microbiome of fish is driven by the interaction of light intensity and genetic background” written by C. Evangelista *et al*. is very well written. It is relatively short, concise, and easy to understand. Applying a very sophisticated mesocosm experiment, the authors tried to quantify the effects of evolution and environment on medaka gut microbiome composition. Said microbial composition was characterized using 16S amplicon sequencing of the V3-V4 region. Evolution was defined by creating two breeding lines where fish were selected for size during 10 generations. Environmental effects were experimentally varied by keeping fish at different abundance (low and high) and then keeping each of those groups at either low or normal light conditions. All in all, a very well-designed set-up that must have taken several months if not years to be developed and carried out.

TITLE/ABSTRACT/INTRODUCTION

Comment 1: The title reflects the content of the article. The word “fish” should be replaced by “medaka” because the results of this current study cannot be generalized for all fishes.
*****Our response: Done (l.1)**

Comment 2: The abstract presents the supported findings of the study. The first sentence of the abstract is too general in my viewpoint and could simply be removed. It ignores the large body of literature investigating the consequences of host genetic background and environmental conditions on gut microbiome composition.
*****Our response: Done (l. 27-28)**

Comment 3: Line 45: is a speculation. Functional importance of the gut microbiome was not investigated in the present study.
*****Our response: True, and the end of the sentence has been removed from the revised version of the manuscript (l. 45-46).**

Comment 4: Key words: “Medaka” could be added, “mesocosm”, “light”, and “fish density”

*****Our response: We have added "mesocosm", "light" and "fish density" as key words (l. 52). However, because "medaka" is now in the title, we have decided not to repeat it in the key word list.**

Comment 5: Line 84: It would be worth considering reading and including the following literature on human-induced selection on fish sizes in the introduction:
https://doi.org/10.1016/j.tree.2016.04.001
https://www.pnas.org/doi/abs/10.1073/pnas.0809235106
https://doi.org/10.1146/annurev-ecolsys-112414-054339
*****Our response: The citations of Darimont et al. 2009, Nusslé et al. 2012 and Heino et al. 2015 have been added here (l. 100) and are in the refence list (l. 630-632, 680-682, 724-726).**

Comment 6: The introduction is not well connected with the discussion section. I only fully understood the rationale of this manuscript after reading the discussion section.
*****Our response: We have revised the entire Introduction based on the Reviewers' comments and details are provided below.**

Comment 7: Lines 109-111: the experimental design is introduced pretty late and does not connect well to the first parts of the introduction.
- Size selected lineages: human impact through fishing is clear
- Why light? Unclear from introduction.
- Why also population density?

*****Our response: Change in fish density was used to reflect the demographic impact of fisheries, and change in light was used to modulate primary production (while avoiding too high growth of filamentous algae, l. 232). Overall, the two treatments were used as proxies of resource availability, as it can drive diet variation between omnivorous individuals and from there, the gut microbiome of fish (Talwar et al. 2018). These have been clarified in the Introduction (l. 39-40, 101-112, 132-134, 151-153).**

Comment 8: Is this microbiome manuscript maybe a side-project of a bigger project where it is laid out more clearly what the motivations behind the experiment were? Renneville *et al*. 2020 seems to be the main study.
*****Our response: The main objective of Renneville et al. (2020) was to examine the phenotypic responses of medaka to size-dependent harvesting performed under controlled laboratory conditions. To do so, they created two lines of medaka in which fish were imposed selection favoring large-sized individuals (LB line) or small-sized individuals (SB line). In the present manuscript, we used SB and LB medaka in an outdoor pond experiment to assess variation in the gut microbiome of these medaka exposed to different environmental conditions. We have reframed the Introduction to clarify these points as well as the distinction between the two studies (l. 115-134).**

Comment 9: Please build some components into the current introduction where you introduce why you would expect light and density to affect the fish gut microbiome composition.

*****Our response: See response to comment 7.**

Comment 10: When reading the methods section, it becomes clear that this study is its own experiment. This was not clear from the introduction.
*****Our response: We hope that the new Introduction has improved the clarity of the manuscript (see responses to comments 5-9).**

MATERIALS AND METHODS

The methods and analysis are described in sufficient detail to allow replication by other researchers. I listed a few places where I would need more detail:

Comment 11: Line 125: how many tank replicates?
*****Our response: There were 20 tanks per line per generation. This has been clarified in the revised version of the manuscript (l. 170).**

Comment 12: Line 131: how many individuals were removed and how often?
*****Our response: At each generation, selection was performed on 212 fish per line on average, and the selection procedure resulted in removing on average 88% of individuals per line. These details are now provided in the line 181 of the revised version of the manuscript.**

Comment 13: Line 146: how were families (sib-families == genotypes) kept separate? How were tank effects separated from family effects? Were fish genotyped?
*****Our response: To keep track of the pedigree throughout the selection experiment, offspring from different breeding pairs were never mixed in the same tank. Thus, the tank and family effects could not be separated in the selection experiment (Renneville et al. 2020), but this was not an issue in our pond experiment. Indeed, in the pond experiment, 180 mature fish were selected to generate 24 populations composed of individuals from the same line (48 populations in total), but from distinct families to limit inbreeding. We have thus removed the confounded family-tank effect. These points have been clarified in the manuscript (l. 175-177, 182-183, 196-197). Please note that the fish were not genotyped.**

Comment 14: Line 230: standardization of samples for sequencing depth, please give more details.
*****Our response: There were substantial differences in sequencing among samples as supported by Fig. S1 (Appendix 1). Therefore, samples were standardized to the median sequencing depth (l. 296.297). This approach is detailed in the R script and the manuscript has been rephrased to: "After standardization of the data, we identified a total of 3,189,868 sequence reads (mean = 30,969 reads per sample) and 627 ASVs for 103 samples." (l.341-342).**

Comment 15: Line 234: not fully clear how family was defined.
*****Our response: See response to Comment 25 below.**

Comment 16: --> Analyses should be run by tank. Within tank replication is most probably pseudo-replication. It is not clear to me whether host families were treated individually in the statistical analyses (= pseudoreplication) or whether tanks were the units of replication.

*****Our response: In the pond experiment, the number of ponds were the units of replication (8 treatment combinations, 48 mesocosms, 6 replicates per treatment combination). This has been clarified in the revised version of the manuscript: "The experiment consisted of a 2 × 2 × 2 full factorial design with size-selected line (LB and SB) crossed with density (high HD and low LD) and light intensity (high HL and low LL). Each treatment combination was replicated six times (48 mesocosm in total; Fig. 1)." (l. 222-227).**

Comment 17: Line 264: Why were no UniFrac distances calculated? Ideally, UniFrac distances are calculated among groups which take into account the phylogenetic relationships of the bacterial taxa in the microbiome. Weighted UniFrac takes into account the relative abundance of species/taxa shared among groups, whereas unweighted UniFrac only considers presence/absence. The latter counts the fraction of branch lengths unique to either community. Your analysis is most closely related to using weighted UniFrac. It is useful for examining differences in community structure. However, it would also be valuable to know what difference low-abundance features make in a community. Your sophisticated experimental design might have the power to also detect subtle differences in less abundant taxa.

*****Our response: We have now reported results from PERMANOVA performed using both weighted and unweighted UniFrac distances, as recommended by the Reviewer (l. 307-315). Outputs from the weighted Unifrac distance PERMANOVA indicated that the gut microbial composition of medaka was influenced by the Line ($F = 2.30$, $P = 0.025$, $R2 = 0.02$) but not by Density ($F = 1.35$, $P = 0.195$, $R2 = 0.013$) or Light ($F = 1.23$, $P = 0.265$, $R2 = 0.012$) or the interactions between Line and the environment (Line × Light and Line × Density). However, results from the unweighted UniFrac distance PERMANOVA revealed that the gut microbial composition of LB medaka depicted greater diversity than that of SB medaka in the high light treatment, while the opposite pattern was found in the low light treatment (Line × Light: $F = 1.66$, $P = 0.020$, $R2 = 0.16$). The Results section of the manuscript has been revised accordingly (l. 351-363, 388-393, 401-408, Fig. 4a).**

I evaluated the R script.

Comment 18: With regard to the rarefaction analysis in the R script, I wonder whether you sequenced deeply enough to capture most of the microbiome composition (code is in the R script)? Why not reporting this in the main manuscript?

*****Our response: The rarefaction curves (Fig. S1) indicate that we captured most species, but that with increasing sequencing depth that we will find more rare species, as indicated by the positive relationship between the number of ASVs and the number of reads per sample (linear model: $P < 0.001$, $R2 = 0.31$; Figure 1 below).**

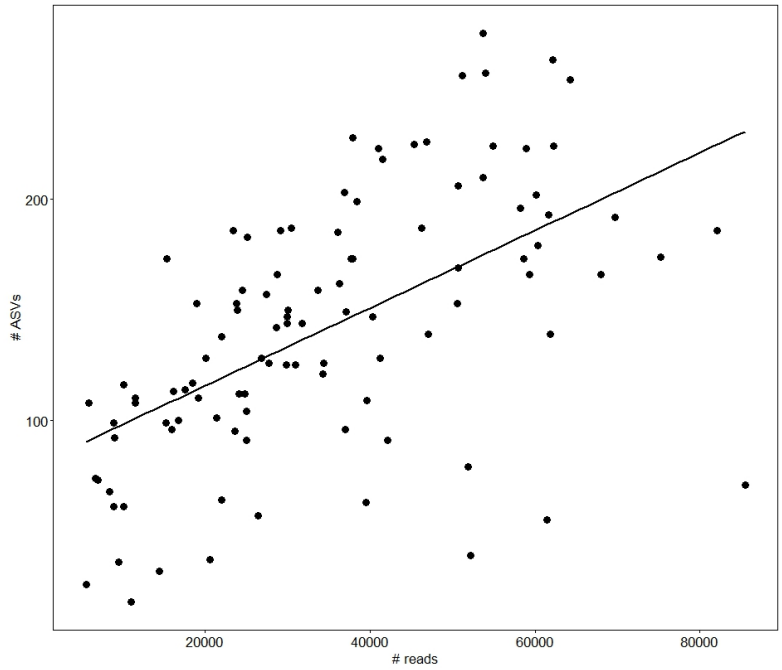


Figure 1 Relationship between the number of ASVs and the number of reads per sample.

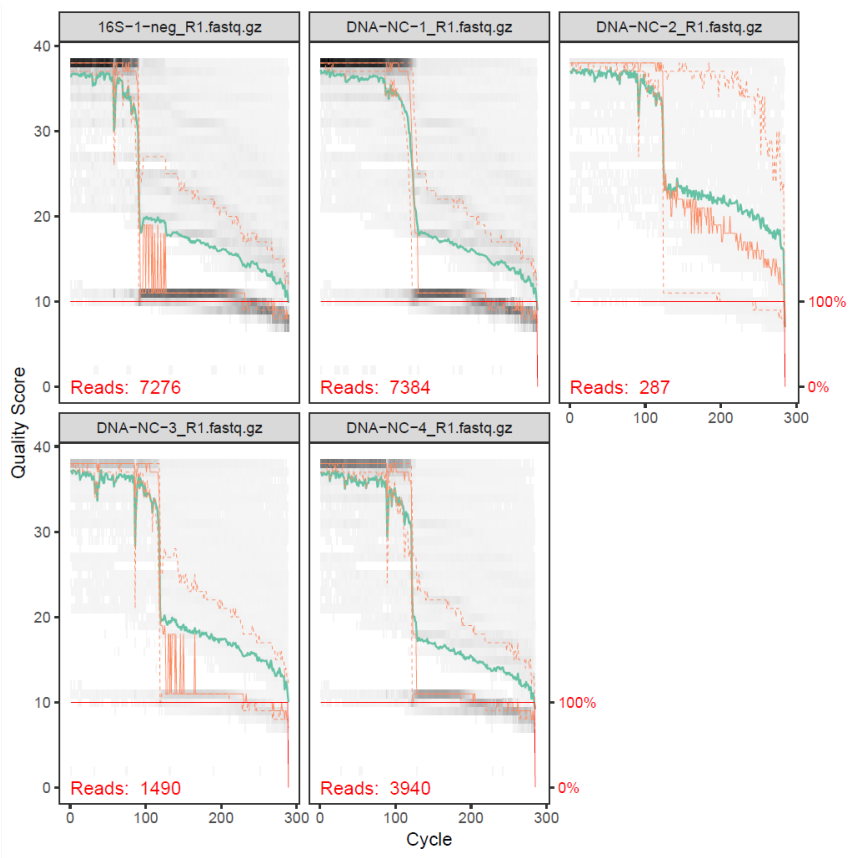
RESULTS

Comment 19: Line 267 and onwards: Relative abundance!! No absolute quantification was performed. Please be very careful in the wording of your text. Features in your dataset can only be compared within the dataset as you did not perform any sort of absolute quantification. Abundance is relative to your dataset.
Our response: Done (l. 346, 355, 356, 359, 371, 374, 4701, etc…).

Comment 20: Table 1: It looks like there is a significant interaction effect between line x light intensity. An interaction effect occurs when the effect of one independent variable on the dependent variable changes across different levels of another independent variable. In other words, the impact of one factor depends on the level of another factor. When there is a significant interaction effect, it indicates that the combined influence of the factors is not adequately captured by the main effects alone. To assess the significance of the individual factors in the presence of an interaction effect, you should conduct follow-up analyses or post-hoc tests. These tests allow you to examine the effects of each factor while controlling for the levels of the other factor. I think the sophisticated experimental design of your study allows you to investigate further and look for pairwise comparisons or simple effects analyses.
Our response: Significant interactions were further investigated using post hoc Tukey's pairwise comparison using the emmeans package (l. 327-329). Results from the post hoc (P-values adjusted for multiple testing using false discovery rate correction) are provided in the text (l. 412, 416).

Comment 21: Line 274: what about the less abundant bacteria? What bacterial taxa were specific to treatment groups? Please see my comment further up in the methods about using unweighted UniFrac distances.
Our response: In the revised manuscript, PERMANOVA has been performed using both weighted and unweighted UniFrac distances and the whole manuscript has been revised accordingly.

Comment 22: Negative controls: Did you include any negative controls in your study? Please include them in the manuscript. This could include: sequencing of PCR water, sequencing blank extractions, or sequencing the water of your mesocosm tanks. Typical contaminants from extraction kits can be highly abundant (*e.g.*, Microbacteriaceae). Fish guts are a typical low bacterial biomass niche and this needs to be incorporated in the analysis. Guidelines can be found here: https://journals.asm.org/doi/10.1128/mSystems.00290-19
Our response: We thank Dr Wilkins for her comment and agree that sampling the water for comparison would have provided a picture of the environmental microbiome to which fish were exposed. Unfortunately, water from the mesocosms was not sequenced (l. 505-508). However, four DNA extraction and one PCR negative controls (i.e., ultra-pure water instead of DNA) were sequenced and subjected to the same sequence filtering criteria along the gut microbiome samples. All negative controls showed indeed as negative, each containing very low number of sequences of very poor quality (see Figure 2 below, the sequencing quality profiles of the negative controls after filtering). The sequences that did make it through processing (~290 sequences for all 5 samples combined) corresponded to only 7 unique sequences, each unique to a negative control sample. Moreover, when looking at the taxonomic classification of these 7 unique sequences, they all match taxa that we do not expect to find in the fish gut samples. Considering this, including negative controls into the data analysis would not be informative. However, we have now reported the details about the negative controls' content mentioned above in the M&M section of the revised manuscript (l. 278-287).



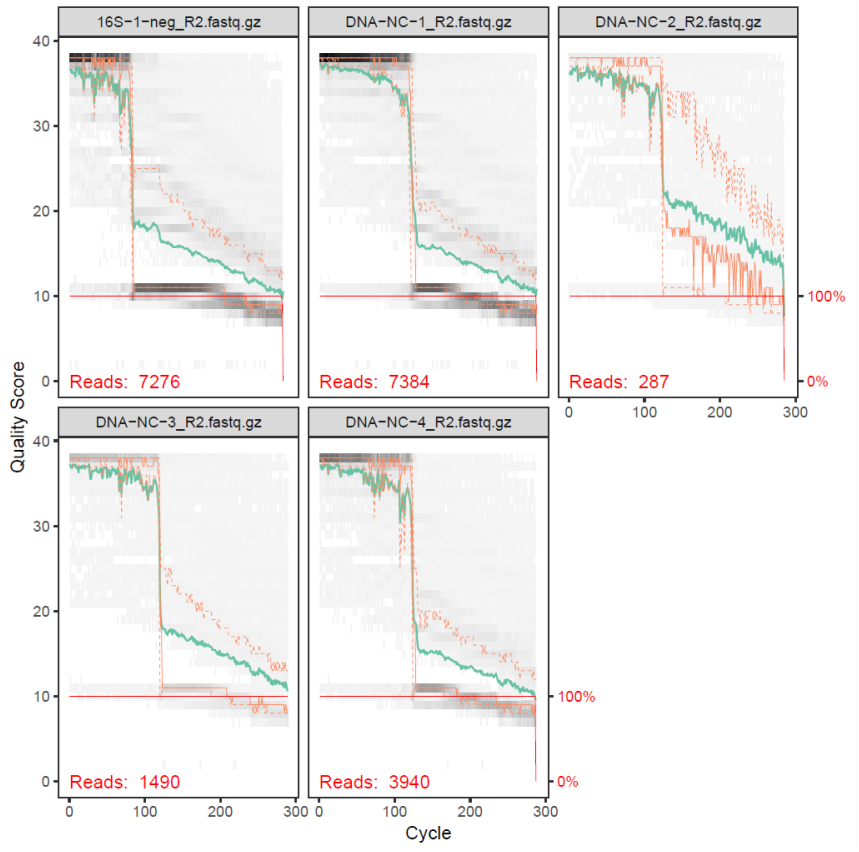


Figure 2 Quality profiles of the five negative controls after filtering (forward and reverse reads are displayed in the upper and lower panel, respectively)

DISCUSSION

Comment 23: Overall, the discussion is very well written. It answered several questions I had after reading the introduction section. The conclusions are adequately supported by the results. After reading the results section, I came up with several questions and hypotheses. These were all addressed in the discussion section. For example Renneville *et al*. 2020 showed that the two lines differ in foraging strategies. Could the difference seen here in microbiome composition represent their preferred diet, which of course is also affected by light conditions? Where is the evidence that differences are caused by host genetics? What mechanisms? Lines 338 – 369 nicely cover these points.
*****Our response: Thank you for the positive feedback on the discussion. We believe that the revised introduction will also help to clarify the purpose of the present study.**

Comment 24: The discussion section (as well as the introduction) did not take into account a few references from relevant recent and past research performed in the field. I suggest including and discussing the following references:

Sullam KE, Essinger SD, Lozupone CA et al. (2012) Environmental and ecological factors that shape the gut bacterial communities of fish: a meta-analysis. Molecular Ecology, 21, 3363–3378.

Sullam KE, Rubin BER, Dalton CM et al. (2015) Divergence across diet, time and populations rules out parallel evolution in the gut microbiomes of Trinidadian guppies. ISME Journal, 9, 1508–1522.

Sevellec M, Pavey SA, Boutin S et al. (2014) Microbiome investigation in the ecological speciation context of lake whitefish (*Coregonus clupeaformis*) using next-generation sequencing. Journal of Evolutionary Biology, 27, 1029–1046.

Ghanbari M, Kneifel W, Domig KJ (2015) A new view of the fish gut microbiome: advances from next-generation sequencing. Aquaculture, 448, 464–475.

Boutin S, Sauvage C, Bernatchez L, Audet C, Derome N (2014) Inter individual variations of the fish skin microbiota: host genetics basis of mutualism? PLoS ONE, 9, 1–17.

Bolnick DI, Snowberg LK, Caporaso JG et al. (2014b) Major histocompatibility complex class IIb polymorphism influences gut microbiota composition and diversity. Molecular Ecology, 23, 4831–4845.

Wilkins LGE, Fumagalli L, and Wedekind C (2016) Effects of host genetics and environment on egg-associated microbiota in brown trout (Salmo trutta). Molecular Ecology 25(19): 388-394.
*****Our response: Thank you these articles, very interesting. We have included some of them in the Introduction/Discussion: Bolnick et al 2014a (l.75), Sullam et al. 2015**

(l. 81, 457, 491), Sevellec et al. 2014 (l. 81, 107, 445), Sullam et al. (2012) (l. 84) and Ghanbari et al. (2015) (l.550).

Comment 25: Overall, beyond the family level, I m curious which core bacteria and functional pathways could be affected by the genetic lines used in this study and their interaction with light and fish density? Since a lot of work has gone into designing and performing this elaborate experiment, it would be nice to go a bit more into the detail and look at the more rare taxa at greater resolution than the family level of bacteria.
*****Our response: We think that analyses of the functional pathways are way beyond the scope of the study as 16S amplicon sequencing is not really tailored for this type of analysis. In addition, since not much is known about medaka's microbiome, 16S sequencing is a straightforward first step towards understanding basic patterns of variation in the microbiome. Using more sophisticated methods such as functional meta genomics could be the logical next step since we still have the samples.**
About doing analyses at lower taxonomic levels, applying bootstrap support for Genus level taxonomic assignment indicates that many of these assignments had low support (median support = 0.52), with more than 1100 ASVs at < 0.1 (which is more than the number of ASVs with good support). At the Family level, the median support is 0.89, which is much better. The sentence has been rephrased to: "All statistical analyses were run with R v.4.2.1 (R Development Core Team, 2022) using the Family level as taxonomic resolution because it was the best taxonomic level for discriminating (median bootstrap support is 0.52 and 0.89 for Family and Genus level taxonomic assignment, respectively)." (l. 301-304).