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

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.

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.

Line 45: is a speculation. Functional importance of the gut microbiome was not investigated in the present study.

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

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

The introduction is not well connected with the discussion section. I only fully understood the rationale of this manuscript after reading the discussion section.

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?

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.

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.

When reading the methods section, it becomes clear that this study is its own experiment. This was not clear from the introduction.

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:

Line 125: how many tank replicates?

Line 131: how many individuals were removed and how often?

Line 146: how were families (sib-families == genotypes) kept separate? How were tank effects separated from family effects? Were fish genotyped?

Lines 230: standardization of samples for sequencing depth, please give more details.

Line 234: not fully clear how family was defined.
→ 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.

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.

I evaluated the R script.

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?

RESULTS

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.

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.

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.

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

DISCUSSION

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.

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.

Overall, beyond the family level, I am 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.