

The bacterial microbiome of symbiotic and menthol-bleached polyps of *Galaxea fascicularis*

Giulia Puntin, Jane Ching-Yan Wong, Till Roethig, David Michael Baker, Michael Sweet, Maren Ziegler <https://doi.org/10.1101/2023.08.23.554380> version 1

Decision for round #1 : *Revision needed*

Recommender

I trust this letter finds you in good health.

I, along with two different reviewers, have had the opportunity to thoroughly review your study and provide feedback on your submission. Reviewers have articulated several concerns about your preprint, and I wholeheartedly concur with their assessments. The reviewers have highlighted issues such as the relatively low level of replication and the lack of clarity in how measurements were replicated. Additionally, Reviewer 2 has offered valuable insights regarding sequencing and assignment.

The primary concerns raised is about the preliminary nature of the study. While your research presents intriguing insights into the core microbiota associated with the *Galaxea fascicularis* coral model, the methodology used appears to hinder the generation of clear, conclusive results. The abstract, for instance, implies an expectation of a decisive conclusion regarding the impact of bleaching on the bacterial microbiome of *G. fascicularis*. However, due to various issues such as the limited number of replicates, sampling from different locations, analysis in different laboratories with slight yet seemingly significant variations in rearing methods, and the absence of comparisons with natural colonies (as pointed out by Reviewer #2), the results come across as over-interpreted. Thus, in my opinion, the study's validity and potential impact must be significantly enhanced with additional results.

After a comprehensive review of your work, I have arrived at the conclusion that I am unable to recommend it for publication in its current state. Below, you will find specific comments that I provide in addition to reviewers comments. Given the methodological limitations, a thorough revision may require the inclusion of additional results.

I cannot make assumptions regarding your ability to supply the requested additional data, so I have opted to request a significant revision rather than an immediate desk rejection. If you are confident in your capacity to furnish additional results and address the queries raised by the reviewers and myself, kindly submit a revised edition of your work. In the event that this proves unfeasible, you will be given the option to withdraw your submission from PCI Microbiology.

We wish to thank the recommender and reviewers for their assessment of our study and their constructive comments. We have addressed the reviewers' comments in a point-by-point response as detailed below in blue font. Please note that in our responses the line numbers refer to the [tracked changes](#) version of the manuscript (word document).

The main motivation for this study was to broaden the knowledge base on the use of the coral species *Galaxea fascicularis* as a model to study symbioses in corals. As in other established model systems, such as *Aiptasia* or *Hydra*, such model organisms are held in captivity in the long-term and only one or few genotypes of these organisms are used to study the phenomenon in question. Because of the large geographic distribution of *G. fascicularis*, we originally endeavored to compare the menthol-bleaching induced changes of colonies from three regions, but due to logistical constraints during the COVID19 pandemic, we were unable to produce additional data from other colonies. While we acknowledge the potential benefits of incorporating additional data to enhance the scope of the study, we hope to make a valuable contribution to coral symbiosis research using the *Galaxea* model system with this study. We have carefully addressed the queries raised by you and the reviewers, and have made comprehensive revisions to underscore the constraints of the data.

Specific comments :

Materials & Methods:

Experimental Design: The experimental design in your study appears to be quite complex, but it is not sufficiently explained. The number of replicates is unclear, which is a critical aspect of any research study. For instance, in the section titled "Sampling for microbial analysis," you mentioned that "n = 15" for both bleached and symbiotic polyps, but it is not clear how this number was determined. Furthermore, the number of polyps collected seems inconsistent with the mentioned number.

We initially sampled 15 polyps per symbiotic state (5 colonies * 3 polyps/colony = 15). Unfortunately, 2 polyps from Hong Kong were damaged during shipping to the UK (where DNA extraction and sequencing were performed for all samples) and were therefore excluded. Additionally, one Red Sea polyp was excluded from alpha and beta diversity calculations (and derived considerations) after rarefaction due to low sequencing depth (lines: 152-154).

To enhance clarity, we made specific amendments to the manuscript. In line 120, we revised the text to read: "At both locations, three polyps per colony (5 colonies: RS1, RS2, RS3, HK1, HK2)(~~3 Red Sea, 2 Hong Kong~~) per state (2 states: symbiotic, bleached)(~~here, symbiotic and bleached~~) were sampled [...]". Additionally, in line 124, we included the following clarification: "Two samples from Hong Kong (1 symbiotic and 1 bleached) were damaged during shipping and therefore excluded from processing".

Moreover, in response to concerns raised by other reviewers and to facilitate better comprehension for readers, we have incorporated a new figure, now labelled Fig. 1, to visually illustrate our experimental design.

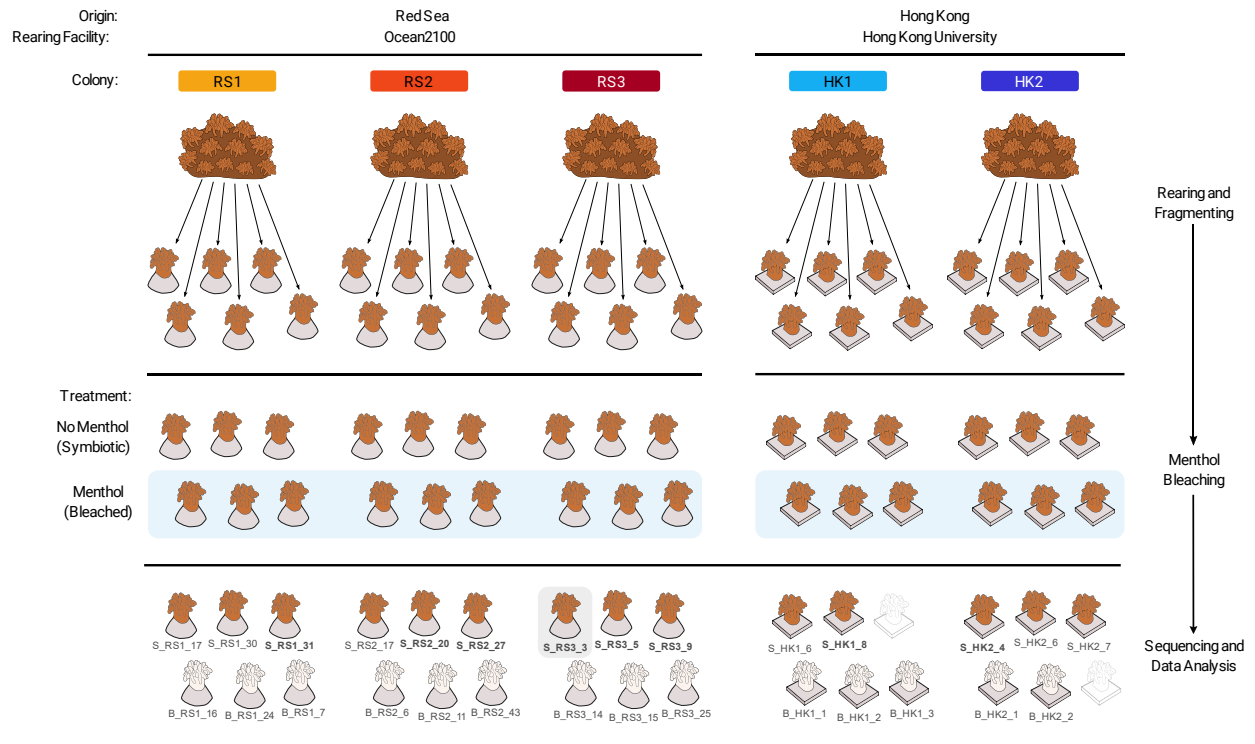


Fig. 1: The statement in Fig. 1 that "Alpha diversity remained similar between symbiotic and menthol-bleached samples across all diversity and richness indices tested" is contradicted by the significant difference in Shannon and Simpson indices in RS1 (Red Sea colony #1). This contradiction should be addressed and clarified.

Thank you for pointing out this incongruence. The reason behind our seemingly contradictory statement lies with the level of grouping used for the comparison, namely whether we considered all colonies together or each colony individually.

When considering all colonies together, we did not find significant differences in any alpha diversity and evenness index between symbiotic and bleached polyps (shown in Fig.1A, Table S1, S2, where all $p > 0.05$). This grouping was chosen because we were interested in understanding general patterns.

When considering each colony individually, colony RS1 stood out for showing consistently low alpha diversity values when symbiotic (across all indices; also visible in Fig.1A). We initially limited our evaluation to a visual assessment (i.e., 95% CI plots, Fig. S2) as we could not statistically test differences between symbiotic and bleached polyps for all colonies due to low replication. Specifically, only colonies RS1 and RS2 have $n = 3$ for both symbiotic states. We nevertheless saw value in reporting this outstanding feature of colony RS1, as it may prove relevant for future development of the Galaxea model.

To address the comment, we now tested the difference between symbiotic and bleached polyps when possible (i.e., for colonies RS1 and RS2). The results align with the observed

pattern in Fig. S2: the difference between symbiotic and bleached RS1 polyps is found to be significant across all tested indices (while these are not significant for RS2).

As such, we have provided the additional results and amended the text and figures when necessary. Specifically:

- Script "09_alpha_diversity_rar1000.R" (<https://zenodo.org/records/10551928>) contains the new code for normality checks and Welch's t-tests.
- We added a new table with the Welch's tests results in the supplementary file as Tab. S3 (previous Tab. S3 was renamed Tab. S4), with caption: "Statistical testing of difference in community diversity and evenness between symbiotic (group2, n2) and bleached (group1, n1) polyps using the unequal variances unpaired t-test (Welch's test). Testing was limited to colonies RS1 and RS2 due to insufficient replication in the remaining colonies (n1 or n2 < 3)".
- We amended the results section as follows (lines 176-184): "When considering all colonies together, alpha diversity remained similar between symbiotic and menthol-bleached samples across all diversity and richness indices tested (incl. observed richness, Chao1, Shannon diversity, Simpson evenness diversity, Pielou's evenness, and Faith's phylogenetic diversity, see Table S1, S2), and regardless of their origin ($P_{t\text{-test}}$ or $P_{\text{Mann-Whitney U-test}} > 0.05$; Fig. 42A). For one colony only (RS1) when considering each individual colony, alpha diversity was remarkably and consistently low in the symbiotic RS1 polyps state, while in their bleached counterparts when bleached it was in range with the other colonies (Fig. 42A, Fig. S2, Tab. S2). Within-colony difference between symbiotic and bleached polyps could only be tested for RS1 and RS2, and it was significant in RS1 across all alpha diversity indices tested ($P_{\text{Welch}} < 0.05$, Tab. S3)."

Additionally, to be more explicit about the statistical limitations, we have amended Fig. S2 to show the raw data point and their range (min – max) instead of the 95% C.I.. The caption now reads: "Comparison of microbial diversity and richness between symbiotic and bleached colonies, displayed as raw data points and ranges ~~95% confidence intervals~~. Differences between symbiotic and bleached polyps could only be tested for RS1 and RS2 due to inadequate replication in the remaining colonies (results in Tab. S3). The difference between symbiotic states is significant for all tested indices in colony ~~Lack of overlap between intervals indicates significant difference ($p < 0.05$), which occurs in colony~~ RS1 across all alpha diversity metrics. RS1, which ~~intervals are~~ is highlighted here with larger ~~line~~ points size for ease of identification."

Results:

Fig. 2A and PERMANOVA: In Fig. 2A, the microbial communities from the Red Sea and Hong Kong colonies appear to be very similar, raising doubts about the significance of the PERMANOVA results. It is essential to reconsider this analysis and possibly perform ANOSIM. Considering the clear heterogeneity of multivariate dispersion in Fig 2A, I doubt that betadisper (PERMDISP2) gave a p-value > 0.05.

Thank you for bringing attention to this matter.

As you correctly noted, PERMDISP2 gave a p-value < 0.05, as reported in the manuscript (lines 197-198, old version lines 206-207). Indeed, PERMANOVA results might have been affected by this difference in dispersion, as well as by the unbalanced sample size (Red Sea n = 17, Hong Kong n = 10), casting doubts on the reliability of its significance (Alekseyenko 2016).

Following your suggestion, we also performed ANOSIM (see new script "07_beta_diversity_ANOSIM.R") which produced similar results: R = 0.2015, P = 0.0186. However, it is worth noting that ANOSIM, like PERMANOVA, is sensitive to violations of homoscedasticity and imbalanced sample size when these occur together (Anderson and Walsh 2013).

Given the characteristics and structure of our data, meaningfully assessing the significance of the difference in community composition between Red Sea and Hong Kong polyps proves challenging. Therefore, we have revised the paragraph to omit the direct comparison in composition between Red Sea and Hong Kong, choosing instead to emphasize the—more evident and better supported—dissimilarity in dispersion. It now reads as follows (lines 196-208): **“Microbial community dissimilarity patterns differed by geographic origin of colonies**

Microbial communities of Red Sea samples showed significantly larger dissimilarities than those from Hong Kong, both within and between colonies (ANOVA on PERMDISP2, F = 118.7, P < 0.0001, Fig. 23A; Mann-Whitney U-test on pairwise Bray-Curtis distances, W = 102, P = 0.0001, Fig. 23B, Fig. S3). Microbial communities were significantly different between colonies from the Red Sea and Hong Kong (PERMANOVA, F = 5.46, P = 0.0003; Fig. 2A). However,

Microbial communities of symbiotic Red Sea polyps clustered by colony, and Red Sea colonies appeared as different from each other as they were from those of Hong Kong colonies. Interestingly, Θ , and one symbiotic colony from the Red Sea did however share had a similar microbial community to with those from Hong Kong (Fig. 23A).” Besides microbial community composition, colonies also differed in dispersion, where Red Sea microbial communities showed significantly larger dissimilarities than those from Hong Kong, both within and between colonies (ANOVA on PERMDISP2, F = 108.3, P < 0.0001, Fig. 2A; Mann-Whitney U-test on pairwise Bray-Curtis distances, W = 102, P = 0.0001, Fig. 2B).

Alekseyenko A V. (2016) Multivariate Welch t -test on distances. *Bioinformatics* 32:3552–3558
<https://academic.oup.com/bioinformatics/article/32/23/3552/2525638>

Anderson MJ, Walsh DCI (2013) PERMANOVA, ANOSIM, and the Mantel test in the face of heterogeneous dispersions: What null hypothesis are you testing? *Ecol Monogr* 83:557–574
<http://doi.wiley.com/10.1890/12-2010.1>

Discussion:

The main takeout is that bleaching induce a very different response in bacterial communities in « HK » compared to « Red Sea ». This is probably linked to the fact that HK and Red Sea experiments were conducted in different places with slightly different conditions.

Unfortunately, it is impossible to test the laboratory/feeding procedure/aquarium effect because this factor has not been replicated which makes it difficult to draw a conclusion. This limitation should be emphasized to provide a more balanced interpretation of your findings.

We agree that emphasizing this limitation is crucial for a meaningful interpretation of our findings. We have therefore amended the text in the Discussion section as follows:

Lines 322-328 now read: "While rearing conditions were largely replicated between facilities, feed type and tank volume differed. Feed can introduce bacteria in the system (Hartman et al. 2020), and uneaten portions could promote microbial growth. Such effects would have been amplified by the smaller volume of the containers used in Hong Kong compared to Red Sea. However, it should be noted that our experimental design did not allow us to directly test these hypotheses. We therefore suggest that future studies incorporate an adequately replicated "facility" factor in their design, as well as food and seawater samples in their analysis to better characterize the influence of rearing conditions on the host microbiome."

Conclusion:

The conclusion section in your paper appears to contrast with the abstract. The abstract fails to clearly convey that the difference between bleached and untreated communities is apparently due to stochastic factors. Instead, it suggests "destabilization and loss of structure of the communities," which comes across as vague and overly wordy.

We appreciate your careful attention to the alignment between the conclusion section and the abstract in our manuscript. We have revisited both these sections to ensure a more accurate and concise representation of our findings.

Abstract: ~~"While t~~The response of the bacterial microbiome to menthol bleaching differed ~~varied~~ between the two facilities, warranting further investigation into the role of rearing conditions. Nevertheless, the changes in community composition observed in both instances appeared to be stochastic, and microbiome destabilization and loss of structure emerged as a unifying response, indicative of a dysbiotic state. Considering the importance of captivity and bleaching treatments for ~~holobiont~~ coral symbiosis research, our results—although preliminary—contribute fundamental knowledge for the development of the Galaxea coral model for symbiosis research."

Conclusions (lines 420-423): ~~"O~~The overall, response to menthol bleaching induced stochastic changes in ~~was a destabilization of the microbiome, indicating dysbiosis. However, captivity also affected~~ the response of the bacterial microbiome to bleaching, with differed nces observed between the two facilities, likely reflecting differences in rearing conditions, which remain to be addressed."

Similarly, the respective part of the **Discussion** was also amended as follows (Lines 293-295): "Menthol bleaching led to stochastic changes in the microbiome of ~~changes in~~ Galaxea. Menthol bleaching was associated with changes in ~~led to a destabilization and loss of structure of the bacterial communities that differed between individual polyps and, produced~~ ing stochastic configurations."

I hope you find these comments helpful in improving the quality of your research.

Sincerely,

Cédric Hubas

by [Cédric Hubas](#), 16 Oct 2023 18:46

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version: 1

Reviewer 1

Review by anonymous reviewer 1, 02 Oct 2023 17:12

In their manuscript, Puntin et al. present very interesting results from their study focusing on the bacterial microbiome of symbiotic and menthol-bleached polyps of *Galaxea fascicularis*.

However some I have some concerns mostly on the way results are presented.

1. I. 5-6: please rephrase

We appreciate the feedback, however, even after consulting with a native English speaker, we were unable to understand the issue with the sentence or the rationale behind the need for its rephrasing. We therefore did not edit the respective text.

2. I. 150 (and elsewhere): What is Simpson evenness? I only know Simpson diversity index.

Thanks for noticing this error. We mistakenly called it "evenness" to highlight that Simpson diversity index places greater emphasis on evenness than on richness (Kim et al. 2017). However, Simpson's evenness is a different index, which is calculated by dividing Simpson's diversity by the observed richness (Smith and Wilson 1996), which we did not use. Therefore, we adjusted the text where necessary.

Kim B-R, Shin J, Guevarra RB, Lee JH, Kim DW, Seol K-H, Lee J-H, Kim HB, Isaacson RE (2017) Deciphering Diversity Indices for a Better Understanding of Microbial Communities. *J Microbiol Biotechnol* 27:2089–2093 <http://www.jmb.or.kr/journal/view.html?doi=10.4014/jmb.1709.09027>

Smith B, Wilson JB (1996) A Consumer's Guide to Evenness Indices. *Oikos* 76:70
<https://www.jstor.org/stable/3545749?origin=crossref>

3. Figure 2B: Where is HK1 symbiotic and HK2 bleached in Fig.2B? Apparently you had only two replicates. Did you mention that earlier because I cannot find it.

HK1 symbiotic and HK2 bleached samples only had two replicates and were consequently omitted from this plot, as explained in the caption at lines 225-226: "(only groups with $n = 3$ considered, but comparable results were found considering groups with $n < 3$, see Fig. S3)."

To address clarity regarding sample replication, we added the following statement at line 124: "Two samples from Hong Kong (1 symbiotic and 1 bleached) were damaged during shipping and therefore excluded from processing."

This aspect is also clarified in the new Figure 1.

4. L. 215: You sampled 30 polyps, and excluded 1 from RS3 symbiotic (which one???) due to low seq. depth, so that makes us 29 polyps. Right? In Fig. 2b above you miss two more polyps. You need a table that will show all your samples with proper encoding and will explicitly explain which was used for every analysis. Also you refer to your samples according to origin (i.e. RS: Red Sea) and a number which indicates the colony (1,2,3). However this is confusing since you do not separate the triplicates you sampled from each colony. I would suggest you add a simple encoding (l.e.a,b,c) since it is confusing (e.g. which RS3 sample was excluded????).

Thank you for the suggestion. The explanation about the two missing polyps has now been added at line 124 as follows: "Two samples from Hong Kong (1 symbiotic and 1 bleached) were damaged during shipping and therefore excluded from processing." The polyp that was excluded after rarefaction due to low sequencing depth is "S_RS3_3" as visible in the supplementary file in "Figure S1- Rarefaction curve".

We understand that a full encoding identifying each individual polyp could be useful. This information is available in the raw data as well as the results in the online repository, however for readability we had omitted them from the main manuscript figures. We have now produced a new figure that provides a graphical overview of the experimental design and analyses in which this information has been included.

Reviewer 2

Review by [Tony Robinet](#), 25 Sep 2023 14:08

Comments on the MS from Giulia et al. sent to PCI microbiol

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The authors aimed at evaluating the behaviour of microbial community in the tropical coral *Galaxea fascicularis* after polyps from the same wild colonies were kept in captivity under controlled conditions only, or under controlled conditions and bleached with menthol.

Bleaching, corresponding to the disappearance of the photosymbiotic Symbiodiniaceae from the polypes, induced a disorganisation in microbiomes in the way that the structure formed by core taxa in symbiotic polypes vanished, turning into a kind of stochastic assemblage of taxa. Authors did not notice any typical signature of bleaching, like would have been the systematic death of some key-taxa.

Authors discussed that, in this study, captivity did reduce the diversity of microbiomes in polypes, compared to those living in non-captive ones, but there were no assessment of wild polypes microbiomes in this study. The comparison relied on data from literature only. However, the captivity effect, i.e. the lack of exogenous wild bacterial flow into bleached polypes, and the potential bacterial flow from food, were appropriately proposed to explain the observed convergence of microbiomes of Red Sea and Hong Kong due to their captivity in similar conditions.

Results are presented by a scientific team who is experienced in coral microbiology, as we can read it in introduction and discussion. Concepts are well defined, literature is recent and abundant, questions are clearly addressed, scripts are clean and working.

A complete study of the bleaching effect would probably have gathered more samples (here only 14 from 5 colonies), sequenced "wild" samples in coral colonies of the same locations where captive ones have been collected, developed a correct sequencing protocol for the Symbiodiniaceae ITS2 (this axis is unfortunately under-explored), and analyzed the unknown microbial contamination brought by feeding (L103: "polyps were fed daily with one small frozen adult *Artemia* each"). We can understand all the reasons explaining why these elements are lacking, but in their absence, I think that this study can be worth to be shared with the scientific community if authors present their results as preliminary, or with these gaps expressed in the abstract, before a complete study can be lead. As well, the title should be clarified by mentioning the fact that "symbiotic" and "bleached" corals were both captive.

We appreciate the reviewer's insightful comments and constructive feedback on our manuscript. We acknowledge the noted limitations in our study and value the suggestions for enhancing clarity and completeness.

Specifically, we modified the **title** to "The bacterial microbiome of symbiotic and menthol-bleached polyps of *Galaxea fascicularis* in captivity", and amended the manuscript text to improve the following aspects:

- We clarify that we characterized microbiome of captive Galaxea, instead of speculating on its responses to captivity:

Abstract: “The coral *Galaxea fascicularis* is an emerging model organism for coral symbiosis research with demonstrated suitability to aquarium rearing and reproduction, and to manipulation of the host-Symbiodiniaceae symbiosis manipulation. However, little is known about the *G. fascicularis* how its microbiome responds to after long-term captivity and how it responds to menthol bleaching —the experimental removal of the Symbiodiniaceae which represents the first step in the coral-algal symbiosis manipulation — remains unexplored. ”)

Conclusions (lines 418-420): “In this study, we provided the first baseline assessment of the response of the *Galaxea* bacterial microbiome to menthol bleaching, and gain initial insights into the potential effects of long-term captivity in this coral species.”

Conclusions (lines 429-431): “A simplified ~~The observed~~ microbiome ~~simplification may~~ could facilitate both characterization and manipulation ~~of the microbiome~~, and it could guide the identification of essential (“core”) members among the retained associates.”

- We highlight the lack of comparison with wild colonies:

Abstract: “We found that captive corals hosted a relatively simple microbiome composed of relatively fewer bacterial taxa, when compared to reports of than typically found in the microbiome of wild corals in the wild. Symbiotic Ppolyps (clonal replicates) from the same colony had similar microbiomes, which were distinct from those of other colonies despite co-culturing in shared aquaria.”

Discussion (lines 335-352): “Due to the absence of direct comparison with wild colonies, we are unable to draw conclusions on whether captivity caused a reduction in bacterial diversity. However, we hypothesize that captivity favours a streamlining of the microbiome, as ~~The reduced bacterial diversity likely resulted from captivity, where~~ stable and homogenous environmental conditions decrease both chances and need for the association with functionally and taxonomically diverse microbial partners. In fact, dDecrease in metabolic diversity and species richness have consistently been reported for tropical reef-building corals reared in closed systems [...]. [...] ~~The observed~~Such effect may also have been exacerbated by the use of filtered seawater during the bleaching phase, which largely reduced the pool of available microbes (Dungan et al. 2021b). Additionally, as colony morphology is a major factor affecting coral microbial communities (Morrow et al. 2022), a loss of the decrease in bacterial species richness might also be ascribed to reduced structural complexity [...]. Although some may see this reduction or simplification of the microbiome has a problem artefact associated with captive corals, simplified microbiomes ~~The reduction of microbial complexity~~ presents the an opportunity to for identifying essential associates and facilitating the development of microbial manipulation protocols to unravel holobiont functioning”

Conclusions (lines 423-428): “Bacterial communities of the captive *Galaxea* colonies were composed of fewer taxa than reported for wild corals, which is in line with decreasing microbial diversity of many captive organisms. ~~Captivity seemingly affected the bacterial microbiome reducing its complexity, where~~ Nevertheless, symbiotic polyps originating

~~from different colonies maintained distinct community assemblies. This, and showed links to host and/or Symbiodiniaceae identity, which we recommend to warrant further investigate investigation.~~

- We stress the importance of further investigating the influence of rearing conditions:

~~**Abstract:** "While t~~The response of the bacterial microbiome to menthol bleaching differed between the two facilities, warranting further investigation into the role of rearing conditions."

~~**Discussion:** (lines 322-328) "While rearing conditions were largely replicated between facilities, feed type and tank volume differed. Feed can introduce bacteria in the system (Hartman et al. 2020), and uneaten portions could promote microbial growth. Such effects would have been amplified by the smaller volume of the containers used in Hong Kong compared to Red Sea. However, it should be noted that our experimental design did not allow us to directly test these hypotheses. We therefore suggest that future studies incorporate an adequately replicated "facility" factor in their design, as well as food and seawater samples in their analysis to better characterize the influence of rearing conditions on the host microbiome."~~

~~**Conclusions:** (lines 420-423) "O~~The overall, response to menthol bleaching induced stochastic changes in was a destabilization of the microbiome, indicating dysbiosis. However, captivity also affected T~~he response of the bacterial microbiome to bleaching, with differed nces observed between the two facilities, likely reflecting differences in rearing conditions, which remain to be addressed."~~

- We clarify the preliminary nature of our study:

~~**Abstract** "Considering the importance of captivity and bleaching treatments for holobiont coral symbiosis research, our results—although preliminary—contribute fundamental knowledge for the development of the Galaxea coral model for symbiosis research."~~

I have no specific comments, the manuscript is well written, only a specific question : Why did you assigned taxa only to genus rank, and then numbered the ASV ? (= the unique sequences, i.e. all variants found on this marker in each species), given that (1) Silva database assignation is quite good down to species rank ; (2) if assignation with qiime is not robust for a given taxa, species is named "unassigned species" ; (3) 16S marker is known to be prone to an unknown number of copies in a same organism, with possible nucleotidic variation between copies, and therefore with the possibility to over-estimate the effective number of different organisms, and thus the reality of some of them ? Or maybe you know that Symbiodiniaceae have only one 16S copy ? Did you try the same analyses at the species level (97% of similarity between sequences)?

Thank you for your positive assessment of our manuscript and for the insightful comments and questions.

We only assigned taxa to the genus level (or above) because this is the lowest reliable taxonomic resolution allowed by our primer set (subregions V5-V6 of the 16S rRNA gene) and sequencing technology (Illumina MiSeq, 2 × 300 bp). Sub-regions of 16S do not capture sufficient variation to discriminate between congeneric taxa, and sequencing of the entire 16S

rRNA gene (~1500 bp) is recommended if interested in species-level classification (Johnson et al. 2019). In agreement with your observation, the classifier that we used (which was trained on the SILVA database) indeed attempted to assign our AVSs down to the species level, so we do have that information (see in the repository `"/out/Gfas_16S/useful_tables/taxonomy_all_nonraref.csv"`). However, most species assigned to our representative sequences (ASVs) were labeled as "Unclassified", "uncultured_bacterium" or similar, providing little value even if we wished to utilize them, and supporting the notion that partial 16S sequencing is inadequate for this taxonomic resolution.

We share the concern regarding the multi-copy nature and intragenomic variation of the 16S rRNA gene, which can potentially impact microbial diversity estimations (Hassler et al. 2022; Pan et al. 2023). However, correcting for these factors is not currently common practice, primarily due to the limited understanding of these phenomena. For example, the prevalence of multiple copies, and the number of copies and variants per genome vary among taxa and taxonomic levels, and remain to be fully characterized (Louca et al. 2018; Pan et al. 2023). While tools are available (e.g., PICRUSt, CopyRighter, PAPRICA), recent methodological evaluations generally caution against their use as they tend to introduce noise (Starke et al. 2021; Gao and Wu 2023). As we acknowledge the potential for corrections with advancing knowledge, we hope that future re-analysis efforts utilize our publicly available raw sequencing data.

Regarding Symbiodiniaceae, we have chosen to rely on the SymPortal analysis framework which is specifically designed to deal with ITS2 intragenomic variants for next-generation sequencing data (Hume et al. 2019).

Finally, we did not analyze our sequences using clustering methods (i.e., 97% similarity threshold) because the use of denoising algorithms (to infer ASVs) is overwhelmingly preferred in the field. Indeed, in other articles, we have encountered the opposite request, where reviewers asked us to re-analyze our initial results, replacing the clustering (OTUs) approach with the denoising (ASVs) approach. See for example ["Additional file 3: Table S2"](#) (Roethig et al. 2020) ["Peer Review File"](#) (Dubé et al. 2021). In both cases, we did not observe any major changes in the resulting microbiome structure and composition.

Dubé CE, Ziegler M, Mercière A, Boissin E, Planes S, Bourmaud CA-F, Voolstra CR (2021) Naturally occurring fire coral clones demonstrate a genetic and environmental basis of microbiome composition. *Nat Commun* 12:6402 <https://www.nature.com/articles/s41467-021-26543-x>

Gao Y, Wu M (2023) Accounting for 16S rRNA copy number prediction uncertainty and its implications in bacterial diversity analyses. *ISME Commun* 3:59 <https://www.nature.com/articles/s43705-023-00266-0>

Hassler HB, Probert B, Moore C, Lawson E, Jackson RW, Russell BT, Richards VP (2022) Phylogenies of the 16S rRNA gene and its hypervariable regions lack concordance with core genome phylogenies. *Microbiome* 10:104 <https://doi.org/10.1186/s40168-022-01295-y>

Hume BCC, Smith EG, Ziegler M, Warrington HJM, Burt JA, LaJeunesse TC, Wiedenmann J, Voolstra CR (2019) SymPortal: A novel analytical framework and platform for coral algal symbiont next-generation sequencing ITS2 profiling. *Mol Ecol Resour* 19:1063–1080 <https://onlinelibrary.wiley.com/doi/10.1111/1755-0998.13004>

Johnson JS, Spakowicz DJ, Hong B-Y, Petersen LM, Demkowicz P, Chen L, Leopold SR, Hanson BM, Agresta HO, Gerstein M, Sodergren E, Weinstock GM (2019) Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis. *Nat Commun* 10:5029 <http://dx.doi.org/10.1038/s41467-019-13036-1>

Kim B-R, Shin J, Guevarra RB, Lee JH, Kim DW, Seol K-H, Lee J-H, Kim HB, Isaacson RE (2017) Deciphering Diversity Indices for a Better Understanding of Microbial Communities. *J Microbiol Biotechnol* 27:2089–2093 <http://www.jmb.or.kr/journal/view.html?doi=10.4014/jmb.1709.09027>

Louca S, Doebeli M, Parfrey LW (2018) Correcting for 16S rRNA gene copy numbers in microbiome surveys remains an unsolved problem. *Microbiome* 6:41 <https://microbiomejournal.biomedcentral.com/articles/10.1186/s40168-018-0420-9>

Pan P, Gu Y, Sun D-L, Wu QL, Zhou N-Y (2023) Microbial Diversity Biased Estimation Caused by Intragenomic Heterogeneity and Interspecific Conservation of 16S rRNA Genes. *Appl Environ Microbiol* 89: <https://journals.asm.org/doi/10.1128/aem.02108-22>

Roethig T, Puntin G, Wong JCY, Burian A, Baker DM (2020) Holobiont nitrogen control and its potential for eutrophication resistance in an obligate photosymbiotic jellyfish. *Microbiome*

Starke R, Pylro VS, Morais DK (2021) 16S rRNA Gene Copy Number Normalization Does Not Provide More Reliable Conclusions in Metataxonomic Surveys. *Microb Ecol* 81:535–539 <https://link.springer.com/10.1007/s00248-020-01586-7>