

Dear Dr. Gobet,

We thank you and the reviewers for your effort and time on the second round of review of our revised manuscript. We provide below a point-by-point response (in red letters) to all these comments. We have addressed all the comments, and we believe that the manuscript has been improved.

On behalf of all the authors,
K. Kormas & S. Stefanos Katsoulis-Dimitriou

Dear authors,

I apologize again for the late reply, and I thank you for your hard work answering the reviewers' and my comments. The abstract and introduction now read very well. I do not think there is a need for another round of reviews, but I feel that there are still few modifications that need to be done on the manuscript before recommending it. After these last modifications, I would be happy to write a recommendation. Please see below some specific comments:

Abstract

L31: Please add a space after each comma: "experimental diets (FO,MI,SP)"

--Corrected.

L37: "fucose, a major cell wall exopolysaccharide" => is it really an exopolysaccharide, please see my comments below on the discussion part?

--Changed, it now reads "a major constituent of the polysaccharide content of several microalgal species". [L. 37-38 of the revised manuscript].

Materials and methods

Although there is more information on the experimental strategy, this is still not very clear to me (and I think it is important to clarify it to understand this study even though this is detailed in another unpublished article for which we do not have access). The following 3 parts need clarification:

Part 1/

L116-124: 40 fishes divided in 4 groups => 10 fishes per nutritional group in triplicate tanks => this means that there were 3 to 4 fishes per tank? Please be more specific.

Part 2/

L139-140: "At the end of the dietary trial, 10 individuals were randomly collected from each dietary group" => so, all fishes were collected, right? Please be more specific.

---No. Each tank had ca. 25 fish from which we took 3 or 4 fish from each replicate tank (10 in total), which were used for this paper.

Part 3/

L143-147: The gut of seven fishes out of the 10 collected were dissected for each nutritional group, right? Please be more specific.

---Yes, correct.

The authors may add a sketch of the experimental strategy to help understand what has been done, it would be helpful.

---We have added the requested information in the relevant part (“Sampling”) and we believe that all of the above are now clarified. We analysed 7/10 fish for keeping 3 samples as backup and for future analysis.

L125: Please correct: “Kg” by “kg”.

--Corrected.

L141-143: The body mass growth parameter L/W^3 of the sampled fish based on weight (W) and length (L) ranged between 0.013 ± 0.001 (FO) and 0.012 ± 0.002 (MI) (Karapanagiotidis & Kormas unpubl. data). Did the authors consider linking the microbiota composition to the body mass growth parameters?

---At first, we apologize for misspelling this ratio, the correct one is W/L^3 . We have run correlations of the most important OTUs abundances with W/L^3 ratios, but there were no statistically significant ones. From our experience, such correlations are more meaningful with other growth parameters (e.g. FCR, SGR etc.) but these values are not available for the specific specimens we analysed.

L151: There is a spelling mistake: “55⁰C” to replace by: “55°C”.

---Corrected.

L160-161: The ‘split.abund’ command (cutoff = 1) was used to exclude the rare sequences
So not possible to calculate Chao1 index

--Chao1 was removed from the manuscript.

L164: Please correct: “from subsequent analys*e*s.”.

--Corrected.

L166: Please correct: “but at the sample time” : at the *same* time?

--Corrected.

L173: Please correct: “The statistical analysis » : The statistical analys*e*s?”

--Corrected.

Results

L189: “319.103 reads » : is it really a dot (decimal) or a comma (thousands)? Please verify all similar numbers in the manuscript.

--Corrected in the whole manuscript.

L197-199: “Also, Taxa_S was calculated for the estimation of the OTUs richness and Chao-1 to estimate the diversity by calculating the potentially missed diversity using the presence of singletons and doubletons (Table 1).”

Please define what measures “Taxa_S” is it raw OTU richness? Another type of alpha diversity measure?

---Taxa_S is the OTUs richness or the number of OTUs, it now reads “Alpha diversity.

Taxa_S (OTUs richness), the Shannon_H and Simpson_1-D indices were calculated to assess the alpha diversity of the gut microbiota of gilthead sea bream in the four groups (Table 1).”

[L. 196-198 of the revised manuscript].

About Chao1, please remove this estimator from the manuscript, as singletons were removed (as written L160-161) and the estimator thus cannot be calculated from this dataset. The

Chao1 estimator uses singletons and doubletons to be calculated (it is true for any type of measure: OTUs, ASVs, individuals).

Please see for instance: Yongcui Deng, Alexander K Umbach, Josh D Neufeld, Nonparametric richness estimators Chao1 and ACE must not be used with amplicon sequence variant data, *The ISME Journal*, Volume 18, Issue 1, January 2024, wrae106, <https://doi.org/10.1093/ismejo/wrae106>

--Chao1 was removed from the manuscript.

L202-203: Please rephrase: “the Bray-Curtis PERMANOVA (permutational analysis of variance)”, for instance: “the permutational analysis of variance (PERMANOVA) calculated from the Bray-Curtis distance matrix”.

--Rephrased as suggested.

L225: The authors chose to italicize only genus and species names, just as a note, it is usual (but not mandatory) to italicize all taxonomic levels for prokaryotes, please see:

« Italic type versus roman type »: <https://lpsn.dsmz.de/text/glossary#italic-type-versus-roman-type>

Also quoting the text from the DSMZ :

« Names used in prokaryotic nomenclature are [☞] Latin or Latinized words and such names are usually printed in italics (or underlined in manuscripts). The [☞] Bacteriological Code (1990 Revision) and its successors set no binding standard in this respect, as typography is a matter of editorial style and tradition not of nomenclature. The name of genera, species, and subspecies are generally printed in italics (or underlined) but for higher categories conventions vary: in Britain they are often in ordinary Roman type, but in America or in France they are often in italics.

It is also interesting to note the following points.

(1) According to Chapter 4 (Advisory Notes) of the Bacteriological Code (1990 Revision), scientific names of taxa should be preferably indicated by a different type face, e.g., italic or by some other device to distinguish them from the rest of the text.

(2) Bacterial names cited in the Bacteriological Code (1990 Revision), irrespective of rank, are consistently printed in italic type.

(3) The preface of the next Code should include the following paragraph: "As in the previous edition, scientific names under the jurisdiction of the Code, irrespective of rank, are consistently printed in italic type. The Code sets no binding standard in this respect, as typography is a matter of editorial style and tradition not of nomenclature. Nevertheless, editors and authors, in the interest of international uniformity, may wish to consider adhering to the practice exemplified by the Code, which has been well received in general and is being followed in an increasing number of microbiological journals."

Consequently, in the "List of Prokaryotic names with Standing in Nomenclature" all bacterial names (except names of [☞] infrasubspecific subdivisions) are in italics to remind the reader that they are Latinized scientific names.”

Italicizing all taxonomic levels for prokaryotes is also likely a way to distinguish prokaryotes from unicellular eukaryotes, please see:

« Nomenclature of unicellular eukaryotes » : https://www.microbiologyresearch.org/marketing/editorial/Nomenclature_Style_Units_2015.pdf

Also quoting the corresponding text:

“Use only correct names of taxa. Although an organism may have a number of correct names, depending on its taxonomic placement, use one particular name consistently; if there are objections to its use, cite this name as a synonym. Taxa above the rank of genus

must be written in times New Roman type font (i.e. not italic). In all taxonomic matters, such as those exemplified for the bacteria, the relevant Code of nomenclature should be followed. For yeasts, authors should use the nomenclature employed in *The Yeasts: a Taxonomic Study*, 4th edn (1998) (Edited by C. P. Kurtzman & J. W. Fell. Amsterdam: Elsevier), and in *Yeasts: Characteristics and Identification*, 3rd edn (2000) (Cambridge: Cambridge University Press). If an author disagrees with this nomenclature, the first use of a scientific name in the text and in the Summary should be followed by the name, in parentheses, as given in *The Yeasts*.”

---This is a long and rather never-ending debate! We choose, as in all of our previous papers and most of the papers we review or handle as editors, to have only genera and species names in italics.

L237: “In all feeding groups the Mycobacteriaceae was most abundant family in the FO”, please rephrase: “In all feeding groups*,* the Mycobacteriaceae was *the* most abundant family in the FO”.

--Corrected.

L252: “(1.67% and 2.44%).” Do you mean % of reads? Please specify. Same comment for L256.

---"% of reads" has been added.

Discussion

L293: Do you mean “not significant compared to the control group”? Please specify or rephrase. Same comment for L308.

---Yes, it now reads “...but not statistically significant different to the control...” [L. 292-293 of the revised manuscript].

L311: “Schizochytrium” to put in italic.

--Corrected.

L324: “is required” to remove? Or maybe rephrase the sentence?

--Sentence rephrased, it now reads “However, differences in diversity alone cannot show the overall effects and it is necessary to investigate the structural changes at the level of bacterial phyla, families, and specific OTUs” [L. 321-323 of the revised manuscript].

L324: there is a misspelling to correct: “phycoshpere”.

--Corrected.

L328-370: If the authors would like to be more specific, Mycobacteria and Vibrio are also known to be able to ferment molecules into SCFAs (which can be further be uptaken by the host). Vibrio are also known to break down complex molecules such as polysaccharides. There may be cooperation between gut bacteria to act as probiotics for the host.

---Regarding *Mycobacterium aurum*, it is reported in BacDive (<https://bacdive.dsmz.de/strain/8379>) that it is negative in glucose fermentation, while we did not locate any paper clearly stating fermentative activity for *M. aurum*, which is discussed here. Regarding *Vibrio*, we have added their potent degradation activity. [L. 360-363 of the revised manuscript].

L371-384: Regarding the story on fucose, the authors may also consider that this monosaccharide is not usual in exopolysaccharides, in the 3 cited articles (with 2 of the cited

articles maybe out of topic as they are on cyanobacteria, not eukaryotic microalgae), this is not very clear. I would just say it is part of the polysaccharide content of microalgae without specifying the origin in the cell. Also, as written by Ahmed et al 2014:

<https://www.tandfonline.com/doi/full/10.1080/09670262.2014.895048#d1e960>: “Fucose is not an unusual component in EPS. It occurs in the glycoconjugates of many microorganisms and is an important component of the cell wall and capsule structures of Gram-negative and Gram-positive bacteria (Maki & Renkonen, 2004, <https://academic.oup.com/glycob/article/14/3/1R/642264>).”

So there is a possibility that this pathway would be intended to degrade bacteria in the gut as well?

---We thank the reviewer for the insightful details on fucose. We have rephrased the part with the “exopolysaccharides”, and it now reads “L-fucose degradation was the only considerably overexpressed bacterial metabolic pathway in the three experimental feeds in comparison to FO. Fucose is a deoxyhexose that is found as a part of the polysaccharide content of microalgae (Bernaerts et al. 2018, Wan et al. 2019).” Whether this pathway could act upon other bacteria, we cannot securely answer or speculate.

This article may be of interest to go on with the algal polysaccharide hypothesis (most algae used in the diets of this study are described):

Bernaerts et al 2018

https://www.sciencedirect.com/science/article/pii/S2211926417310421?casa_token=sUp5hbt1z6MAAAA:6pe9ltKluxS6zsz7FFIbvzLOoeTmImq4AeTTOazSff_64HI5GILfkAhOtRIjtbtQ0sWG6hEERoA

--Changed; it now reads “Fucose is a deoxyhexose that is found as a part of the polysaccharide content of microalgae (Bernaerts et al. 2018, Wan et al. 2019)” [L. 373-375 of the revised manuscript].

L395: There seem to be a word missing: “with the highest number of unique OTUs compared to FO *for* which,”.

--Corrected.

L394-397: Past and present tenses are used in the same sentence, please rewrite the sentence.

--Corrected.

Figures and tables

Table 1: Please remove Chao1, see comments above.

--Chao1 was removed from the manuscript.

Figure 1, L710-711: “PI samples are separated from FO and SP samples.” Please rephrase.

---We deleted this sentence from the legend as this is more of a result which is mentioned in the “Results” section. [L. 712-713 of the revised manuscript].

Figure 2: Why do the bars corresponding to each diet to not reach 100%? Also add a title to the y axis, maybe “Read relative abundance”?

L716: Please specify: “Reads relative abundance”.

L717: “calculated based on the average samples reads” of the 7 guts per diet? Please specify.

---They do not reach 100% because families with relative abundance < 1% are not in the diagram. “Reads Relative Abundance (%)” added to the y axis. The legend now reads “**Figure 2.** Taxonomic composition of the bacterial families in each dietary group. Relative abundances were calculated adding the relative abundance (calculated based on the average

samples reads of the seven samples per diet) of all OTUs belonging to each family. Only the families with relative abundance > 1% were considered for the diagram. Families with relative abundance < 4% are depicted in grey. FO: Fish Oil, MI: *Microchloropsis* + *Isochrysis*, SP: *Schizochytrium* + *Phaeodactylum*, PI: *Phaeodactylum* + *Isochrysis*.”

Figure 3

L727: “closest relative (Nucleotide BLAST) at the genus level.” Do you mean the species level?

--Corrected.