General Response to the Reviewers

Dear Associate Editor and Referees,

We sincerely thank the Associate Editor Dr. Pierre Amato, and the three anonymous referees for their careful evaluation of our manuscript and for their detailed and constructive comments. The reviews identified a common and fundamental concern regarding the limited, unbalanced, and temporally heterogeneous sampling design. We agree that the original manuscript overstated the inferential capacity of the dataset, particularly regarding comparisons among lakes and interpretations involving preservation status, anthropogenic pressure, lake size, spatial heterogeneity, vertical stratification, temporal variation, and predicted functional differences.

Obtaining additional contemporaneous biological replicates was not feasible during the present revision. We therefore did not attempt to defend the original statistical framework or present the six samples as representative biological replicates of the four lakes. Instead, we substantially revised the conceptual framing, analyses, figures, Results, Discussion, and Conclusions, recasting the study as an exploratory and descriptive baseline characterization of bacterial communities detected in six water samples from four Colombian Andean lakes.

Under this revised framework, we made the following major changes:

1. The individual water sample was redefined as the observational unit, rather than the lake, preservation category, or anthropogenic-pressure category.

2. Unsupported inferential analyses were removed, including lake-level and group-level statistical comparisons, Spearman correlations, co-occurrence network analyses, differential-abundance testing of predicted functions, and other analyses requiring biological replication.

3. The former temporal-comparison subsection for Fúquene and Tota was removed. Samples collected on different dates or in different years are no longer treated as temporal replicates, and no claims are made regarding seasonal change, temporal stability, or interannual variation.

4. Only descriptive analyses supported by the dataset were retained, including sequencing summaries, sample-level coverage-standardized diversity estimates, taxonomic composition, relative-abundance patterns, Bray–Curtis dissimilarities, and ordination plots presented without inferential testing or causal interpretation.

5. Statements attributing the observed patterns to preservation status, anthropogenic pressure, nutrient availability, eutrophication, seasonality, lake size, spatial heterogeneity, water-column depth, or other environmental drivers were removed or substantially weakened. These variables are now discussed only as mechanisms reported in the broader freshwater literature and as hypotheses requiring future replicated investigation.

6. The absence of concurrent physicochemical measurements is now stated explicitly. No environmental–microbiological correlation analysis was conducted because nutrient concentrations, dissolved oxygen, chlorophyll-a, pH, temperature, conductivity, organic-matter indicators, and related variables were not measured synchronously with microbiological sampling. We expanded the description of the sampling design and included all recoverable sample-specific metadata, including collection date, location, lake zone, and sequencing information. Where broader lake-level environmental information from published or institutional sources is included, it is clearly identified as contextual, non-contemporaneous

information and is not used for statistical association, causal attribution, or reconstruction of conditions at the time of sampling.

7. PICRUSt2 results were reframed as descriptive predictions of potential genomic functions. Differential-abundance testing and preservation-status comparisons were removed. The revised analysis presents the most abundant predicted pathway categories, a descriptive Bray–Curtis ordination of predicted functional profiles, and sample-level weighted NSTI values. These outputs are explicitly described as predictions based on ASV phylogenetic placement and reference genomes, rather than direct evidence of gene presence, expression, metabolic activity, pathogenicity, or ecosystem processes.

8. The *Mycobacterium* analysis was substantially reduced and reframed. It is now presented only as an exploratory phylogenetic placement of genus-assigned ASVs, motivated by the occurrence of water-associated nontuberculous mycobacteria. The limited species-level resolution of the V3–V4 16S rRNA gene region is explicitly acknowledged, and no definitive species identifications, pathogenicity claims, or ecological interactions are inferred. The maximum-likelihood tree was moved to the Supplement.

9. The manuscript was comprehensively rewritten and reorganized. The revised title is: "Exploratory characterization of bacterial communities and predicted functional profiles in six water samples from four Colombian Andean lakes using 16S rRNA gene amplicon sequencing."

10. The revised supplementary material was deposited in Zenodo and assigned a new DOI (https://doi.org/10.5281/zenodo.21647587). The manuscript's Data Availability statement, supplementary-material references, and associated links were updated accordingly to ensure public access to the revised supporting data and files.

The Abstract, objectives, Methods, Results, Discussion, Conclusions, figures, supplementary material, terminology, and taxonomic formatting were revised so that the scope and claims are proportional to the available evidence. The revised supplementary files were deposited in Zenodo under a new DOI, and all corresponding references and data-access information in the manuscript were updated. The revised title explicitly identifies the exploratory character of the study, the number of samples and lakes represented, and the 16S rRNA gene amplicon sequencing approach.

After reviewing the manuscript categories offered by Biogeosciences, we did not reclassify the manuscript as a BG Letter merely to accommodate its reduced scope, because that category is intended for particularly important results and major advances. Subject to the Associate Editor's assessment, we have retained the manuscript as a shorter and substantially revised Research Article focused on its value as a preliminary baseline survey of poorly characterized Colombian Andean lake bacterial communities.

We recognize that this general response does not replace the required individual replies. Accordingly, we provide separate point-by-point responses to every referee comment and indicate how each concern was addressed in the revised manuscript.

We greatly appreciate the reviewers' recognition that the dataset may retain value as an initial characterization of microbial communities in understudied Andean lakes. We believe that the extensive restructuring has removed unsupported statistical and ecological claims, clarified the distinction between measured results and contextual hypotheses, and produced a more rigorous, transparent, and appropriately cautious manuscript.

Sincerely,

Andrés Gómez-Palacio
on behalf of all authors

Anonymous Referee #1

The authors used 16S rRNA gene amplicon sequencing combined with PICRUSt2 functional prediction to systematically analyze the bacterial community structure, co-occurrence patterns, and potential metabolic functions of four high-altitude lakes (F ú quene, Tota, Calderona, Colorado) with different conservation states in the eastern Andes Mountains of Colombia. Research has found that lakes with greater human influence, such as Fúquene, exhibit higher microbial richness and diversity, while lakes with better protection, such as Calderona and Colorado, have more stable communities but lower diversity; Spatial heterogeneity (such as inflow rivers and coastal zones) and vertical stratification further shape community composition. Functional prediction reveals potential differences in bacterial communities in pathways such as carbohydrate metabolism and exogenous substance degradation under different protection states. This study provides baseline data for understanding the response of microorganisms in Andean mountain lakes to anthropogenic stress, and has certain ecological and conservation biological value.

However, despite the practical significance of the research topic, there are several significant deficiencies in the rigor of experimental design, data analysis, and generalizability of the conclusions. Taking all factors into consideration, I believe that the manuscript is currently not sufficient for publication in this journal. I suggest making significant revisions and reconsidering it.

The main issue with this manuscript is the severe lack of sample size and sampling design, which greatly undermines the reliability of all statistical inferences and ecological conclusions. In addition, the explanation of functional prediction results is too bold and lacks necessary cautious wording.

> ***Response:***
> *We thank the reviewer for the careful assessment of our manuscript and for recognizing the ecological and conservation relevance of providing baseline information on bacterial communities in Colombian Andean lakes. We agree that the limited, unbalanced, and temporally heterogeneous sampling design substantially restricts the statistical inference and generalizability of the original study. We also agree that several interpretations of the PICRUSt2 results were stated too strongly.*
>
> *In response, we have substantially reframed the manuscript as an exploratory and descriptive baseline study rather than a statistically replicated comparison of lakes or conservation categories. The revised title is now:*
>
> *"Exploratory characterization of bacterial communities and predicted functional profiles in six water samples from four Colombian Andean lakes using 16S rRNA gene amplicon sequencing."*
>
> *Throughout the revised manuscript, we now treat each water sample as an individual observational unit and no longer present the samples as biological replicates representative of their respective lakes. We have removed analyses and conclusions that were not*

adequately supported by the available sample size, including inferential comparisons among lakes or conservation categories, Spearman correlation analyses, and bacterial co-occurrence network analyses. We have also revised the interpretation of alpha diversity, community composition, spatial variation, and depth-related patterns so that these are presented only as descriptive observations and hypotheses for future investigation.

In addition, the PICRUSt2 results are now explicitly described as predictions of potential genomic functions inferred from 16S rRNA gene profiles. We no longer interpret these predictions as evidence of actual metabolic activity or as statistically demonstrated functional differences associated with anthropogenic pressure or conservation status. All corresponding statements in the Abstract, Results, Discussion, and Conclusions have been revised using more cautious language.

Detailed responses to the reviewer's specific comments and the corresponding manuscript changes are provided below.

1.Rows 91-130, Table 1: The sample size is extremely small and the statistical power is seriously insufficient. This study only involved 4 lakes, and the number of sampling points for each lake was extremely small (2 samples from Fúquene, 2 samples from Tota, and 1 sample each from Calderona and Colorado). Such a small sample size cannot support any meaningful inter group statistical tests (such as comparisons between different conservation states), nor can it distinguish individual differences in lakes from conservation state effects. The so-called 'significant differences' (such as lines 237-241) are likely driven by a single outlier.

Suggest the author to significantly increase the sampling intensity. Each lake should have multiple sampling stations (at least 3-5) and repeat sampling in different seasons. If it cannot be achieved, this study should be honestly positioned as a "preliminary exploratory case study" and avoid making generalizations in the title, abstract, and conclusion..

Response:
We thank the reviewer for highlighting this fundamental limitation. We agree that the original sampling design was too limited, unbalanced, and temporally heterogeneous to support inferential comparisons among lakes or preservation categories. In particular, the two samples from Fúquene and the two samples from Tota were collected at different times and cannot be considered true biological or temporal replicates, while Calderona and Colorado were each represented by a single sample. Therefore, the dataset cannot distinguish lake-specific variation from effects associated with preservation status or anthropogenic influence.

Because additional contemporaneous sampling is not feasible within the present revision, we followed the reviewer's recommendation and substantially reframed the manuscript as an exploratory and descriptive case study. The title, Abstract, Introduction, Methods, Results, Discussion, and Conclusions have been revised accordingly.

In the revised Methods section, we now explicitly state that the dataset comprises six individual water samples: two from Fúquene, two from Tota, and one each from Calderona and Colorado. We also clarify that samples collected at different dates or locations were treated as individual observations rather than biological or temporal replicates. The preservation categories are now presented only as descriptive environmental context and are no longer treated as statistically replicated explanatory factors.

Table 1 and its caption have also been revised to clarify the sampling dates, origin of each dataset, sequencing depth, number of ASVs, and the descriptive nature of the preservation classifications. In addition, the previously published Tota sample collected in 2018 is now clearly identified as an previously published raw-read dataset that was reprocessed using the same bioinformatic workflow rather than as a replicate collected within the present sampling campaign.

We have removed unsupported intergroup statistical comparisons and no longer report significant differences among lakes or preservation categories. Richness, diversity, taxonomic composition, community dissimilarity, and predicted functional profiles are now interpreted descriptively at the sample level. Generalizations regarding the effects of preservation status, anthropogenic pressure, sampling depth, or spatial position have been removed or reformulated as hypotheses requiring future replicated and contemporaneous sampling.

We acknowledge that a more robust study would require multiple independent stations per lake and repeated sampling across seasons. This limitation is now stated explicitly throughout the revised manuscript.

2.Rows 116-120, Table 1: Pseudo repetition and time confusion. Two samples from F ú quene come from two different periods in 2019, one sample from Tota comes from 2018 and the other from 2019, while Calderona and Colorado only have one sample from 2019. This uneven and mixed time factor makes it difficult to distinguish the impact of "conservation status", "lake characteristics", and "sampling time" on community variation. For example, the high diversity of Fúquene may be due to its two samplings capturing seasonal fluctuations, rather than its low conservation state.

It is recommended that all lakes be sampled at the same time. If historical data (such as Tota's 2018 data) must be used, it should be analyzed separately as a control, rather than mixed with other 2019 data for comparison.

***Response**:*
We agree with the reviewer that the original presentation created temporal confusion and could be interpreted as pseudoreplication. The two Fúquene samples were collected during different periods of 2019, the two Tota samples originated from different years, and Calderona and Colorado were each represented by only one sample. Consequently, variation associated with lake identity, environmental context, and sampling time cannot be disentangled.

We have revised the sampling description and Table 1 to make the temporal structure of the dataset explicit. The five samples collected in 2019 are now identified as the primary exploratory dataset, although we acknowledge that they were not all collected contemporaneously. Each sample is therefore treated as an individual observation rather than as a biological or temporal replicate, and samples are no longer pooled by lake or preservation category for inferential comparisons.

We also removed the term "internal control" for the Tota sample collected in December 2018. This sample, obtained from the previously published BioProject PRJNA720890, is now described as a historical reference sample. It was reprocessed using the same bioinformatic workflow but analyzed separately from the 2019 cross-lake dataset. It was excluded from cross-lake summaries and ordination analyses involving the 2019 samples and is presented only as a descriptive within-lake reference for Tota.

Accordingly, we no longer attribute the comparatively high observed diversity of the Fúquene samples to their preservation status or anthropogenic influence. The revised manuscript explicitly acknowledges that these observations may reflect temporal variation, sampling location, lake-specific characteristics, or other unmeasured environmental factors. Determining the relative contribution of these factors would require contemporaneous and seasonally replicated sampling across all four lakes.

3.Lines 28-29, 85-88, 197-208, 354-370, 467-50: Overinterpretation of functional prediction (PICRUSt2) results. Although the author pointed out the limitations of PICRUSt2 in the methods section (lines 85-88), deterministic language such as "predicted metabolic potential variables" and "pathways related to... were identified" is frequently used in the results and discussion section. Especially when directly associating KEGG pathway annotations such as "infectious diseases" with potential pathogenic risks in environmental samples (lines 368-370), it can easily lead to misunderstandings. These are only computer predictions based on genome homology, not evidence.

Suggest revising this type of expression throughout the entire text. The word 'identified' should be changed to 'predicted to be present based on 16S rRNA gene induced metagenomics'; Change 'variation in... pathways' to' variation in the predicted abundance of genes associated with... pathways'. For the "infectious diseases" pathway, it must be clearly stated that this is only an artificial product annotated in the database and does not represent any pathogenic activity.

***Response**:*
We thank the reviewer for identifying this important issue. We agree that several expressions in the original manuscript overstated the evidential value of the PICRUSt2 outputs and could be interpreted as demonstrating the presence or activity of specific metabolic pathways. PICRUSt2 provides computational predictions based on the phylogenetic placement of 16S rRNA gene ASVs and the genomic content of reference taxa; it does not directly detect genes, gene expression, metabolic activity, or ecological process rates.

We have therefore revised the terminology throughout the title, Abstract, Methods, Results, Discussion, figure captions, and Conclusions. Expressions such as "pathways were identified," "metabolic functions were detected," and "variation in metabolic pathways" were replaced with wording such as "pathways were predicted to be represented based on 16S rRNA gene-derived functional inference" and "variation in the predicted relative abundance of genes associated with particular pathways." The PICRUSt2 outputs are now consistently described as predicted functional profiles or inferred genomic potential rather than demonstrated metabolic capacity.

The functional-prediction Methods subsection was also revised to state explicitly that the predictions depend on available reference genomes and may not capture strain-level variation, genes absent from reference databases, gene expression, or actual biochemical activity. In view of the limited sampling design, inferential differential-abundance tests of predicted pathways among lakes or preservation categories were removed, and the functional profiles are now summarized descriptively for each individual sample.

We also removed the previous interpretation linking KEGG categories labelled "infectious diseases" with potential pathogenic risk in the lakes. The revised manuscript now clarifies that such labels are broad database annotation categories containing genes or homologous functions that may occur in many non-pathogenic organisms. Their predicted representation

does not demonstrate the presence of pathogens, virulence, infection risk, or pathogenic activity in the sampled environments.

These changes ensure that the PICRUSt2 results are presented strictly as exploratory, hypothesis-generating predictions requiring validation through shotgun metagenomics, transcriptomics, or direct functional measurements.

4.Lines 189-196, 319-338: Species level identification is unreliable. The author constructed a phylogenetic tree based on the V3-V4 16S rRNA gene fragment (approximately 460bp) and conducted species level identification of Mycobacteria. As is well known, the resolution of 16S rRNA genes at the species level is limited, especially for highly similar NTM groups. The author himself acknowledges this (lines 326-328), but still provides a detailed species level discussion below (lines 444-456).

***Response**:*
We agree with the reviewer that the V3–V4 region of the 16S rRNA gene does not provide sufficient resolution for reliable species-level identification within Mycobacterium, particularly among closely related nontuberculous mycobacteria. We therefore removed all definitive species-level assignments and substantially reduced and reframed the analysis as an exploratory phylogenetic placement. In the revised text, species names are mentioned only to identify the reference sequences contained within particular clades and are not assigned to the environmental ASVs. The Results now explicitly state that these placements indicate phylogenetic affinity but do not establish species identity. We also removed the former detailed species-level discussion and revised the Discussion to avoid conclusions concerning pathogenicity, virulence, organism viability, host infection, or health risk. The maximum-likelihood tree has been moved to Supplementary Figure S2, and the general community analyses are reported only at the phylum, family, genus, and ASV levels.

Suggest either deleting all species level identification and discussions based on this fragment, and keeping the analysis at the genus level; Alternatively, it should be clarified that these are only "tentative species level assignments" and it is recommended to confirm them through whole genome sequencing or specific marker genes in the future.

***Response**:*
We thank the reviewer for this recommendation. We adopted the first option and removed all definitive species-level identifications and species-specific interpretations based on the V3–V4 fragment. The Mycobacterium analysis is now retained only as an exploratory phylogenetic placement of environmental ASVs relative to named reference sequences. Species names are used solely to identify the reference sequences or clades included in the tree and are not assigned to the environmental ASVs. The revised Results explicitly state that the observed placements indicate phylogenetic affinity but do not establish species identity. The detailed species-level discussion was removed, and the general interpretation is restricted to the genus level. We also added that definitive identification would require future analysis of cultured isolates using whole-genome sequencing or validated higher-resolution marker genes. The maximum-likelihood tree was moved to Supplementary Figure S2.

5.The article discusses multiple driving factors such as nutrients, oxygen, and temperature, but lacks environmental physicochemical data.

During the discussion, it was repeatedly mentioned that factors such as "nutrient inputs," "temperature," and "oxygen gradients" shape microbial communities, but the entire text did not provide any synchronously measured environmental variable data (such as total

phosphorus, total nitrogen, dissolved oxygen, chlorophyll-a, pH, etc.). This makes all inferences about environmental drivers unfounded. This is a fatal flaw.

It is recommended to supplement at least basic on-site hydrochemical parameters. If the data has been lost, it should be explicitly stated in the discussion that environmental microbiological correlation analysis cannot be conducted and the relevant inferences should be significantly weakened.

Response:

We thank the reviewer for identifying this important limitation. No physicochemical variables were measured concurrently with the microbiological sampling, including nutrient concentrations, dissolved oxygen, chlorophyll-a, pH, temperature, conductivity, or indicators of organic matter. Therefore, these data cannot be added retrospectively, and relationships between microbial-community composition and environmental conditions cannot be evaluated in the present study.

The Discussion has been extensively revised accordingly. Statements attributing the observed microbial patterns to nutrient availability, eutrophication, temperature, oxygen gradients, hydrological conditions, lake size, spatial heterogeneity, preservation status, or anthropogenic pressure were removed or substantially weakened. These environmental processes are now mentioned only as mechanisms documented in previous freshwater studies and are clearly distinguished from factors directly evaluated in our dataset. The differences observed among the six samples are now interpreted strictly as descriptive sample-level patterns rather than as responses to measured environmental gradients.

We also added an explicit statement that the absence of concurrent physicochemical measurements prevents environmental–microbiological correlation analyses and limits identification of the processes underlying the observed variation. This limitation is now incorporated into both the Discussion and Conclusions, together with a recommendation that future studies combine replicated microbiological sampling with simultaneous physicochemical measurements.

6.Lines 175-188, 286-296: The limitations of co-occurrence network analysis have not been fully discussed. The network is constructed based on only 6 samples (N=6), with 20 nodes and 226 edges. Calculating correlation on such extremely sparse datasets results in extremely unstable results, almost entirely driven by individual samples. Although the author mentioned in lines 186-188 and 526-527 that statistical associations do not equal ecological interactions, they did not emphasize the fundamental issues brought about by sample size.

It is not recommended to conduct network analysis under the current sample size. Alternatively, the author should explicitly downgrade this section to 'exploratory visualization' and warn readers that the results cannot be extrapolate

Response:

We agree with the reviewer that a co-occurrence network based on only six samples would be statistically unstable and highly sensitive to individual observations. We therefore removed the network analysis in its entirety, including the correlation calculations, network figure, corresponding Results subsection, and related Discussion statements. The revised manuscript no longer presents taxon–taxon associations or interprets them as ecological relationships. Community structure is now described using taxonomic composition, coverage-standardized alpha diversity, and descriptive Bray–Curtis ordination only.

The statement "Temporary stability" in lines 297-306 is not valid.

Response:
We agree with the reviewer. The statement suggesting temporal stability was not supported by the sampling design because each sampling date was represented by only one sample. We therefore removed the claim of temporal stability and deleted the former subsection focused on temporal bacteriome composition. Differences between the available sampling dates for Fúquene and Tota are now described only as sample-level variation, without temporal inference.

Only two time points of data (Fúquene and Tota twice each) claim 'temporary stability in dominant taxa'. Two time points can only describe changes and cannot prove stability.

Response*:*
We agree with the reviewer that two sampling events per lake, each represented by a single sample, are insufficient to demonstrate temporal stability. The former statement claiming "temporal stability in dominant taxa" has therefore been removed, together with the separate subsection on temporal bacteriome composition. Differences between the two available samples from Fúquene and Tota are now reported only as descriptive sample-level variation and are not interpreted as evidence of temporal stability, temporal trends, or consistent change.

Suggest deleting the word 'stability' and replacing it with 'temporary variation between two sampling events'.

Response:
We agree with the reviewer that the term "stability" was not supported by the sampling design. Rather than replacing it with "temporal variation," we removed the entire subsection on temporal bacteriome composition because each sampling event was represented by only one sample and the study was not designed to assess temporal patterns. Differences between the two available samples from Fúquene and Tota are now mentioned only descriptively within the general taxonomic composition section and are not interpreted as evidence of temporal stability or temporal change.

7.It is recommended to conduct detailed language polishing and formatting proofreading throughout the entire text.

Response: *We thank the reviewer for this recommendation. The manuscript was extensively revised throughout, not only for language and formatting but also for scientific framing, methodological accuracy, presentation of results, interpretation, and consistency with the revised analyses.*

Anonymous Referee #2

This study explores the diversity of bacterial communities and their functional potential in several Andean lakes subject to varying levels of anthropogenic pressure. Using amplicon sequencing and functional prediction based on 16S rRNA gene taxonomy, the authors observed greater bacterial diversity in lakes exposed to greater anthropogenic influence than in better preserved environments, as well as differences in the representation of metabolic pathways related to carbohydrate metabolism and xenobiotics degradation. They also observe some spatial heterogeneity and vertical stratifications within the lakes.

This work may be useful as a preliminary study of the condition of the lakes and will be all the more valuable if sampling continues over the years. However, the present study has several significant weaknesses. Firstly, the number of samples is unfortunately too small to allow for statistical analysis: six in total for four lakes, and for Lakes Tota and Fúquene, the two samples taken were from different seasons or years. Secondly, most of the statistical tests presented in the study are not valid given the number of samples, in particular Spearman's correlation. Thirdly, the authors make several claims regarding the results and their significance that the dataset and analyses cannot support.

I therefore recommend that the manuscript be thoroughly revised, that the analyses of the dataset be redone, and that the article be rewritten accordingly. The study would benefit greatly from increasing the size of the dataset, with at least true triplicates per lake, collected simultaneously. If more extensive sampling is not possible, a more descriptive preliminary study is possible, but the analyses of the dataset must be thoroughly revised, with appropriate use of the statistical tests, which will then be limited. Finally, I would have liked to have metadata concerning the samples, such as weather conditions and certain characteristics of the lakes (pH, temperature, etc.). With the exception of the 16S rRNA gene sequencing data, all metadata is missing from the study. These factors could influence bacterial communities and affect the interpretation of the results.

In its current form, the article doesn't meet the journal's publication criteria.

> ***Response:***
> *We thank the reviewer for this careful assessment. We agree that the original sampling design does not support formal statistical testing or strong conclusions about lake identity, preservation status, anthropogenic pressure, sampling depth, or temporal variation. We have therefore substantially revised the manuscript as a descriptive exploratory study.*
>
> *Specifically, we removed inferential analyses that were not appropriate for the dataset, including correlation-based tests, and no longer report p-values or claims of statistically significant differences. Diversity, taxonomic-composition, ordination, and functional-prediction results are now summarized descriptively at the individual-sample level. We also clarified that the two samples from Fúquene and the two samples from Tota were collected in different periods and are not biological or temporal replicates.*
>
> *We further revised the Abstract, Introduction, Materials and Methods, Results, and Discussion to avoid causal or unsupported interpretations. Apparent differences among samples are now described as sample-level patterns only, and we explicitly state that the study was not designed to test the effects of lake identity, preservation status, anthropogenic pressure, sampling depth, spatial position, or temporal variation.*
>
> *We also agree that the lack of physicochemical and weather metadata limits interpretation. We have added this limitation to the manuscript and state that environmental drivers such as pH, temperature, conductivity, dissolved oxygen, nutrients, and recent precipitation could not be evaluated directly. We now present these results as a baseline inventory and hypothesis-generating framework for future studies involving replicated, contemporaneous sampling and environmental measurements.*

Major comments:

The abstract will need to be revised in accordance with the new analyses and the revision of several statements, such as L25: "vertical stratification driven by …" I have not found any analysis of this kind in the manuscript.

***Response*:**
We agree with the reviewer. We have therefore revised the abstract substantially to align it with the revised descriptive framework of the manuscript.

The new abstract now presents the study as an exploratory survey of bacterial community composition and predicted functional potential in six water samples. We removed unsupported statements about vertical stratification and causal environmental drivers. We also clarified that, because the sampling design was limited, unbalanced, and not fully contemporaneous, the observed patterns cannot be attributed conclusively to lake identity, preservation status, anthropogenic pressure, sampling depth, or spatial position within the lakes.

In addition, we revised the description of PICRUSt2 results to avoid overinterpretation. The abstract now states that PICRUSt2 predictions represent inferred genomic potential based on 16S rRNA gene composition and should not be interpreted as direct evidence of gene presence, gene expression, microbial metabolic activity, or ecosystem process rates. Finally, the abstract now emphasizes that replicated and contemporaneous sampling, together with environmental measurements, shotgun metagenomics, and direct functional assays, will be required to evaluate the environmental processes shaping bacterial community composition and functional potential in these lakes.

L59-61: If all these described processes have already been extensively documented, what novelties your manuscript is bringing? This sentence or the objective of the study needs to be reworded.

***Response**:*
We agree with the reviewer that the previous wording did not clearly define the novelty of the manuscript. We revised this section to distinguish between ecological processes that are already well documented in Colombian lakes and the specific contribution of the present study. The revised text now clarifies that, although previous work has begun to characterize microbial communities in Colombian highland lakes, comparable ASV-based metataxonomic surveys across multiple Colombian Andean lentic systems remain scarce. We therefore frame the manuscript as one of the first exploratory inventories of bacterial community composition and predicted functional potential across contrasting highland lakes in this region, rather than as a study demonstrating new anthropogenic-pressure effects.

L120: In what way is the sample taken from Lake Tota in 2018 considered an internal control? How was it taken into account in the analyses? Further details are required.

***Response**:*
We agree with the reviewer that the previous description of the December 2018 Tota sample as an "internal control" was unclear and inappropriate. We have removed this terminology from the manuscript. The 2018 Tota sample was not treated as an internal control, biological replicate, temporal replicate, or evidence of temporal change.

We clarified that this sample was retrieved from Forero-Pineda et al. (2021) and reprocessed in the present study using the same bioinformatic workflow applied to the 2019 samples. It was included only to provide a historical reference for Tota under a comparable ASV-based

analytical framework. Because it was non-contemporaneous with the 2019 samples, it was analyzed separately and excluded from ordination analyses and cross-lake summaries of the five-sample 2019 dataset.

L116-118: Unfortunately, the dataset is too limited to reveal any variation between the conditions (at least three samples per site, taken on the same date, would be required). Furthermore, the experimental protocol and the selection of samples need to be reconsidered. We have a mix of conditions: a single sample for two lakes, and two samples for two other lakes, but two of these are from the same year but a different season, and two others are from a different year. No trends can be observed with such experimental protocol.

***Response**:*
We agree with the reviewer that the dataset is too limited and unbalanced to reveal trends among conditions or to test lake-level, seasonal, temporal, or preservation-status effects. We have revised the Materials and Methods to clarify the sampling structure and to avoid presenting the samples as replicated comparisons.

The revised text now states that the primary exploratory dataset comprised five samples collected in 2019: two from Fúquene in April and September, one each from Calderona and Colorado in October, and one from Tota in November. We further clarify that, because these samples were collected at different times and were not obtained under a balanced, replicated design, each sample was treated as an individual observational unit. No sample was considered a biological or temporal replicate of another, and no condition-level trends were inferred from this sampling design.

Accordingly, the analyses and interpretation have been revised throughout the manuscript to present sample-level descriptive patterns only, without statistical testing or claims of trends driven by lake identity, preservation status, season, depth, or temporal variation.

L117: I found the term "semester" rather vague and would recommend specifying the date or month instead (or the season if there are several samples). This makes it even more difficult to identify a seasonal trend, or is this aspect relevant given the local climate?

***Response:** We agree with the reviewer that "semester" was vague. We replaced this term with the specific sampling months. We also clarified that, in the Colombian Andean region, seasonal variation is more closely related to rainfall regime than to temperate climatic seasons. However, because the samples were not replicated across months or lakes, rainfall-related or seasonal patterns were not evaluated. The manuscript now states that each sample was treated as an individual observational unit, that no sample was considered a biological, seasonal, or temporal replicate of another, and that no seasonal trends were inferred from this sampling design.*

L139: I would have liked a brief explanation as to why this particular protocol was chosen. Why was a home-made protocol chosen rather than a commercial kit, which would have allowed for a better comparison of the data with that from other publications? Is this due to technical reasons and the type of samples?

***Response**:*
We thank the reviewer for this suggestion. We have added a brief explanation for the use of the CTAB/SDS-based extraction protocol. The protocol was chosen because it had been used for all filters included in the study, including the previously published Tota 2018 sample. We also clarified that CTAB/SDS-based extraction is suitable for environmental filter material

containing mixed microbial biomass and potential organic inhibitors. We agree that commercial kits can facilitate comparisons among studies, but in this case consistency across the samples included in the present dataset was prioritized.

L138-149: Please elaborate a little further on the description of the methodology here. Was a pre-amplification PCR carried out prior to sequencing? Were the PCR and MiSeq sequencing steps carried out in-house or performed by a sequencing platform? If in-house, which kits were used?

***Response**:*
We thank the reviewer for requesting additional methodological detail. We have revised this section to clarify which steps were performed locally and which were performed by the sequencing provider. Specifically, we now state that amplification of the 16S rRNA gene V3–V4 region was performed locally using primers 341F and 805R, whereas library preparation, indexing, quality control, and Illumina MiSeq sequencing were performed by Macrogen Inc.

Because the downstream library-preparation and sequencing workflow was performed by the external sequencing provider, we did not add kit-specific details unless explicitly documented in the sequencing report. We also clarified that the historical Tota sample was generated using the same DNA extraction protocol, primer pair, target region, sequencing platform, and library-preparation procedure as the 2019 samples.

L160: I think there's a sentence missing here to explain how the ASVs were trained, and what parameters were used for the clustering?

***Response**:*
Response: We thank the reviewer for pointing out that the ASV-inference procedure required further clarification. We have revised the Methods to explain that sequence variants were inferred using the DADA2 denoising framework and were not clustered into operational taxonomic units at a fixed sequence-similarity threshold. After quality filtering, forward and reverse reads were dereplicated, error models were learned separately for each read direction, and exact amplicon sequence variants were inferred using the DADA2 core algorithm. Because the retained forward and reverse reads did not provide sufficient overlap, the denoised reads were concatenated using mergePairs with justConcatenate = TRUE. A sequence table was then constructed, and chimeric sequences were assessed using the consensus method implemented in removeBimeraDenovo. The corresponding filtering and denoising parameters have now been reported explicitly in the revised Methods.

L177: Spearman's correlation test, like correlation tests in general, requires a considerable number of samples to produce reliable results. Please review your analysis.

***Response**:*
We agree with the reviewer that Spearman's correlation-based co-occurrence analysis is not appropriate for this dataset because the number of samples is too small to support reliable correlation inference. We have therefore removed the co-occurrence analysis from the manuscript, including the associated Spearman correlation test, network results, figure/table references, and interpretation.

The revised manuscript no longer presents co-occurrence patterns or correlation-based associations among taxa. Instead, microbial community structure is summarized descriptively at the individual-sample level using taxonomic composition, alpha-diversity

summaries, and descriptive ordination. We also state explicitly in the Methods that no correlation analyses were performed and that no claims of statistically significant correlations or co-occurrence relationships are reported.

L210: I am not entirely sure how the differential analysis carried out here with 2 samples per treatment group (or less?). Please revise your analysis to ensure you only use statistical tests that are appropriate for your dataset.

***Response**:*
We agree with the reviewer that the previous ALDEx2 differential-abundance analysis was not appropriate for the sampling design, because the dataset lacked sufficient replication within lakes or preservation-status categories. We therefore removed the ALDEx2 analysis from the manuscript, including the associated statistical results, p-values, and interpretations.

The revised Methods now state that PICRUSt2 outputs were used only for descriptive visualization of sample-level predicted functional profiles. Predicted pathway abundances were converted to within-sample relative abundances, and no pooling by lake or preservation status and no inferential differential-abundance tests were conducted. We also revised the Results and Discussion to avoid claims of statistically significant differences in predicted functional pathways.

L233: Please could you explain and clarify in the 'Methodology' section how you filtered out ASVs 'with no name available'? Did this filtering apply only to unclassified ASVs, or also to those with multiple affiliations at certain taxonomic levels?

***Response**:*
We thank the reviewer for identifying this ambiguity. We have revised the Methods to clarify that the phrase "ASVs with no name available" was imprecise. ASVs lacking an assignment at the phylum level were excluded from the bacterial-community summaries, as were sequences classified as chloroplasts or other non-target eukaryotic lineages. In contrast, ASVs assigned at the phylum level but unresolved at the family, genus, or species level were retained and represented as unclassified categories at the corresponding rank. Taxonomic assignment was performed hierarchically using the RDP training set with assignTaxonomy; unresolved lower ranks were recorded as NA or unclassified rather than treated as multiple taxonomic affiliations. We have revised the Methods and Results accordingly.

L236-242: I don't think it's possible to observe a statistically significant difference between just two samples. This echoes my general comments on the proper use of statistical tests.

***Response**:*
We agree that a statistically significant difference cannot be reliably inferred from only two individual samples without biological replication. We therefore removed the corresponding statistical comparisons, p-values, significance symbols, and post hoc tests from the manuscript and figures. The Results were revised to describe differences among samples only descriptively. We also now state explicitly that inferential testing was not performed because the study comprised only six samples and temporal sampling was uneven among lakes.

L271: I believe the authors are referring to 'alpha diversity' in this section? Please use standard terminology for diversity analysis. Once again, the authors must be very cautious when interpreting the results of statistical tests carried out on such a small sample.

***Response**:*
We agree and have revised the terminology throughout this subsection. Coverage-standardized Hill numbers are now explicitly described as measures of alpha diversity, whereas Bray–Curtis dissimilarities and principal coordinates analysis are described as measures of beta diversity. We also removed inferential statistical tests and restricted the interpretation to descriptive patterns among individual samples because of the small and unevenly replicated dataset.

L281-283: This is an overstatement that is not sufficiently supported by your results and statistical tests. It is not possible to demonstrate temporal or spatial heterogeneity by comparing just two samples. The same applies to several of the claims made in the discussion.

***Response**:*
We agree and have revised the terminology throughout this subsection. Coverage-standardized Hill numbers are now explicitly described as measures of alpha diversity, whereas Bray–Curtis dissimilarities and principal coordinates analysis are described as measures of beta diversity. We also removed inferential statistical tests and restricted the interpretation to descriptive patterns among individual samples because of the small and unevenly replicated dataset.

L215-295: As mentioned above, I recommend either omitting this part of the analysis or re-running it using a larger sample size. The Spearman's correlation test has been misapplied here.

***Response**:*
We agree with the reviewer that the limited and unevenly replicated dataset does not support reliable inferential statistical comparisons. We therefore comprehensively revised the analytical framework and removed all hypothesis-testing procedures that treated the six samples as sufficient biological replication. Specifically, we removed the Kruskal–Wallis and post hoc comparisons of ASV abundances, the statistical comparisons between sampling dates, the ALDEx2 differential-abundance analysis and associated FDR thresholds and effect sizes, the Spearman correlation-based co-occurrence network, and any related p-values or significance statements. We also did not apply PERMANOVA to the Bray–Curtis dissimilarities. The revised Results now present taxonomic composition, coverage-standardized alpha-diversity estimates, Bray–Curtis ordination, and predicted functional profiles descriptively at the individual-sample level. The corresponding Methods, figures, Results, and Discussion were revised accordingly, and the limitations imposed by the small and uneven sampling design are now stated explicitly.

L305: once again, it is not possible to draw this conclusion from a sample of this size.

***Response**:*
We agree with the reviewer that the limited sampling design does not support conclusions regarding temporal stability or temporal variation. We therefore removed the former subsection entitled "Temporal bacteriome composition in Tota and Fúquene aquatic systems" and deleted the statement suggesting temporal stability in dominant taxa. Differences between the two available sampling dates for Fúquene and Tota are now mentioned only descriptively within the taxonomic-composition section, with an explicit

statement that each date was represented by a single sample and that no temporal inference was made.

L319: Why this interest? Please provide a more detailed explanation of the interest in these taxa and mention it in the introduction.

***Response**:*
We thank the reviewer for requesting a clearer justification. We expanded the Introduction to explain that the interest in these taxa extends beyond human health and is framed within a One Health context. The genus Mycobacterium includes environmental nontuberculous mycobacteria associated with humans, cultured and wild fish, reptiles, and other aquatic organisms, while other detected genera, such as Leptospira and Legionella, include lineages of potential human or animal-health relevance. We nevertheless emphasize that genus-level detection based on the V3–V4 region cannot demonstrate the occurrence of pathogenic species, viable organisms, host infection, virulence, or health risk. Accordingly, the analysis is presented only as exploratory phylogenetic placement and taxonomic observation.

L380: Please reformulate the sentence by removing the word "significant". This applies to statistical tests, and your results do not support such a claim.

***Response**:*
We agree with the reviewer. The term "significant" was inappropriate because the limited and unbalanced sampling design did not support reliable inferential comparisons of predicted functional profiles. We therefore removed the ALDEx2 differential-abundance analysis, the associated p-values and false-discovery-rate results, and all statements referring to statistically significant functional differences. Predicted pathways are now described only as sample-level patterns inferred by PICRUSt2.

L381: I do not see how these results could be linked to the size of the lake and its spatial heterogeneity. Please rephrase your argument and be careful not to overstate your point.

***Response**:*
We agree with the reviewer. Lake size and within-lake spatial heterogeneity were not evaluated by the sampling design and therefore cannot be invoked as explanatory variables. The sentence was removed and the opening of the Discussion was rewritten to present the study as an exploratory description of bacterial community composition in six water samples from four Andean lakes. References to lake size and spatial heterogeneity as factors explaining the observed patterns were removed throughout the Discussion and Conclusions.

The revised text now interprets differences strictly at the sample level and explicitly states that the observed variation cannot be attributed causally to lake size, spatial heterogeneity, anthropogenic pressure, preservation status, sampling period, water-column depth, or any other unmeasured environmental driver.

L400: Please modify terms such as "stable", "heterogeneity", "significant", etc. The results don't support these claims.

***Response**:*
We agree. Terms implying inferential support or ecological properties not evaluated by the study, including "stable," "community stability," "heterogeneity," "significant," "resilience," and "dynamic," were removed or replaced with explicitly descriptive language.

The revised Discussion now states that community composition and coverage-standardized diversity "differed descriptively among the six samples." Calderona and Colorado are described only as showing lower observed richness and diversity and high relative abundances of a limited number of taxa. No claims are made regarding community stability or statistically significant differences.

We also added a specific clarification that assessment of community stability, resistance, or resilience requires a defined disturbance and an adequately replicated temporal design, which was not available in this study.

L401-403: overstatement

***Response**:*
We agree and removed this interpretation entirely. The revised manuscript does not state that preservation status or anthropogenic pressure caused the observed diversity patterns. Although Fúquene showed comparatively higher richness and diversity than Calderona and Colorado, this observation is now reported only as a descriptive sample-level pattern.

The Discussion explicitly states that no concurrent physicochemical, hydrological, or land-use variables were measured and that the limited and unbalanced sampling design prevents causal evaluation of preservation status, anthropogenic pressure, or other environmental drivers.

L421-423: Based on what? Your dataset can't support such statement.

***Response**:*
We agree. The statement attributing differences between the Tota samples to seasonal change was removed. The revised Discussion clarifies that the two Tota samples represent only two sampling dates and that one corresponds to a historical December 2018 sample reprocessed alongside the primary 2019 dataset. These samples therefore do not constitute replicated temporal or seasonal sampling.

The observed differences are now interpreted only as variation between individual samples and not as evidence of seasonal dynamics, temporal instability, or responses to nutrient, temperature, oxygen, hydrological, or biological gradients.

L429-441: See my previous comments about co-occurrence and Spearman test. I suggest strongly to remove this part.

***Response**:*
We agree and removed the co-occurrence analysis entirely from the revised manuscript. The network analysis, Spearman correlations, associated figure, Methods description, Results, Discussion, and Conclusions were deleted.

Accordingly, statements regarding shared niches, indirect microbial interactions, ecosystem resilience, conservation predictions, and a specialized relationship between Mycobacterium and Polynucleobacter were also removed. The revised manuscript does not infer ecological interactions from taxonomic co-occurrence.

L474-476: overstatement

***Response**:*
We agree. Statements linking predicted functional profiles to preservation status, anthropogenic pressure, or nutrient availability were removed. The revised functional analysis is entirely descriptive and presents variation among individual samples without assigning samples to preservation-status groups for inferential comparison.

The Discussion now explains that PICRUSt2 predictions are derived from ASV phylogenetic placement and available reference genomes and therefore may partly reflect differences in taxonomic composition. However, they are not interpreted as evidence of gene presence, expression, metabolic activity, or statistically supported associations with environmental conditions.

Because nutrient concentrations and other physicochemical variables were not measured concurrently, the predicted functional patterns are not attributed to eutrophication, nutrient availability, or other environmental gradients.

L492-498: I suggest removing this part too. I have serious doubt about the robustness of such differential abundance analyses. The size of the dataset doesn't support it.

***Response**:*
We agree and removed the differential-abundance analysis from the revised manuscript. The ALDEx2 analysis, adjusted p-values, group comparisons, and all claims of significant differences among preservation-status categories were deleted from the Methods, Results, figures, Discussion, and Conclusions.

The revised Figure 2 now presents only:

- *sample-level weighted NSTI values.*
- *the 30 pathways with the highest total predicted abundances;*
- *a descriptive Bray–Curtis PCoA of the complete predicted functional profiles;*

No inferential differential-abundance tests or PERMANOVA were conducted. Functional variation is interpreted descriptively at the individual-sample level, and the two sampling events available for Fúquene and Tota are not treated as biological replicates or evidence of temporal patterns.

Figures:

Fig1. Panel a does not really provide any information and could be incorporated into a table, for example Table1. The captions for panels e and f are difficult to follow. I would recommend using a more common visualisation, such as the one in panel d.

Fig2. Panel a does not currently contribute anything to the analysis or discussion. I would recommend removing this figure or replacing it with a box plot containing a larger number of samples.

Fig4. The captions are not readable.

Response:
We thank the reviewer for these recommendations. The figures were comprehensively reorganized in the revised manuscript. The former Figure 1 was replaced by a new composite figure containing only the principal taxonomic and diversity results. The uninformative panel from the previous version was removed from the main figure, and

sequencing-depth and rarefaction information is now presented separately in Supplementary Figure S1. The less intuitive panels were replaced by more conventional visualizations, including stacked relative-abundance profiles, coverage-standardized Hill diversity, and Bray–Curtis principal coordinates analysis. The Figure 1 caption was rewritten to describe each panel explicitly.

The former Figure 2 was removed. The co-occurrence network was deleted because the six-sample dataset was insufficient for a reliable correlation-based network analysis, and the comparisons between sampling dates were removed because the study design did not support conclusions regarding temporal variation or stability. The remaining informative diversity results were incorporated into the revised Figure 1.

The former Figure 3, showing the exploratory maximum-likelihood placement of Mycobacterium ASVs, was moved to Supplementary Figure S2. This change reduces the emphasis placed on an analysis based on the limited species-level resolution of the V3–V4 region.

Consequently, the revised manuscript now contains two main figures: Figure 1, summarizing taxonomic composition and alpha and beta diversity, and Figure 2, presenting the descriptive PICRUSt2 functional predictions. Supplementary Figure S1 presents sequencing-depth and rarefaction assessments, whereas Supplementary Figure S2 presents the exploratory Mycobacterium phylogeny.

Minor comments:

L93-94: "the sea level, and is the largest remaining…"? This comment applies to several places in the manuscript. The text will need to be checked to ensure it does not contain errors of this kind.

Response:
We thank the reviewer for identifying this grammatical error. The sentence was removed during the broader revision of the study-area description. The revised text now reads: "The study included six water samples from four Andean lentic systems in the Eastern Cordillera of Colombia: Fúquene Lagoon, Tota Lake, Calderona Lagoon, and Colorado Lagoon (Table 1)."

L126: Please avoid giving the reference twice and write instead "Paver and coworkers (2020).

Response:
We agree and revised the citation accordingly. Similar instances of duplicated narrative and parenthetical citations were also checked and corrected throughout the manuscript to ensure consistent reference formatting.

L138: Please correct the title by removing "Assembly". I don't think any assembly step was done here.

Response:
We agree. No sequence-assembly step was performed in this analysis. The subsection title was corrected to: 2.2. DNA extraction and 16S rRNA gene amplicon sequencing

Anonymous Referee #3

The article presents a study analyzing the microbiome of four different lakes located in the Eastern Cordillera of Colombia. The underlying premise is that two of the lakes are relatively well preserved, whereas the other two are more heavily affected by human activities and environmental degradation, exhibiting higher levels of eutrophication and contamination.

The objective of the study is interesting because it focuses on the analysis of the 16S rRNA gene to characterize the bacterial communities inhabiting the lakes and compare their composition. The study also includes a functional analysis based on these amplicons. Although this analysis is presented as a prediction of potential functional activities, it might provide valuable insights into the possible ecological roles and metabolic functions of the bacterial communities identified.

> ***Response***:
> *We thank the reviewer for the positive assessment of the study objective and for recognizing the value of combining 16S rRNA gene-based bacterial community characterization with predicted functional profiling. In the revised manuscript, we have clarified that the study is exploratory and descriptive, based on six water samples from four Colombian Andean lakes. We also clarified that the preservation-status categories were assigned qualitatively from available environmental information and were not treated as statistically replicated explanatory factors.*
>
> *In addition, we revised the Abstract, Introduction, and Methods to emphasize that PICRUSt2 outputs represent predicted genomic potential inferred from 16S rRNA gene data, rather than direct evidence of gene presence, gene expression, metabolic activity, or ecosystem process rates. Thus, the functional analysis is now presented as a hypothesis-generating component of the study.*

Mayor comments:
The authors acknowledge the limited number of samples available for comparing the lakes. However, it is unclear why only one sample was collected from two of the lakes, whereas two samples were collected from the other two lakes.

> ***Response***:
> *We thank the reviewer for pointing out that the unequal number of samples among lakes required clarification. The original sampling effort was constrained by access, logistics, public-order conditions, and available resources. Subsequent plans to expand the dataset through additional balanced sampling were delayed by pandemic-related restrictions and continued access limitations.*
>
> *We also clarified that, for this reason, the dataset was treated as an exploratory collection of individual water samples rather than as a replicated lake-level comparison. Accordingly, the revised manuscript does not use the dataset to test lake-level effects or preservation-status effects, and all results are interpreted descriptively at the sample level.*

The manuscript includes an entire section devoted to a phylogenetic analysis of sequences assigned to the genus Mycobacterium. A clearer justification is needed for focusing specifcally on this genus, and, more importantly, for emphasizing this analysis when the authors

themselves acknowledge that the results should be interpreted with caution due to the limited taxonomic resolution at the species level.

***Response**:*
We thank the reviewer for highlighting the need for a clearer rationale and a more proportionate treatment of this analysis. We revised the Introduction to explain that Mycobacterium was selected because nontuberculous mycobacteria are common inhabitants of natural and engineered aquatic environments and include lineages of potential relevance to human, veterinary, aquaculture, and wildlife health. However, we agree that the V3–V4 region of the 16S rRNA gene does not provide sufficient resolution for reliable species-level identification within this genus.

Accordingly, we substantially reduced the emphasis placed on this analysis. All definitive species-level assignments and species-specific interpretations were removed, and the analysis was reframed as an exploratory phylogenetic placement of Mycobacterium ASVs relative to named reference sequences. Species names are now used only to identify the reference sequences included in the tree and are not assigned to the environmental ASVs. The Results explicitly state that the observed placements indicate phylogenetic affinity but do not establish species identity, pathogenicity, viability, virulence, host infection, or health risk. In addition, the maximum-likelihood tree was moved from the main figures to Supplementary Figure S2, and the corresponding discussion was shortened. Definitive taxonomic identification would require future analysis using cultured isolates, whole-genome sequencing, or validated higher-resolution marker genes.

- Discussion contains several very general statements that do not provide specific insights. For example, in lines 423–428, the authors mention that "contributing factors such as nutrient fluctuations, temperature, light..." influence the microbial communities. Are these factors relevant only to Lake Tota or to all the lakes studied? If they apply to all lakes, do the authors have measurements for any of these environmental variables collected during sampling?

***Response**:*
We thank the reviewer for this observation and agree that the former wording could be interpreted as attributing the observed microbial patterns to environmental variables that were not measured. No physicochemical variables were collected concurrently with microbiological sampling, including nutrient concentrations, dissolved oxygen, chlorophyll-a, pH, temperature, conductivity, organic-matter indicators, or hydrological variables. Therefore, environmental–microbiological correlations could not be performed.

The Discussion has been revised extensively to remove unsupported explanations based on nutrient fluctuations, temperature, oxygen conditions, light availability, rainfall, evaporation, phytoplankton, zooplankton, or seasonal dynamics. These factors are now mentioned only as mechanisms documented in previous freshwater studies and are explicitly distinguished from variables evaluated in the present study. We also removed the former statement that seasonal changes influenced bacterial abundance and diversity in Tota. The two Tota samples are now described as individual observations from two sampling dates, one of which was a historical December 2018 sample, and not as replicated temporal or seasonal sampling.

The revised text states explicitly that differences among samples cannot be attributed to eutrophication, nutrient availability, anthropogenic pressure, preservation status, lake size, spatial heterogeneity, sampling period, water-column depth, or other unmeasured environmental drivers.

- Similarly, in line 499, the authors refer to the presence of infectious bacteria associated with diseases and their potential importance for public health. However, earlier in the manuscript (lines 368–370), they state that these functional assignments should not be interpreted literally because they are inferred based on gene homology. This represents a contradiction that should be clarified.

***Response**:*
We agree and have removed the interpretation that KEGG pathways labelled as infectious diseases indicate the presence of infectious bacteria or public-health risk. The revised manuscript now explains that organism-, disease-, drug-resistance-, and eukaryote-associated KEGG labels reflect the structure and nomenclature of the reference database and the occurrence of homologous genes within annotated pathways.

These labels are no longer interpreted as evidence of the named organisms, pathogenicity, virulence, antimicrobial resistance, infection, or corresponding biological processes in the sampled lakes. The PICRUSt2 results are now described only as a descriptive approximation of predicted genomic potential based on ASV phylogenetic placement and available reference genomes, rather than as direct evidence of gene presence, expression, metabolic activity, or ecological function.

We also revised the interpretation of Leptospira, Legionella, and Mycobacterium. Their detection is now reported strictly at the taxonomic resolution supported by the short 16S rRNA gene region. The manuscript explicitly states that these assignments do not establish pathogenic species, viable cells, transmission, infection, or public-health risk.

- The Discussion ends with an entire paragraph describing the study's limitations. The points raised there essentially demonstrate that additional sampling is needed and that both the phylogenetic and functional results should be interpreted cautiously. However, throughout the manuscript, these analyses are given considerable emphasis. Rather than focusing so extensively on the study's limitations, the authors should instead emphasize the conclusions that can be robustly supported by the type of analysis performed, namely 16S rRNA gene sequencing.

***Response**:*
We agree and reorganized both the Discussion and Conclusions. The former extended limitations paragraph was removed, and the principal limitations are now integrated directly into the relevant interpretative sections. This allows each result to be discussed together with the specific constraint affecting its interpretation without ending the Discussion with an extensive catalogue of methodological weaknesses.

The revised Discussion places primary emphasis on findings directly supported by the 16S rRNA gene data: the predominance of common freshwater bacterial phyla, descriptive differences in taxonomic composition and coverage-standardized diversity among samples, genus-level detection of selected low-abundance taxa, and exploratory phylogenetic placement of Mycobacterium-related ASVs. Species-level claims based on the V3–V4 region were removed, the co-occurrence analysis was deleted entirely, and the functional analysis was reduced to descriptive PICRUSt2 predictions without differential-abundance testing or environmental attribution.

The Conclusions were also rewritten to focus on the descriptive baseline provided by the six samples. A concise final statement identifies the need for replicated sampling, concurrent

physicochemical measurements, metagenomic sequencing, and targeted taxonomic validation, while avoiding unsupported conclusions about preservation status, temporal dynamics, ecological interactions, pathogenicity, or environmental causation..

Minor comments

- The Introduction would benefit from a more detailed description of the potential differences in the geological origins of the lakes, as this is stated to be an important factor in line 111. However, no information is provided to support this claim beyond Table 1. Could these geological differences explain the distinct microbial community compositions observed among the lakes?

***Response**:*
We thank the reviewer for noting that the geological context was insufficiently described. We expanded the Introduction to include all four water bodies. Fúquene and Tota are described using published geomorphological and paleolimnological evidence, Calderona is identified as a high-elevation glacial lake, and Colorado is described more cautiously as a lower-elevation, rainfall-influenced wetland because we found no sufficiently robust geological source supporting a formal "pluvial-origin" classification. We also clarify that these contrasting settings could influence microbial habitats indirectly through basin morphology, sediments, hydrology, residence time, and water chemistry. However, because these variables were not systematically measured and the study lacked adequate replication, geological or hydrological origin cannot be identified as the cause of the observed microbial differences.

- In the sampling methodology, the materials used for sample collection are not described in sufficient detail. For example, the manufacturer of the "Bottle 1 Sequi disk" (line 123) is not specified, nor is the type of filter used for sample filtration (line 124).

***Response**:*
We thank the reviewer for identifying the insufficient description of the sampling materials. We revised the Methods to clarify that water was collected at a depth of 5 m using a custom-made, non-commercial Schindler–Patalas-type sampler constructed from a thoroughly cleaned plastic bottle; therefore, no commercial manufacturer or model applies. We also added the diameter and color of the Secchi disk, the diameter and opening size of the prefilter, and the membrane material, filtration area, manufacturer, and catalog number of the Sterivex units. The preservation reagent and transport conditions were also specified.

- In the DNA extraction section, could the authors briefly explain the purpose of CTAB in the extraction protocol? (lines 140–141).

***Response**:*
We thank the reviewer for this suggestion. We revised the DNA extraction section to clarify that CTAB acts as a cationic detergent that contributes to cell lysis and, in the presence of high salt, facilitates the removal of polysaccharides and other co-extracted contaminants that could inhibit downstream molecular analyses.

- Lines 145–146: Were the primers used adopted from previous studies, or were they specifically designed by the authors for this study? No reference is provided to support their use.

***Response**:*

We thank the reviewer for noting the missing reference. We have added the original DADA2 citation after the description of chimera removal using removeBimeraDenovo.

- Line 160: please include a reference after "removeBimeraDenovo".

***Response**:*
We thank the reviewer for identifying the missing reference. The primers were not designed specifically for this study but were adopted from Klindworth et al. (2013). We have added the corresponding citation and clarified the primer names, sequences, targeted V3–V4 region, and basis for their selection.

- Line 162: Why was a threshold of 100 reads selected instead of a lower or higher value? This appears to be a rather arbitrary cutoff, and it may be relatively high, potentially affecting the detection of taxa that are present at low abundance and influencing the identification of taxa specific to each lake.

***Response**:*
We thank the reviewer for raising this concern. Upon re-examining the original DADA2 analysis script, we found that the manuscript incorrectly reported the application of a 100-read abundance threshold. No such ASV-level threshold was applied in the original pipeline. We have therefore removed this statement and revised the Methods to describe the actual filtering procedure. ASVs lacking a phylum-level assignment and non-target chloroplast or eukaryotic sequences were excluded, whereas ASVs unresolved at lower taxonomic ranks were retained as unclassified categories. We also revised the description of the DADA2 workflow to clarify the read-trimming parameters, error-model learning, concatenation of non-overlapping paired reads, and consensus-based chimera assessment.

- Line 173: Please rewrite this sentence, as it is difficult to understand what the word "respectively" refers to.

***Response**:*
We thank the reviewer for identifying the unclear wording. We rewrote the sentence to define each Hill-number order explicitly: q=0 as ASV richness, q=1 as exponential Shannon diversity, and q=2 as inverse Simpson diversity. We also separated the description of alpha-diversity estimation from the descriptive Bray–Curtis beta-diversity analysis.

- Lines 235-242: The authors compare ASV abundances among the lakes. Could they clarify whether the comparison is based on the abundances of the same ASVs across lakes, or whether it is simply an overall comparison to determine which lake contains ASVs with higher relative abundances?

***Response**:*
We thank the reviewer for requesting clarification. In the former version, the analysis compared the overall distributions of ASV read abundances among individual samples; it was not a matched comparison of the abundances of the same ASVs across lakes. We recognize that this approach treated ASVs within each sample as replicate observations and did not provide an appropriate test of differences among lakes. We therefore removed this analysis, including the associated pairwise comparisons, p-values, significance statements, and figure elements. The revised Results now describe ASV richness, taxonomic relative abundance, alpha diversity, and beta diversity only at the individual-sample level, without inferential comparisons among lakes or sampling dates.

- Line 238: Although the text states that no comparison was performed between Colorado and Fúquene, Figure 1 includes a comparison between Colorado and Fúquene 2. Is this a typographical error?

***Response**:*
We thank the reviewer for identifying this inconsistency in the former version. After reconsidering the statistical approach, we removed the ASV-abundance pairwise comparisons in their entirety because the limited sampling design did not support reliable inferential testing among lakes or sampling dates. Consequently, the revised Figure 1 contains no comparison brackets, p-values, or significance annotations, and the corresponding statements were removed from the Results. Community differences are now presented descriptively at the individual-sample level.

- Lines 235-242: The manuscript also states that statistically significant differences were detected at $p < 0.001$, whereas Figure 1 reports $p< 0.0001$. In addition, the number of asterisks varies from two to four across the different comparisons, but the corresponding p-value thresholds are not explained. The figure should either be revised or the statistical significance levels should be described more clearly.

***Response**:*
We thank the reviewer for identifying the inconsistency between the p-values reported in the text and those shown in the former Figure 1, as well as the absence of definitions for the significance symbols. After reconsidering the statistical design, we removed all inferential ASV-abundance comparisons from the manuscript because the limited and uneven sampling did not support reliable hypothesis testing. Accordingly, the revised Figure 1 contains no p-values, asterisks, significance brackets, or pairwise comparisons, and the corresponding statements were deleted from the Results. Differences among samples are now presented descriptively only.

- Regarding the diversity analysis, line 276 states that the inverse Simpson index was calculated, whereas the figure legend (line 312) refers to the Simpson index. Please clarify which diversity index was actually used.

***Response**:*
We thank the reviewer for identifying this inconsistency. The analysis used Hill numbers, for which $q=2$ corresponds to inverse Simpson diversity, not the Simpson index. We have revised the figure legend and the corresponding Methods and Results text to use the term "inverse Simpson diversity" consistently throughout the manuscript.

- Línea 280: The authors state that Calderona shows a gradual decline. However, the decline is still substantial, although not as pronounced as in the other lakes. Moreover, no statistical analysis is presented to support the claim that the pattern differs from those observed in the other lakes. Please clarify how this conclusion was reached.

***Response**:*
We thank the reviewer for identifying this overinterpretation. The statement that Calderona showed a more gradual decline was based only on visual inspection of the Hill-number profile and was not supported by a formal statistical comparison. We therefore removed this claim. The revised Results now describe the alpha-diversity patterns among individual samples descriptively and do not assert that Calderona differed significantly from the other lakes.

- Line 329-332: The authors mention the detection of the bacterial genera Leptospira and Legionella, but they do not indicate in which samples these genera were detected. This information can only be found in the supplementary table. Since these genera are highlighted in both the Results and the Discussion because of their potential significance, the manuscript should clearly state in which samples they were detected and their relative abundances. Furthermore, line 462 states that Legionella proliferates under specific environmental conditions, but these conditions are not described. Do the environmental conditions of the lakes included in this study meet those requirements?

> ***Response****:*
> *We thank the reviewer for this observation. Although the sample-specific occurrence and relative abundances of Leptospira and Legionella were already provided in Table S1, we agree that they should also be reported explicitly in the Results because these genera were discussed in the main text. We therefore added the samples and corresponding relative abundances for both genera. Leptospira was detected in the two Fúquene and two Tota samples, whereas Legionella was detected in all six samples and reached its highest relative abundance in Calderona. We also removed the statement suggesting that environmental conditions in the sampled lakes favored Legionella proliferation, because the relevant environmental variables were not measured contemporaneously. The revised manuscript treats these findings only as genus-level taxonomic observations and does not infer species identity, viability, pathogenicity, proliferation, or health risk.*

- Typos: In lines 67 and 69, the publication year is missing from the citation to Sessitsch et al. Additionally, in line 238, the word "Furthermore" appears to contain a typographical error.

> ***Response****:*
> *We thank the reviewer for identifying these typographical errors. They were corrected in the revised version.*