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.*