Clouds influence the functioning of airborne microorganisms, by R. Péguilhan et al.

Author response to comments by Referee #1.

All referee comments are shown in black, our author responses in blue; suggested new manuscript text is indicated in red; text citations are *in italic*.

In the manuscript "Clouds influence the functioning of airborne microorganisms", by Péguilhan, et al., the authors explore the metabolic activity of cloud-borne microbes in comparison to airborne microbes using metagenomic and metatranscriptomic approaches. A thorough analysis has been conducted to explore possible mechanisms once microbes experiencing humid conditions in clouds compared to open air, with respect to stress response, metabolism, etc. However, some theories, such as the "birch effect," lack robust support, and I find the presented data unconvincing, as I specify below.

We thank the Referee for evaluating our work and for their suggestions to improve the manuscript. We provide a point-by-point response to the comments below.

Sample collection:

1- What were the negative controls for the cloud and air samples? This should be clarified in the methodology and presented as SI data.

We agree that this basic information regarding controls was missing, and we acknowledge for it. We will include the related following text in the Materials and Methods section about sample collection (Section 2.1):
"*Negative controls consisted of unexposed collection liquid, and of collection liquid exposed to the sampling tank for 10 minutes, sampler off. These were taken immediately before sampling, and processed in parallel of samples. For atmospheric samples, ….*". and "*Samples and controls were processed immediately after sampling …*".
And, in Section 2.2 (Nucleic acid extraction and shotgun sequencing):
"*Only trace amounts of DNA could be obtained from negative controls (7.3 ng of DNA on average, 11.4 ng at maximum), and these were, thus, not processed for sequencing. In contrast, the total amounts of DNA and RNA recovered from environmental samples ranged from 42.6 to 838.7 ng and 22.5 to 244.8 ng, respectively. The corresponding total DNA and RNA concentrations in the air volumes sampled, as inferred from concentrations in the extracts, ranged from 0.03 to 0.73 ng DNA.m^{-3} and from 0.026 to 0.42 ng RNA.m^{-3}, respectively (Table S1)*".
In addition, we detected a mistake related with conversion factors in the concentrations of DNA and RNA as reported per volume of air in Table S1, and this will be corrected. This does neither have impacts on the statistics (non-parametric) nor on the conclusions.

2- As the manuscript cannot include seasonality, authors should constrain their samples to a specific season. Specifically, the cloud sample during springtime could impact diversity and abundance and introduce seasonal-related impact.

We aimed at evaluating statistically the influence of a specific environmental variable on the functioning of airborne microorganisms, *i.e.* the presence of condensed water, which is expected to represent one of the major limitations for microbial activity in the atmosphere. We agree with the Referee that the lack of systematic data is a weakness of our study and does not

allow any conclusions about possible temporal variability on the functioning of airborne microorganisms, but such an aim is not the motivation of our study.
The diversity of microorganisms in the air is known to be highly variable over short temporal and spatial scales (Fierer et al., 2008). Every air sample differs from others in one or the other temporal, spatial, or environmental variables. We tried to minimize this variability by contrasting samples collected at a single location (puy de Dôme Mountain station), using unique sampling methods. This site is conveniently exposed to ranges of meteorological conditions including the alternance of clear conditions and clouds, and also to variations of temperature, humidity, position respect to the boundary layer, air mass origin, etc. At this site, clouds are inherently more frequent during the cold season than during summer (Baray et al., 2019). To follow our hypothesis that condensed water may affect microbial functioning, we categorized the samples among two categories discriminated by the presence, or not, of liquid water (*i.e.*, RH >100%, LWC >0). In our study, clear atmosphere was collected in July, September and November, and clouds in March, July, October and November, so at similar periods of the year except for the March sample. This one particular cloud sample was not an outlier in our dataset, so except for prejudices there is no specific rationale for excluding it. We will develop this in the concluding paragraph in Section 5, as:
"*Our study focused in particular on the potential impact of clouds on microbial functioning, and it relies on samples collected on a single site, using unique sampling methods in order to avoid introducing site effects and methodological bias. We could qualitatively show that there are differences in microbial gene expressions in samples collected in cloud-free vs cloudy air masses. We used 'water availability' as a proxy to distinguish the two air mass types. However, cloudy air masses also differ from those outside clouds in a multitude of other environmental factors which are expected to play roles on aeromicrobiome's functioning, and they still need to be evaluated (Amato et al., 2023). Such variables include temperature, solar radiation, chemical composition, etc, and they are linked not only to clouds but also to altitude, location, day/night cycles and season. The synergy, temporal dynamics and arrangement of these variables (shocks, cloud cycles, freezing events, combination of chemicals, etc…) could also participate in shaping the aeromicrobiome in even more complex ways.*".

3- Table 1 - What was the time of sampling? It is not specified whether samples were collected during day or night.

All the samples were collected during daytime, typically from ~9:00 AM to 3:00 PM local time. This information was indeed missing, so we will add it in Table 1, and mention it in the Methods Section 2.1:
"*A total of nine cloud and six clear air events were sampled in 2019 and 2020, for periods of about two to six consecutive hours, during daytime.*".

Discussion:

4- P. 15, L. 56: I'm afraid authors are overstating their findings, suggesting this is the first time demonstrating the impact of atmospheric conditions on microbial functioning in the atmosphere (See Bryan et al, 2019, and others).

This comment relates to our statement that "*For the very first time, we demonstrate that atmospheric conditions influence multiple facets of microbial functioning in the natural atmosphere*."
The study by (Bryan et al., 2019) did not report microbial functioning, but rather structural biodiversity from amplicons, and evidence for viability from cultures and ATP. General

indicators of microbial viability and potential metabolic activity such as cultures, ATP or RNA are frequently reported in atmospheric samples (Amato et al., 2007, 2017; Fahlgren et al., 2010; Hill et al., 2007; Šantl-Temkiv et al., 2018; Vaïtilingom et al., 2012; Wirgot et al., 2017), but they do not provide any information about the actual biological functions themselves. We therefore disagree with Referee's comment.
The impact of environmental conditions on the survival of aerosolized microorganisms has long been studied in atmospheric simulation chambers (Ehrlich et al., 1970; Wright et al., 1969). Similarly, the influence of environmental variables on the metabolic activity of airborne cells was actually first demonstrated in simulation chamber, where the presence of volatile organic compounds could be linked with elevated ribosome content in bacteria cells (Krumins et al., 2014). In the natural environment, (Wirgot et al., 2017) observed a positive correlation between the concentrations of H_2O_2 and ATP in clouds, suggesting some extent of causality on the metabolism, but no indication regarding the biological functions actually involved. To our knowledge, so far only (Amato et al., 2019) reported biological functioning of microorganisms in the natural atmosphere, without a priori (using untargeted methods), on samples captured into a fixative agent. This study did however not investigate possible relationships with environmental conditions, specifically the presence of clouds, which is the purpose of the present study.
Hence, unless we are missing an important reference, we decide to maintain our statement that our study is the first to bring evidence for natural atmospheric conditions influencing multiple airborne microbial biological functions, but we rephrase it as follows:
"*For the first time, we demonstrate that multiple microbial functions are directly influenced by atmospheric conditions, specifically by the presence of clouds*.".

5- Section 4.1: it is problematic to deduce from higher RNA:DNA levels that the metabolic levels in clouds are higher. Especially as the annotated genes in MT are not significantly different between the two environments, as seen in Table S1. Instead, it seems that the levels of DNA gene annotation in clouds are the factor that results in a higher RNA:DNA ratio in clouds.

We admit that our text may not have been fully clear. The amount of nucleic acids (in mass) and the number of annotated genes (in number of unique hits of the sequences against the database) are independent variables. The conclusion that clouds exhibit higher levels of microbial metabolic activity is based on the respective amounts of RNA and DNA extracted from samples, not from the numbers of annotated genes. The RNA-to-DNA concentration ratio in samples ranged from 0.21 to 3.62 overall, and this was significantly higher in clouds than in clear atmosphere, as shown in Fig 1A and Table S1. To avoid confusion, we will specify in the Discussion RNA-to-DNA "*concentration*" ratio:
"*Higher concentration of RNA, respect to DNA, in clouds than in cloud-free air suggests higher levels of microbial metabolic activity (Baldrian et al., 2012; Salazar et al., 2019). This remains to be assessed quantitatively through more direct activity measurements."*

6- Thus, the Birch effect doesn't seem likely to explain your findings. Instead, I suggest considering an environmental switch of specific genes as related to the environmental conditions.

The Birch effect corresponds to a pulse of CO_2 emission from dry soils following rainfall, due to the promotion of microbial activity by water (Griffiths and Birch, 1961). We observed signs of increased biological activity in relation with liquid water. Hence this analogy appears sound with regards to what is known in soils. We did not evaluate it here, but the release of CO_2 from

biological activity in clouds is expected to be negligible compared to soils due to much lower biomass (Ervens and Amato, 2020; Vaïtilingom et al., 2013).
The current text in the manuscript clearly refers to, in particular, the increase of biological activity during the Birch effect, but we will make it clearer in the Discussion Section "*A 'Birch effect' up in the sky*", as:
"*The switch of functional gene expression observed in microorganisms between clear atmosphere and clouds therefore suggests a phenomenon similar to the "Birch effect" that occurs in dry soils in response to rewetting by rain, where the sudden influx of water triggers a burst of microbial activity*".
As it is stated in the current text, the impact in terms of gas fluxes is necessarily low: "*As with soils – but necessarily to a much smaller extent because of the low biomass – the resurgence of microbial activity in clouds may lead to the release of gaseous biogenic compounds* […] *which is also supported by the overrepresentation of ammonium utilization process*".

7- Moreover, if a birch effect occurs, I suspect it would be linked with spore-forming species, and the transformation from the dormant to the vegetative form would be characterized by key genes that should be presented to support the proposed theory.

Indeed, elements in support of spore germination are discussed in Section 4.2 "Airborne fungal spores initiate germination in clouds". We will extend this to include mentions of specific biomarkers overrepresented in cloud metatranscriptomes, as:
"*In agreement with the overrepresentation of numerous transcripts, it is likely that fungal spores initiate germination in clouds. These include translation initiation and elongation factors affiliated to several taxa of fungi (elF4E, eEF3 and others) (Osherov and May, 2001; Van Leeuwen et al., 2013; Li et al., 2022), chitin deacetylase (Leroch et al., 2013) and other regulatory protein genes such as areA (Kudla et al., 1990).*".

8- Section 4.3: This section appears to rely more on generalizations than on solid data. I recommend either omitting this part or revising it for clarity and support.

The possibility that microorganisms could multiply in atmospheric water (clouds, fog), supported by dissolved nutrients and liquid water, was suggested earlier from others (Fuzzi et al., 1997; Sattler et al., 2001). We agree that this section about biomass production is not sufficiently supported by data in our work, so we will merge this section with the next Discussion Section "*Utilization of nutrients and interactions with chemistry*", and modify the text accordingly as:
"*Microbial activity is driven by the balance between water availability and accessibility to substrates (Skopp et al., 1990). Although not evaluable here, the amounts of water retained by efflorescent aerosols below water vapor saturation may be sufficient to sustain microbial activity, down very low values of relative humidity (Cruz and Pandis, 2000; Ervens et al., 2024). In clouds, i.e., above saturation levels, the large amounts of available water make it even conceivable that bacterial multiplication occurs. Bulk cloud water indeed contains enough nutrients to sustain microbial growth including carboxylic acids, aldehydes, sugars, amino-acids, ammonium, nitrate, etc. (Amato et al., 2007; Bianco et al., 2016, 2018, 2019; Deguillaume et al., 2014; Renard et al., 2022), and the level of microbial activity at 0°C was shown to be compatible with it (Sattler et al., 2001). Field observations indicate that fog carries higher biomass than clear atmosphere (Fuzzi et al., 1997; Saikh and Das, 2023), while estimations suggest that microbial mass may double during cloud's lifetime (Ervens and Amato, 2020). The fact that statistically only 1 out of ~10 000 droplets contains a microbial cell in aerially suspended water, as opposed to bulk water, potentially causes a very efficient and rapid*

depletion of nutrients in these small biotic volumes (Khaled et al., 2021) (~10^{-6} µl for 20 µm diameter droplets, so a cell concentration of at least ~10^{9} cells mL^{-1} in biotic droplets), which exposes cells to starvation and may limit metabolic processes (Gray et al., 2019).
The overrepresentation of transcripts related to carbon, ammonium and nitrate utilization in clouds supports that carbon and nitrogen biological processing occurs ….".

9- Figure 1A: Change "clear situation" to "clear atmosphere/open air". Also seen in Fig. 4 and across the manuscript.

We thank the Referee for pointing this out. Where necessary, "*clear situation*" will be modified as "*clear atmosphere*". The following figures will thus be updated: Fig 1, Fig 3, Fig 4, and Fig S3, S8, S9, S11, as well as Tables 1 and S1.

References cited:

Amato, P., Parazols, M., Sancelme, M., Mailhot, G., Laj, P., and Delort, A.-M.: An important oceanic source of micro-organisms for cloud water at the Puy de Dôme (France), Atmospheric Environment, 41, 8253–8263, https://doi.org/10.1016/j.atmosenv.2007.06.022, 2007.

Amato, P., Joly, M., Besaury, L., Oudart, A., Taib, N., Moné, A. I., Deguillaume, L., Delort, A.-M., and Debroas, D.: Active microorganisms thrive among extremely diverse communities in cloud water, PLOS ONE, 12, e0182869, https://doi.org/10.1371/journal.pone.0182869, 2017.

Amato, P., Besaury, L., Joly, M., Penaud, B., Deguillaume, L., and Delort, A.-M.: Metatranscriptomic exploration of microbial functioning in clouds, Scientific Reports, 9, 4383, https://doi.org/10.1038/s41598-019-41032-4, 2019.

Amato, P., Mathonat, F., Nuñez Lopez, L., Péguilhan, R., Bourhane, Z., Rossi, F., Vyskocil, J., Joly, M., and Ervens, B.: The aeromicrobiome: the selective and dynamic outer-layer of the Earth's microbiome, Frontiers in Microbiology, 14, 2023.

Baldrian, P., Kolařík, M., Stursová, M., Kopecký, J., Valášková, V., Větrovský, T., Zifčáková, L., Snajdr, J., Rídl, J., Vlček, C., and Voříšková, J.: Active and total microbial communities in forest soil are largely different and highly stratified during decomposition, ISME J, 6, 248–258, https://doi.org/10.1038/ismej.2011.95, 2012.

Baray, J.-L., Bah, A., Cacault, P., Sellegri, K., Pichon, J.-M., Deguillaume, L., Montoux, N., Noel, V., Seze, G., Gabarrot, F., Payen, G., and Duflot, V.: Cloud Occurrence Frequency at Puy de Dôme (France) Deduced from an Automatic Camera Image Analysis: Method, Validation, and Comparisons with Larger Scale Parameters, Atmosphere, 10, 808, https://doi.org/10.3390/atmos10120808, 2019.

Bianco, A., Voyard, G., Deguillaume, L., Mailhot, G., and Brigante, M.: Improving the characterization of dissolved organic carbon in cloud water: Amino acids and their impact on the oxidant capacity, Scientific Reports, 6, https://doi.org/10.1038/srep37420, 2016.

Bianco, A., Deguillaume, L., Vaïtilingom, M., Nicol, E., Baray, J.-L., Chaumerliac, N., and Bridoux, M.: Molecular Characterization of Cloud Water Samples Collected at the Puy de Dôme (France) by Fourier Transform Ion Cyclotron Resonance Mass Spectrometry, Environ. Sci. Technol., 52, 10275–10285, https://doi.org/10.1021/acs.est.8b01964, 2018.

Bianco, A., Deguillaume, L., Chaumerliac, N., Vaïtilingom, M., Wang, M., Delort, A.-M., and Bridoux, M. C.: Effect of endogenous microbiota on the molecular composition of cloud water: a study by Fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR MS), Scientific Reports, 9, 7663, https://doi.org/10.1038/s41598-019-44149-8, 2019.

Bryan, N. C., Christner, B. C., Guzik, T. G., Granger, D. J., and Stewart, M. F.: Abundance and survival of microbial aerosols in the troposphere and stratosphere, ISME J, 13, 2789–2799, https://doi.org/10.1038/s41396-019-0474-0, 2019.

Cruz, C. N. and Pandis, S. N.: Deliquescence and Hygroscopic Growth of Mixed Inorganic−Organic Atmospheric Aerosol, Environ. Sci. Technol., 34, 4313–4319, https://doi.org/10.1021/es9907109, 2000.

Deguillaume, L., Charbouillot, T., Joly, M., Vaïtilingom, M., Parazols, M., Marinoni, A., Amato, P., Delort, A.-M., Vinatier, V., Flossmann, A., Chaumerliac, N., Pichon, J. M., Houdier, S., Laj, P., Sellegri, K., Colomb, A., Brigante, M., and Mailhot, G.: Classification of clouds sampled at the puy de Dôme (France) based on 10 yr of monitoring of their physicochemical properties, Atmos. Chem. Phys., 14, 1485–1506, https://doi.org/10.5194/acp-14-1485-2014, 2014.

Ehrlich, R., Miller, S., and Walker, R. L.: Relationship Between Atmospheric Temperature and Survival of Airborne Bacteria, Appl Microbiol, 19, 245–249, 1970.

Ervens, B. and Amato, P.: The global impact of bacterial processes on carbon mass, Atmospheric Chemistry and Physics, 20, 1777–1794, https://doi.org/10.5194/acp-20-1777-2020, 2020.

Ervens, B., Amato, P., Aregahegn, K., Joly, M., Khaled, A., Labed-Veydert, T., Mathonat, F., Nuñez López, L., Péguilhan, R., and Zhang, M.: Ideas and perspectives: Microorganisms in the air through the lenses of atmospheric chemistry and microphysics, EGUsphere, 1–18, https://doi.org/10.5194/egusphere-2024-2377, 2024.

Fahlgren, C., Hagström, Å., Nilsson, D., and Zweifel, U. L.: Annual Variations in the Diversity, Viability, and Origin of Airborne Bacteria, Appl. Environ. Microbiol., 76, 3015–3025, https://doi.org/10.1128/AEM.02092-09, 2010.

Fierer, N., Liu, Z., Rodríguez-Hernández, M., Knight, R., Henn, M., and Hernandez, M. T.: Short-Term Temporal Variability in Airborne Bacterial and Fungal Populations, Appl Environ Microbiol, 74, 200–207, https://doi.org/10.1128/AEM.01467-07, 2008.

Fuzzi, S., Mandrioli, P., and Perfetto, A.: Fog droplets—an atmospheric source of secondary biological aerosol particles, Atmospheric Environment, 31, 287–290, https://doi.org/10.1016/1352-2310(96)00160-4, 1997.

Gray, D. A., Dugar, G., Gamba, P., Strahl, H., Jonker, M. J., and Hamoen, L. W.: Extreme slow growth as alternative strategy to survive deep starvation in bacteria, Nat Commun, 10, 890, https://doi.org/10.1038/s41467-019-08719-8, 2019.

Griffiths, E. and Birch, H. F.: Microbiological Changes in Freshly Moistened Soil, Nature, 189, 424–424, https://doi.org/10.1038/189424a0, 1961.

Hill, K. A., Shepson, P. B., Galbavy, E. S., Anastasio, C., Kourtev, P. S., Konopka, A., and Stirm, B. H.: Processing of atmospheric nitrogen by clouds above a forest environment, J. Geophys. Res., 112, D11301, https://doi.org/10.1029/2006JD008002, 2007.

Khaled, A., Zhang, M., Amato, P., Delort, A.-M., and Ervens, B.: Biodegradation by bacteria in clouds: an underestimated sink for some organics in the atmospheric multiphase system, Atmospheric Chemistry and Physics, 21, 3123–3141, https://doi.org/10.5194/acp-21-3123-2021, 2021.

Krumins, V., Mainelis, G., Kerkhof, L. J., and Fennell, D. E.: Substrate-Dependent rRNA Production in an Airborne Bacterium, Environ. Sci. Technol. Lett., 1, 376–381, https://doi.org/10.1021/ez500245y, 2014.

Renard, P., Brissy, M., Rossi, F., Leremboure, M., Jaber, S., Baray, J.-L., Bianco, A., Delort, A.-M., and Deguillaume, L.: Free amino acid quantification in cloud water at the Puy de Dôme station (France), Atmospheric Chemistry and Physics, 22, 2467–2486, https://doi.org/10.5194/acp-22-2467-2022, 2022.

Saikh, S. R. and Das, S. K.: Fog-Induced Alteration in Airborne Microbial Community: a Study over Central Indo-Gangetic Plain in India, Appl Environ Microbiol, 89, e01367-22, https://doi.org/10.1128/aem.01367-22, 2023.

Salazar, G., Paoli, L., Alberti, A., Huerta-Cepas, J., Ruscheweyh, H.-J., Cuenca, M., Field, C. M., Coelho, L. P., Cruaud, C., Engelen, S., Gregory, A. C., Labadie, K., Marec, C., Pelletier, E., Royo-Llonch, M., Roux, S., Sánchez, P., Uehara, H., Zayed, A. A., Zeller, G., Carmichael, M., Dimier, C., Ferland, J., Kandels, S., Picheral, M., Pisarev, S., Poulain, J., Acinas, S. G., Babin, M., Bork, P., Boss, E., Bowler, C., Cochrane, G., de Vargas, C., Follows, M., Gorsky, G., Grimsley, N., Guidi, L., Hingamp, P., Iudicone, D., Jaillon, O., Kandels-Lewis, S., Karp-Boss, L., Karsenti, E., Not, F., Ogata, H., Pesant, S., Poulton, N., Raes, J., Sardet, C., Speich, S., Stemmann, L., Sullivan, M. B., Sunagawa, S., Wincker, P., Acinas, S. G., Babin, M., Bork, P., Bowler, C., de Vargas, C., Guidi, L., Hingamp, P., Iudicone, D., Karp-Boss, L., Karsenti, E., Ogata, H., Pesant, S., Speich, S., Sullivan, M. B., Wincker, P., and Sunagawa, S.: Gene Expression Changes and Community Turnover Differentially Shape the Global Ocean Metatranscriptome, Cell, 179, 1068-1083.e21, https://doi.org/10.1016/j.cell.2019.10.014, 2019.

Šantl-Temkiv, T., Gosewinkel, U., Starnawski, P., Lever, M., and Finster, K.: Aeolian dispersal of bacteria in southwest Greenland: their sources, abundance, diversity and physiological states, FEMS Microbiol Ecol, 94, https://doi.org/10.1093/femsec/fiy031, 2018.

Sattler, B., Puxbaum, H., and Psenner, R.: Bacterial growth in supercooled cloud droplets, Geophysical Research Letters, 28, 239–242, 2001.

Skopp, J., Jawson, M. D., and Doran, J. W.: Steady-State Aerobic Microbial Activity as a Function of Soil Water Content, Soil Science Society of America Journal, 54, 1619–1625, https://doi.org/10.2136/sssaj1990.03615995005400060018x, 1990.

Vaïtilingom, M., Attard, E., Gaiani, N., Sancelme, M., Deguillaume, L., Flossmann, A. I., Amato, P., and Delort, A.-M.: Long-term features of cloud microbiology at the puy de Dôme (France), Atmospheric Environment, 56, 88–100, https://doi.org/10.1016/j.atmosenv.2012.03.072, 2012.

Vaïtilingom, M., Deguillaume, L., Vinatier, V., Sancelme, M., Amato, P., Chaumerliac, N., and Delort, A.-M.: Potential impact of microbial activity on the oxidant capacity and organic carbon budget in clouds, PNAS, 110, 559–564, https://doi.org/10.1073/pnas.1205743110, 2013.

Wirgot, N., Vinatier, V., Deguillaume, L., Sancelme, M., and Delort, A.-M.: H2O2 modulates the energetic metabolism of the cloud microbiome, Atmos. Chem. Phys., 17, 14841–14851, https://doi.org/10.5194/acp-17-14841-2017, 2017.

Wright, D. N., Bailey, G. D., and Goldberg, L. J.: Effect of Temperature on Survival of Airborne Mycoplasma pneumoniae, J Bacteriol, 99, 491–495, 1969.