aerobiology

*Article*

# Biological Characterisation of Hailstones from Two Storms in South Brazil

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**Citation:** Mantoani, M.C.; Quintino, T.B.; Emygdio, A.P.M.; Guerra, L.C.C.; Dias, M.A.F.S.; Dias, P.L.S.; Rodrigues, F.; Silva, D.M.C.; Duo Filho, V.B.; Rudke, A.P.; et al. Biological Characterisation of Hailstones from Two Storms in South Brazil. *Aerobiology* **2023**, *1*, 98–108. https://doi.org/10.3390/aerobiology1020008

Academic Editor: Chad J. Roy

Received: 27 October 2023
Revised: 5 December 2023
Accepted: 11 December 2023
Published: 13 December 2023



**Abstract:** Although studies focusing on the physicochemical properties of aerosols/clouds have not been performed extensively, even less attention has been given to hailstones and their biological composition. Here, we present the results of the physical and microbiological characterisation of 20 hailstones collected in Southern Brazil originating from two storms. Nearly half of the hailstones (9 out of 20, or 45%) did not contain any cultivable bacteria or fungi. A total of 18 bacterial species were found in hailstones from both storms, and the genus *Bacillus* was found in 5 out of the 11 hailstones, with *Bacillus cereus* being the most frequent bacterial species. Fungi, on the other hand, were only present in four hailstones derived from a single storm, with three fungal species identified and *Epicoccum nigrum* being the most frequent fungal species. HYSPLIT modelling indicated the different flow of air masses from the Amazon and Pacific Ocean that contributed to the loading of microorganisms found in the clouds at the time of the two storms. Our findings suggest that ca. 50% of hailstones have cultivable bacterial or fungal species, which came mainly from the local landscape with intrusions of air masses derived from the Amazon and the Pacific Ocean.

**Keywords:** citizen science; cloud formation; HYSPLIT; ice nucleation activity; primary biological aerosol particles

## 1. Introduction

Aerosols can influence the formation and development of clouds, playing an important role in global climate regulation by affecting cloud optical properties and lifetime, as well as the water cycle [1]. The chemical and physical properties of aerosols have been studied for a long time [2,3]. More recently, however, primary biological aerosol particles (hereafter, PBAPs), such as bacterial and fungal cells and spores, have gained much attention due to their importance to cloud properties, rain, snow, and hail formation [4–8]. When such PBAPs act as ice-nucleating particles (hereafter, INP), they affect ice water content in clouds, in particular at temperatures greater than −10 °C [9,10], which, in turn, impacts climate regulation and local-to-regional water regimes [11].

Clouds, in some respects, can be extreme environments characterised by low pressure and pH, with a mixture of organic and inorganic components that are toxic to the living fraction of PBAPs [12,13]. Nevertheless, not only are bacteria and fungi found in such adverse cloud environments, but they impact cloud physicochemical properties and rain [1,14,15]. These microorganisms can act in three ways in cloud formation and precipitation processes: (i) enhancing the phase change from vapour to liquid by acting as cloud condensation nuclei; (ii) accelerating coalescence via large particles; and (iii) enhancing the phase change from vapour or liquid to ice by acting as INP [1]. Whilst this implies that PBAPs involved in these processes would be found within hailstones derived from storm clouds, to date, only a few studies have investigated the microbial content of hailstones [16–19].

Among other sources, PBAPs primarily originate from soil, vegetation, and aquatic surfaces, while the atmosphere serves as their transient habitat [20]. The inherent complexity in atmospheric community assembly depends utterly on land-cover and land-use patterns [21]. In addition, factors such as humidity and dispersal capacity are essential for PBAPs transportation, impacting on the atmospheric processes depending on them [6,13]. Although studies focusing on storm clouds have been carried out previously, there is a lack of studies investigating PBAPs' presence in hailstones. Furthermore, the inherent difficulty of sampling associated with unpredictable nature of storm clouds makes it hard to sample hailstones and investigate their biological content. Thus, citizen science can be a useful tool that allow us access to hailstone samples from diverse locations, augmenting the chances of collecting more samples and/or spanning a bigger area of sampling [22].

In this context, to better understand the processes of hailstone formation and their relationship with ice nucleation by microorganisms, here, we provide the first work that characterises the physical properties and the microbiological composition of hailstones collected using a network of volunteer citizens in Brazil. For this, we obtained access to hailstone samples from two winter storms collected through a citizen science project of farmers in Southern Brazil, an area belonging to the Atlantic Forest biome that has been neglected in PBAPs research and lacks aerobiology knowledge [23]. We then investigated the microbiological content of the hailstones collected, as well as the atmospheric systems that acted in the same region, analysing back trajectories using HYSPLIT models. This enabled us to verify the contribution of air currents to the transport of the PBAPs, underpinning the onset of the storms in the studied region.

## 2. Materials and Methods

### 2.1. Area of Study

Hailstones were sampled in the northwest region of the state of Santa Catarina in Southern Brazil (Figure 1). The region, which belongs to the Upper Paraná River Basin, is characterised by an agricultural landscape [24] and is among the top regions with worst storms in the world [25,26]. Due to the main synoptical system that occurs there, especially because it is the gateway to the Mesoscale Convective Systems and the Low-Level Subtropical Jet [27–29] for Brazil and South America, this region has received significant attention from the scientific community in the past few years [30–32].

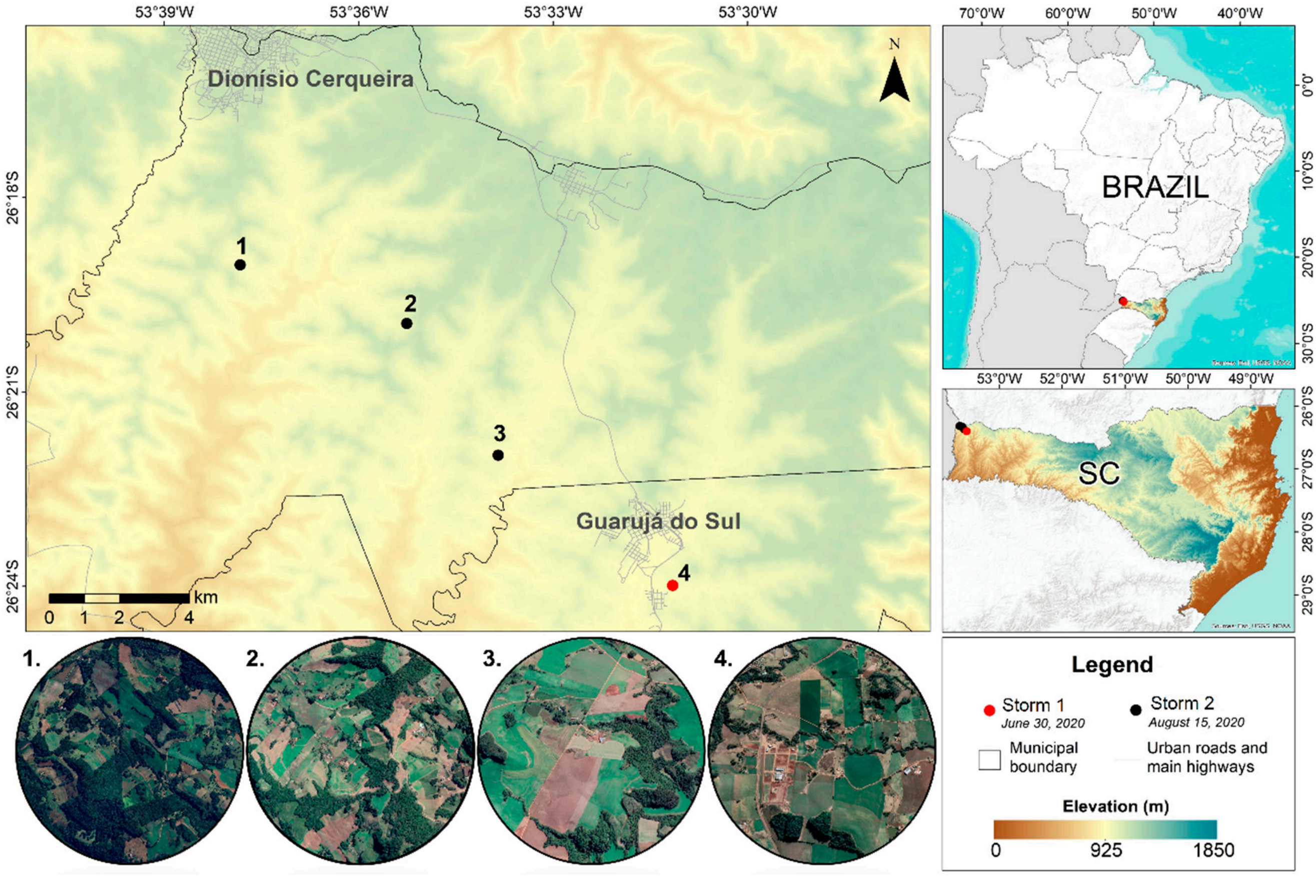


**Figure 1.** Map of the area of study showing the first sampling of hailstones carried out on 30 June 2020 (red dot) and the second sampling performed on 15 August 2020 (black dots), at Guarujá do Sul and Dionísio Cerqueira, respectively, in the state of Santa Catarina, Southern Brazil.

### *2.2. Hailstones Collection and Physical Analysis*

The two hailstorms occurred during the winter of 2020, on 30 June, in the municipality of Guarujá do Sul, and on 15 August, in the municipality of Dionísio Cerqueira. Once the storms ceased, hailstones were collected from the ground by our citizen science network composed of volunteer farmers and stored in high-density polyethylene containers inside a freezer to prevent defrosting [31]. Frozen samples were shipped to the University of São Paulo (USP) and stored in a freezer at −20 °C until analysis. A total of 20 hailstones were collected, 9 from the first storm (Guarujá do Sul) and 11 from the second (Dionísio Cerqueira).

Prior to sterilising their surfaces, hailstones were weighted and measured. As per Šantl-Temkiv et al. (2012), measurements were carried out inside a cold room (10 °C) to avoid ice melting and loss of material. For mass determination, hailstones were weighted three times using a fine analytical scale, providing us with an average of the final weight for each hailstone [16,31]. To estimate the diameter, we used a calliper ruler and measured the hailstones three times at three different orthogonal angles, as the hailstones were not perfectly round. An average of the three values was taken to estimate the diameter of each hailstone. To determine the average volume, hailstones were considered spheres [33] and the following formulae was used: Volume = $(4\pi r^3)/3$. Finally, the density of each hailstone was obtained by dividing their masses by their volumes.

### 2.3. Biological Characterisation of Hailstones

Before the biological characterisation, following Šantl-Temkiv et al. (2012) [16], hailstones were decontaminated. For that, they were rinsed with a mixture of 1% benzalkonium chloride and 70% ethanol, followed by a final rinse with sterile deionized water. After this step, each hailstone was put inside a sterilised falcon tube and left at room temperature (*c.* 25 °C) until melted. This meltwater was then used for the subsequential analyses. For bacteria, the culture medium R2A was used [16–18], whereas for fungi, a modified Dicloran Rosa Bengal culture medium was used [34,35]. Plates were inoculated using a spread 100 µL of hailstone meltwater in triplicates. Inoculated plates were incubated inside a biological incubator at *c.* 30 ± 2 °C for up to seven days for isolation and identified (Adolfo Lutz Institute Mycology Laboratory, São Paulo, Brazil).

To identify bacteria and fungi, as per Mantoani et al. (2023) [35], we used a Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS; MALDI Biotyper, Bruker Daltonics, EUA) [36]. This technology is based on interpretations of specific spectral masses of each microorganism, through the reading of the time of flight of laser-excited ribosomal proteins in a lipid matrix [36]. The instrument's calibration is performed using a standard strain (*E. coli* 16S), containing known spectra that ensure the sensitivity of the test and identification. Through the results provided in the score, it is possible to identify genus (scores between 1.7 and 1.9) and species (scores above 2.0). Samples from plates were transferred into tubes containing soybean tryptone (TSA) and incubated for 24 h at 28 ± 2 °C. Afterwards, a small amount of each colony was inoculated into 300 µL of ultrapure water. The solution was then homogenised completely before adding 900 µL of 99% ethanol. Subsequently, the solution was centrifuged at 18,000 RPM for two minutes and the supernatant was discarded. Each microtube was then left opened for five minutes to allow ethanol to evaporate. After this period, 50 µL of 70% formic acid was added, and the samples were vortexed for one minute. Then, 50 µL of acetonitrile was added and the tubes were vortexed again for one minute. Subsequently, the samples were centrifuged at 18,000 RPM for two minutes. Once ready, 1 µL of the supernatant combined with 1 µL of matrix was used for the readings [37].

### 2.4. Atmospheric Air Mass Trajectories and Hailstorms—HYSPLIT Model

The analysis of air masses trajectories, which are potentially the origin of biological species found inside the hailstones, was evaluated by verifying the air masses that arrived in the sites where the samples were collected. This allowed us to check the contribution of air masses to the microbial loading of the hailstones. For this, we applied the HYSPLIT (Hybrid Single-Particle Lagrangian Integrated Trajectory; available at: http://ready.arl.noaa.gov/HYSPLIT.php; accessed on 31 August 2023) model, which calculates the trajectories of air masses [38,39]. To calculate the 96 h back trajectories, the geographic coordinates of the sampling sites were used (26.40° S, 53.52° W for Guarujá do Sul and 26.33° S, 53.59° W for Dionísio Cerqueira). A total of six trajectories were generated in the 4-day period. Moreover, different heights (1000 m, 2500 m, and 5000 m above ground level) were analysed considering that cloud-droplets in the mixed-phase region of deep convection in vigorous convective storms are initiated by aerosols that are entrained laterally from the free troposphere and originating from remote sources [40] and because such in-cloud activation is ubiquitous in deep convections systems [41]. The 12:00 UTC of the sampling days was chosen as the starting time for the back trajectories. The method of calculation of vertical movement used the model vertical velocity, and the input meteorological data were taken from the National Oceanic and Atmospheric Administration (NOAA) Global Forecast System (GFS) dataset at 0.25° horizontal resolution. Figure 2 shows the backward trajectories for both storms.

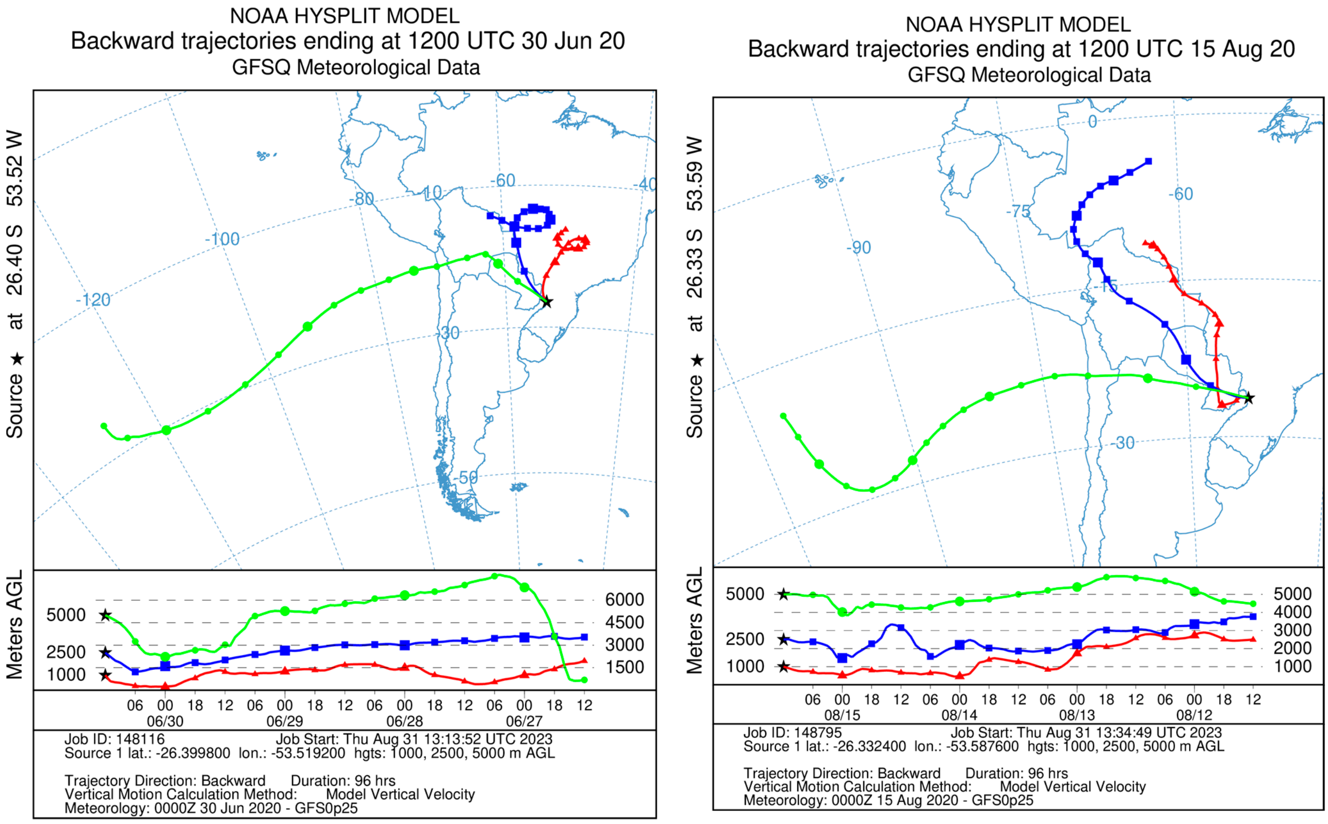


**Figure 2.** Ninety−six hours of backward trajectories modelled by HYSPLIT for the two air masses sampled in the study. Line colours indicate different altitudes of air masses: red line represents 1000 m, blue line refers to 2500 m, and green line represents 5000 m. Each point on the trajectory represents a period of 6 h.

### *2.5. Statistical Analysis*

Data were transformed (i.e., log X + 1) to meet the assumptions of normality (Shapiro–Wilk) and homoscedasticity (Levene). ANOVA followed by Tukey's HSD post hoc test was applied to check for differences regarding hailstone mass, size, volume, density, bacterial and fungal CFUs, and number of species. Regression analysis was used to correlate the physical hailstone parameters with the biological ones. All analyses were performed with a significance level of $\alpha = 0.05$, using R 4.3 [42].

## 3. Results

### *3.1. Hailstones Morphology*

Values of mass, size, volume, and density of the analysed hailstones are shown in Table 1. On average, the first storm that occurred in Guarujá do Sul presented hailstones that were smaller in mass, size, and volume than the hailstones derived from the second storm that occurred in Dionísio Cerqueira. The density of hailstones of the first storm was bigger than the second storm. Finally, the size of the hailstones collected in Dionisio Cerqueira was the only physical parameter assessed in our study that showed a significant positive correlation with the number of bacterial species ($r^2 = 0.573$; $p < 0.05$). No other significant correlations were found for the other physical parameters.

**Table 1.** Physical and microbiological characteristics of the 20 hailstones collected in the two storms at Guarujá do Sul (GS; 30 June 2020; n = 9 ± SE) and Dionísio Cerqueira (DC; 15 August 2020; n = 11 ± SE) in the state of Santa Catarina, Southern Brazil. Legend: TOTAL = all 20 hailstones analysed collectively; CFU = colony forming units; Richness = number of bacterial or fungal species found in hailstones. Lowercase letters indicate significant statistical differences (ANOVA; $p < 0.001$) between the two storms.

| Storm | Mass (g) | Size (cm) | Volume ($cm^3$) | Density ($g/cm^3$) |
|---|---|---|---|---|
| **GS** | 0.339 ± 0.027 [b] | 0.699 ± 0.010 [b] | 0.179 ± 0.007 [b] | 1.869 ± 0.095 [a] |
| **DC** | 0.550 ± 0.025 [a] | 1.026 ± 0.012 [a] | 0.568 ± 0.020 [a] | 0.970 ± 0.034 [b] |
| **TOTAL** | 0.455 ± 0.030 | 0.879 ± 0.038 | 0.393 ± 0.046 | 1.374 ± 0.112 |
| | **Bacterial CFU** | **Fungal CFU** | **Richness Bacteria** | **Richness Fungi** |
| **GS** | 3.22 ± 1.99 | 0.00 ± 0.00 | 0.78 ± 0.32 | 0.00 ± 0.00 |
| **DC** | 11.46 ± 5.03 | 5.46 ± 4.96 | 1.82 ± 0.52 | 0.46 ± 0.21 |
| **TOTAL** | 7.75 ± 2.99 | 3.00 ± 2.74 | 1.35 ± 0.34 | 0.25 ± 0.12 |

### *3.2. Hailstones Microbiological Composition*

We found cultivable bacterial and/or fungi in 11 out of 20 hailstones (or 55%). Bacteria were four-fold more frequent than fungi and were derived from eleven hailstones, whereas fungi were only present in four hailstones all belonging to a single storm (Dionísio Cerqueira). The average number of bacterial CFUs was double (7.75 ± 2.99) that of fungi (3.00 ± 2.74). In addition, a total of eighteen bacterial species were identified (Table 2), as compared to only three fungal species. The most frequent bacterial and fungal species were *Bacillus cereus* and *Epicoccum nigrum*, present in four and three hailstones, respectively. No differences between the number of bacterial ($F_{1,18} = 1.26$; $p = 0.276$) and fungal ($F_{1,18} = 2.29$; $p = 0.148$) CFUs, and the number of bacterial ($F_{1,18} = 1.81$; $p = 0.195$) and fungal ($F_{1,18} = 4.29$; $p = 0.053$) species were observed between the two storms. Again, only for the Dionísio Cerqueira storm, the number of bacterial species was positively correlated with the number of fungal species ($r^2 = 0.485$; $p < 0.05$). No other significant correlations were found for any other biological parameters analysed.

**Table 2.** List of bacteria and fungi species identified in 11 out of 20 hailstones collected during the two storms in Southern Brazil. Species are organised by frequency (as a percentage of appearance in all samples) and alphabetical order.

| Bacteria | Frequency (%) | Fungi | Frequency (%) |
|---|---|---|---|
| *Bacillus cereus* | 20 | *Epicoccum nigrum* | 15 |
| *Priestia megaterium* | 15 | *Curvularia lunata* | 5 |
| *Bacillus licheniformis* | 10 | *Fusarium incarnatum* | 5 |
| *Curtobacterium flaccumfaciens* | 10 | | |
| *Cytobacillus horneckiae* | 10 | | |
| *Methylobacterium rhodesianum* | 10 | | |
| *Arthrobacter gandavensis* | 5 | | |
| *Arthrobacter koreensis* | 5 | | |
| *Bacillus marisflavi* | 5 | | |
| *Bacillus pumilus* | 5 | | |
| *Brevundimonas vesicularis* | 5 | | |
| *Gordonia rubripertincta* | 5 | | |
| *Lysinibacillus fusiformis* | 5 | | |
| *Oceanobacillus* sp. | 5 | | |
| *Paenibacillus* sp. | 5 | | |

**Table 2.** *Cont.*

| Bacteria | Frequency (%) | Fungi | Frequency (%) |
|---|---|---|---|
| *Pantoea agglomerans* | 5 | | |
| *Peribacillus simplex* | 5 | | |
| *Pseudomonas chlororaphis* | 5 | | |

*3.3. HYSPLIT Modelling*

The results of 96 h back trajectories using HYSPLIT modelling indicate that the air masses of the two storms came mainly from the Pacific Ocean and the Northern regions of Brazil, such as the Amazon Forest (Figure 2). While the 1000 and 2500 m air masses came from inner the South American continent, the 5000 m air mass came from the middle of the Pacific Ocean.

## 4. Discussion

*4.1. Hailstones Morphology*

Values of the physical characteristics of the hailstones analysed in our study were, in general, lower than those reported in previous studies [16–18]. For instance, hailstone masses and volumes found by Šantl-Temkiv et al. (2012) [16] were, on average, 34-fold (17.26 g) and 14-fold (20.70 $cm^3$) greater in comparison to our samples, respectively. In addition, Michaud et al. (2014) [18] collected hailstones ranging from 1–4 cm in diameter, whereas the largest hailstone in our study was 1.1 cm, with an average of 0.9 cm. These differences can be partially explained by location, since the abovementioned studies were performed in environments with different climatic conditions than our subtropical area of study (Šantl-Temkiv et al., 2012 [16], in Southern Central Europe, and Michaud et al., 2014 [18], in Montana, USA), which could favour faster melting and the loss of biological material. Finally, due to the nature of this type of study that involves the manual handling of the hailstones, there may be inherent differences in the results obtained, so extrapolations should be carried out with care.

*4.2. Hailstones Microbiological Composition*

The assemblage of bacteria that was isolated from the different hailstones contained similar bacterial taxa to those previously reported in other studies [16,17,19] with prevalence of the genus *Bacillus*. Surprisingly, the appearance of *Oceanobacillus* indicates that airmasses travelling long distances and passing over the ocean contributed to the microbial load of the hailstones analysed, which was confirmed by the HYSPLIT modelling. In comparison to values reported in the literature, the number of bacterial CFUs observed in our samples was lower (average of eight) than reported elsewhere [16–18], which could be a result of different sampling protocols. Concerning fungi, most samples (i.e., 80%) did not have any fungi species inside them. This might be related to the low survival of fungi in the atmosphere, to the inherent difficulty of cultivation, and/or to the fact that fungal PBAPs deposit faster due to their larger sizes, causing them to fall to the ground before being trapped inside hailstones.

*4.3. HYSPLIT Modelling and Microbial Load of Hailstones*

The similarity between the microbial composition in hailstones from both storms can be explained by the similar air mass origins, which predominately came from the Northern and Western regions of Brazil (Figure 2). For both storms, air masses from the Amazon (1000 m and 2500 m) and from the Pacific Ocean (5000 m) were predominant. This atmospheric flow from the northern part of Brazil is linked to the Low-Level Jets, which are known to bring moisture from the Amazon region, favouring the formation of hailstorms in the study region [27–29]. Despite the contribution of ocean air masses to the microbial loading of the hailstones, local contributions also occurred.

The landscape of the region where samples were collected is heavily agricultural and composed of many crops (e.g., wheat, soybean) [24]. Since soil and plants are recognised as sources of PBAPs [17,43], there is also a local contribution of such sources to the microbial loading of the hailstones as well. Beal et al. (2022) [32] found through the chemical analysis of fine particles that soil and agricultural activities in the region where the hailstones were collected are the main sources of $PM_{2.5}$. Moreover, the presence of cultivable bacteria (i.e., *Bacillus* and *Methylobacterium*) is consistent with a soil and plant-surface origin from the local region and from long-range transport from air masses coming from northern region of Brazil as well. Reinforcing this idea, Beal et al. (2020) [30] analysed the climatology of hailstones in the studied region and found a combined action of different atmospheric systems, which involves the transport of moisture from tropical regions merged with local air masses.

### *4.4. Importance of Biological Ice Nucleation for Hailstone Formation*

Despite many decades of studies, understanding the ice nucleation process in clouds remains one of the most challenging gaps in atmospheric sciences. The many investigations on the topic have incorporated field measurements, laboratory experiments, and mathematical modelling [44–46]. Most studies have focused on heterogeneous ice nucleation (involving a particle), which is the relevant process for the mixed-cloud phase (water and ice), between 0 °C and −37 °C [47]. Such investigations include mineral dust particles, soot, bioaerosols (bacteria, fungal spores, and pollen), as well as inorganic/organic acids formed from the gas phase in the troposphere [48–52]. Of the number of particles suspended in the troposphere, only a small fraction has the potential to act as INP inside clouds. This is a small population, but it is of great importance, as without it, there would be no ice formation in most of the cloud column. Still, it is important to highlight that the presence of ice is only significant for altitudes above the isotherms of −15 to −20 °C [53], depending on the study, as it is for temperatures lower than this that most INP (generally soil mineral particles) is activated. And it is precisely here that PBAPs play an important role in the formation of clouds, i.e., because empirical evidence has shown that this type of particle (pollen, bacteria, and fungal spores) is highly efficient as IN for temperatures above −15 °C and even very close to 0 °C [54–57], favouring hail formation and occurrence.

## 5. Conclusions

The results obtained through this work constitute an advance in research involving the microbiological composition of the hailstones in Southern Brazil, a region that is greatly affected by these meteorological events. It was possible to observe that the distribution of bacterial and fungal species within hailstones were not equal. Of the total 18 bacterial species identified, *Bacillus cereus* was the most frequent, confirming that the genus *Bacillus* as one of the most common to be found in hailstones. Fungi, on the other hand, were only present in four hailstones derived from a single storm with three fungal species identified and *Epicoccum nigrum* as the most frequent fungal species. Using HYSPLIT modelling, we showed that air masses came from the Amazon and from the Pacific Ocean, which likely contributed to the microbiological composition of the hailstones. Our findings suggest that ca. 50% of hailstones have cultivable microbial cells inside them, with bacterial species being four-fold more abundant than fungi, and that such microorganisms came mainly from the local landscape with intrusions of air masses derived from the Amazon and the Pacific Ocean.

**Author Contributions:** M.C.M., T.B.Q., L.D.M., J.A.M. and F.L.T.G. conceived and designed the research; T.B.Q., D.M.C.S., V.B.D.F., A.P.R., R.A.A. and L.D.M. performed the experiment and collected and analysed samples; M.C.M., T.B.Q., L.C.C.G., A.S. and S.M.B. analysed the fieldwork data; M.C.M., T.B.Q., A.P.M.E., L.C.C.G., M.A.F.S.D., P.L.S.D., F.R., D.M.C.S., V.B.D.F., A.P.R., R.A.A., L.D.M., J.A.M., A.S., S.M.B., F.C., T.Š.-T., V.P. and F.L.T.G. wrote and edited the manuscript; M.C.M., T.B.Q. and F.L.T.G. led the writing of the manuscript. All authors have read and agreed to the published version of the manuscript.

**Funding:** This work was supported by the São Paulo Research Foundation (*Fundação de Amparo à Pesquisa do Estado de São Paulo*—FAPESP, grants: 2016/06160-8 to F.L.T.G. and 2020/14143-1 to M.C.M.) and by the Brazilian Coordination for the Improvement of Higher Education (*Coordenação de Aperfeiçoamento de Pessoal de Nível Superior*—CAPES, grant no. 88887.373123/2019-00 to T.B.Q.). T.Š.-T. was supported by the Danish National Research Foundation (DNRF106, to the Stellar Astrophysics Centre, Aarhus University), the AUFF Nova programme (AUFF-E-2015-FLS-9-10), the Novo Nordisk Foundation (NNF19OC0056963), and the Villum Fonden (23175 and 37435).

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Data will be made available upon request.

**Acknowledgments:** The authors thank the support received from local landowners and city councils of Guarujá do Sul and Dionísio Cerqueira. We also thank the EAE-LAPAR team for help in the field and the funding agencies for support.

**Conflicts of Interest:** The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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