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1 **Survival of extremophilic yeasts to the stratospheric environment on balloon flights**
2 **and laboratory simulations**

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16 **Running title:** Survival of extremophilic yeasts in the stratosphere

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17 **ABSTRACT**

18 The high-altitude atmosphere is a harsh environment with extremely low
19 temperatures, low pressure, and high UV irradiation. For this reason, it has been proposed
20 as an analogue for Mars, presenting deleterious factors similar to the surface of this planet.
21 We evaluated the survival of extremophilic UV-resistant yeasts isolated from a high-
22 elevation area in the Atacama Desert to stratosphere conditions. As biological controls,
23 intrinsically resistant *Bacillus subtilis* spores were used. Experiments were performed in
24 two independent stratospheric balloon flights and with an environmental simulation
25 chamber. Three different conditions were evaluated: (1) desiccation; (2) desiccation plus
26 exposure to stratospheric low pressure and temperature; (3) desiccation plus exposure to the
27 full stratospheric environment (UV, low pressure and temperature). Two strains,
28 *Naganishia* (*Cryptococcus*) *friedmannii* 16LV2 and *Exophiala* sp. 15LV1 survived full
29 exposures to the stratosphere, in larger numbers than *B. subtilis* spores. *Holtermanniella*
30 *watticus* 16LV1, however, suffered substantial loss of viability upon desiccation and did
31 not survive the stratospheric UV exposure. The remarkable resilience of *N. friedmannii* and
32 *Exophiala* sp. to the extreme, Mars-like conditions of the stratosphere confirms its potential
33 as eukaryotic models for astrobiology. Additionally, our results with *N. friedmannii*
34 strengthen the recent hypothesis that yeasts belonging to this genus are fit for aerial
35 dispersion, which might account for the observed abundance of this species in high-
36 elevation soils.

37

38 **IMPORTANCE**

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39 Studies of eukaryotic microorganisms under conditions of astrobiological relevance,
40 as well as the aerial dispersion potential of extremophilic yeasts, are still lacking on the
41 literature when compared to works with bacteria. Using stratospheric balloon flights and a
42 simulation chamber, we demonstrate that yeasts isolated from an extreme environment are
43 capable of surviving all stressors found in the stratosphere, including intense UV
44 irradiation, scoring an even higher survival than *B. subtilis* spores. Notably, the yeast *N.*
45 *friedmannii*, which displayed one of the highest tolerances to the stratospheric environment
46 in the experiments, was recently proposed to be adapted to airborne transportation, although
47 such hypothesis had not yet been tested. Our results strengthen such assumption and can
48 help to explain the observed distribution and ecology of this particular yeast species.

49 **Keywords:** Yeasts, *Naganishia friedmannii*, *Cryptococcus*, Aerobiology, Astrobiology,
50 Atacama, Extremophiles, Stratosphere

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51 **INTRODUCTION**

52 Dispersal of microorganisms in the atmosphere contributes to shaping microbial
53 biodiversity around the world, including extreme habitats like high-elevation soils and the
54 Antarctic continent (1-3). Atmospheric transportation allow cells to be carried over long
55 distances beyond geographic barriers, playing a key role in the distribution and ecology of
56 microbes (1). Additionally, microorganisms suspended in the air can impact important
57 meteorological and atmospheric processes, such as cloud formation, precipitation, and
58 cloud water chemistry (4, 5). Due to their importance, airborne microbial communities at
59 different environments and altitudes are widely studied by molecular and cultivation-based
60 methods (6-11). Complementing these aerobiological investigations on the ecology of
61 airborne microbes, it is also important to study the survival potential of microorganisms in
62 actual atmospheric conditions in order to better understand how life adapts to air
63 transportation and exposure to the high altitudes (12, 13).

64 Microbial cells suspended in the atmosphere many kilometers above the ground
65 must cope with several stressing factors, such as the solar radiation, low temperatures, and
66 desiccation (14). Microorganisms are not restricted to the troposphere, the lowest layer of
67 the atmosphere where most weather events occur (up to an altitude of 12 km at the tropics,
68 reaching 18 km near the equator). Aerosol particles, including cells, can be carried further
69 above into the stratosphere by natural meteorological phenomena like thunderstorms (12,
70 15-18). At this atmospheric layer, more severe environmental conditions prevail (18).
71 Pressures can reach values below one-hundredth of that at sea level, temperatures drop
72 down to as low as -70°C, and high UV irradiation is present. With a shorter atmospheric

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73 path through absorbing gases (particularly ozone), the unfiltered UV radiation is more
74 intense at the stratosphere, mainly at the damaging UV-B range (280-320 nm) (19).

75 Due to the harshness of the stratospheric environment, some authors have proposed
76 to explore it as an analogue of Mars' surface on Earth (20, 21). This planet may have
77 harbored habitable conditions in its geological past, including superficial liquid water, but
78 has since lost most of its atmosphere and is now dominated by a cold and dry climate (22,
79 23). With a thin, rarefied atmosphere composed mostly of CO_2, its present surface is mostly
80 unprotected from the Sun's UV radiation. Mars is considered an important target for
81 astrobiology and the exploration of its potential to have harbored life through time is the
82 goal of future space missions. In this context, the study of organisms under Mars-like
83 extreme conditions is additionally relevant to planetary protection research (13, 24).

84 Information on the viability of microbial cells during airborne transportation, and
85 survival to the combined high-altitude atmospheric stressing factors (or for cells shielded
86 from solar radiation), are questions that can be addressed experimentally, expanding our
87 current knowledge about adaptation of airborne microbes. Additionally, investigating the
88 capacity of microorganisms to endure these conditions can help the discovery of novel or
89 more efficient cell protection mechanisms, improving our knowledge about life limits upon
90 multiple simultaneous stressing conditions (25, 26), such as the ones faced by airborne
91 microbes, especially in the stratosphere. Although several works have reported fungi and
92 yeasts collected from air (6, 27, 28), as well as in high-altitude atmospheric samples (8, 17,
93 29), there are few studies that have evaluated the potential of these organisms to survive the
94 stressors found on these environments.

95 In this work, we have investigated the tolerance to atmospheric and stratospheric
96 conditions of cold-adapted, UV resistant yeasts previously isolated from the slopes of an

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97 Atacama Desert volcano at 4000 to 5000 m above sea level (30). Interestingly, this
98 particular type of high-elevation environment, with freezing temperatures and high UV
99 intensities, has itself been proposed to represent another Mars analogue site on Earth (30-
100 33). The yeast strains chosen were the black-pigmented *Exophiala* sp. 15LV1, and the
101 seemingly non-pigment producing *Naganishia friedmannii* 16LV2 (formerly *Cryptococcus*
102 *friedmannii*) and *Holtermaniella watticus* 16LV1. Intrinsically resistant *Bacillus subtilis*
103 spores were used as biological controls. For the experiments, two stratospheric balloon
104 flights were performed, as well as controlled laboratory tests with a simulation chamber.
105 The results obtained are discussed from astrobiological and aerobiological perspectives.

106 **RESULTS**

107 The desiccated microorganisms were exposed directly to stratospheric conditions
108 (Fig. 1A and 1B) and its viability was estimated by CFU enumeration. Both balloon flights
109 lasted for an average of 110 min, reaching up to 25 km of altitude (Fig. 1C and 1D).
110 Additional images of the balloon launches are shown in Fig. S1. and microorganisms tested
111 in Fig. S2. Results of the two independent flights are shown in Fig. 2.

112 As can be observed, the results obtained with the two balloon experiments were
113 similar. Desiccation and exposure to stratospheric conditions without UV nearly did not
114 affect *B. subtilis* spore's viability, as expected. However, near 5 logs of inactivation
115 occurred in spores exposed to solar UV. The yeasts *N. friedmannii* and *Exophiala* sp.
116 15LV1 were partially affected by the initial desiccation and to the further exposure to
117 stratospheric conditions without UV. However, for the yeast *H. watticus*, desiccation alone
118 was already enough to reduce viability by 2 logs (99% viability drop) (Fig. 2A and 2B).
119 When considering the full stratospheric exposure (all conditions combined, including UV

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120 irradiation), *N. friedmannii* lost almost 99% of viability at the first flight and 90% at the
121 second flight, whereas *Exophiala* sp. lost 90% and 99% of its viability at these respective
122 experiments. That is, both of those yeasts suffered a 1 to 2 logs viability drop during their
123 passage through the stratosphere. *H. watticus* was completely inactivated after UV
124 exposure in both flights. Although data is not available for the first balloon flight, we
125 observed at the second flight, in a parallel experiment, that desiccated cells of the yeast
126 *Saccharomyces cerevisiae* BY4743 also did not survive exposure to the solar UV radiation
127 (Fig. S3).

128 In addition to the exposures during balloon flights, we also performed simulations
129 of the stratospheric environment using a simulation chamber (AstroCam) capable of
130 reproducing the conditions found at the stratosphere (Fig. 3A). Details of the sample
131 preparation and experimental configuration are shown in Fig. 3B. Due to the characteristics
132 of the simulation chamber (see Materials and Methods for details), cells were desiccated
133 and exposed over silicon chips, instead of PTFE strips used on the balloon flights. The
134 overall survival behavior observed on the balloon experiments was maintained in the
135 simulation assays: *N. friedmannii* and *Exophiala* sp. survived even after UV exposure,
136 whereas no *H. watticus* CFU were recovered after irradiation (Fig. 3D). Despite these
137 similarities, data obtained with the simulation chamber differed from the two different
138 balloon exposure assays, as the desiccation and desiccation plus stratospheric conditions
139 treatments were more detrimental to yeasts cells in our simulation when compared to the
140 balloon experiments (Fig. 3D). At the simulation chamber, the decompression rates as well
141 the cooling speed and conditions are different from the ones at the balloon flights.
142 Therefore, we decided to test whether these parameters were responsible for the observed
143 differences.

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144 Differential decompression rates did not affect the survival of the desiccated yeasts
145 and *B. subtilis* spores (Fig. 4B). However, when cooling down samples in a desiccator
146 covered with dry ice, higher survival rates (similar to the ones observed during balloon
147 flights) were observed for the yeasts cells, whereas a significant reduction in viability was
148 observed at the simulation chamber. These results indicate that the cooling system at the
149 AstroCam is more lethal to yeasts than the low temperature exposure at the balloon flights.
150 Viability of *Bacillus subtilis* spores remained virtually unaffected when exposed to
151 stratospheric parameters while shielded from solar radiation, whereas for all yeasts, even
152 the most resistant ones, a decrease in cell viability during stratospheric conditions without
153 UV irradiation was observed (Fig.2, Fig 3 and Fig. 4). Therefore, when considering a
154 scenario where cells are shielded from UV, spores scored a better survival when compared
155 to yeasts. However, when considering a scenario were cells are fully exposed to all
156 stratospheric conditions including solar radiation, *N. friedmannii* and *Exophiala* sp. 15LV1
157 scored a greater survival than *B. subtilis* spores (Fig. 2A and 2B), even in the simulation
158 chamber assays (Fig. 3D) in which viability was already impaired due to desiccation and
159 exposure to low temperatures (Fig. 4D). *H. watticus* had a large reduction in its viability,
160 even without solar radiation exposure and suffered total inactivation when exposed to UV,
161 demonstrating that this yeast does not seem to be adapted to aerial transport and
162 stratospheric exposure, even considering UV-shielded cells.
163
164 **DISCUSSION**
165 **Yeast survival to stratospheric environment.**

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166 In order to endure atmospheric transportation, microorganisms must deal with
167 intense UV, dehydration, reduced pressures and low temperatures (12, 18). Desiccation is
168 the first challenge faced by these yeasts, but not for metabolically inert *B. subtilis* spores,
169 which are extremely resistant to this type of stress (34). In fact, the spores' viability was
170 observed to be unaffected by desiccation and exposure to additional low pressures and
171 temperatures found in atmospheric high altitudes (Fig. 2 and 3D). The yeasts *N.*
172 *friedmannii* and *Exophiala* sp. were shown to have a reasonable resistance towards
173 desiccation even as vegetative cells. Survival, as obtained in our results (using a suspension
174 of washed cells), was only achieved by using stationary-phase cultures, high cell densities,
175 and slow dehydration rates, a factor already known to improves the resistance of yeasts
176 towards desiccations (35). Even the model yeast *Saccharomyces cerevisiae*, considered
177 desiccation-tolerant, requires high cell densities at the stationary phase to survive this type
178 of treatment (35, 36). This differs from *B. subtilis* spores, which can tolerate even abrupt
179 desiccation events.

180 *N. friedmannii* 16LV2 and *Exophiala* sp. 15LV1 achieved a significant survival
181 after direct exposure to the high-altitude atmospheric environment, including UV
182 irradiation, even higher than *B. subtilis* spores (Fig. 2A and 2B). These yeasts were already
183 shown to possess high resistance to UV-C, UV-B, and environmental UV radiation (UV-A
184 + UV-B) in laboratory conditions (30). Considering that UV is the most deleterious factor
185 to cells exposed to high altitudes (12), broad UV resistance probably is a key factor in
186 tolerating the stratospheric environment and might account for the survival observed for our
187 yeasts (Fig. 2A and 2B). While *Exophiala* sp. survival may be partially explained by its
188 strong pigmentation, containing both melanin and carotenoids, *N. friedmannii* 16LV2 is

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189 completely pale in our growth conditions, as observed by cell coloration (Fig. S2) and
190 previous Raman spectroscopy analysis (30). It is supposed that other still uncharacterized
191 protection systems may play a role in this yeast's UV tolerance. The exception of our group
192 of extremophilic yeasts was *H. watticus* 16LV1, which presented a large loss of viability
193 upon desiccation, increased loss of viability after exposure to low pressure and temperature,
194 and complete loss of viability after UV exposure (Fig. 2). It is important to highlight that
195 our cells were desiccated from suspensions in 0.9% (w/v) NaCl solution, after washing, to
196 eliminate any possible interference and protection of the components of the growth media
197 in cell survival. In addition, exposures to solar radiation at the stratospheric environment
198 were performed directly, without cover or protection by any component.

199 Our results using the simulation chamber rendered somewhat different results when
200 compared with the data obtained with the balloon flights (Fig. 3D). The laboratory
201 simulation was somewhat more lethal for the yeasts than the real exposure to the
202 stratosphere (Fig. 2 and 3D), although *N. friedmannii* and *Exophiala* sp. still recorded a
203 higher survival than *B. subtilis* spores at the full simulation with UV exposure. The main
204 observed difference is due to the increased lethality during desiccation plus stratosphere
205 treatment, which reduced the viability of the tested yeasts in 1 to 1.5 logs at the simulation
206 chamber (Fig. 3D), compared to 0.2 to 0.3 logs reductions at the balloon flights (Fig. 2).
207 For *H. watticus*, the effects of such treatment were even more severe, in which cells were
208 completely inactivated (Fig. 3D), whereas for the balloon flights, the maximum observed
209 drop was 0.5 logs (Fig. 2).

210 We tested to see if the observed difference may be accounted by the fact that the
211 decrease in pressure is more abrupt than the one observed at the balloon flight (a couple
212 minutes in the chamber, when compared to over 50 minutes at the balloon flights).

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213 However, we measured no difference in survival of desiccated samples exposed to different
214 decompression rates (Fig. 4A and 4B). Therefore, we aimed to investigate if the cooling
215 system of the AstroCam was responsible for the observed differences. The maximum
216 cooling rates at the chamber are actually slower than the observed at the balloon flight (Fig.
217 4C). Interestingly, we observed that the yeasts' survival inside the cooled desiccator is
218 similar to the observed at the balloon flights (Fig. 4D and Fig. 2), whereas the cooling
219 system used in the Astrocam was significantly more lethal to the yeast cells (Fig 4D).
220 Therefore, we believe that the cooling system of the Astrocam accounts to the observed
221 difference between the balloon flights and the simulation, and the cells were actually
222 exposed to a more stressful situation than the at the stratospheric balloon flights.

223 There are two possible explanations for these changes: the first one is due to the
224 slower cooling rate, which could favor formation of ice crystals, which are more
225 detrimental to cells. The second explanation is related to a particularity of the Astrocam
226 cooling system: only the sample holder (Fig. 3A) is cooled, and the remaining parts of the
227 simulation chamber actually remain at room temperature. Due to such characteristic, ice
228 crystals start to form and cover the desiccated cells, even at the low relative humidity inside
229 the chamber (See material and methods). We observed that this ice crystal formation over
230 the samples increases with time and might be responsible for the increased lethality
231 observed at the simulation chamber. Independently of the explanation, an interesting
232 observation can be made: *B. subtilis* spores seem to be indifferent to such variations to low
233 temperature stresses, but yeasts cells viability is likely to be more affected.

234 Importantly, these data highlights that surviving desiccation and low-temperature is
235 a key factor dictating the final survival of yeasts to stratosphere exposure, whereas for *B.*
236 *subtilis* spores, these factors seem less important (Fig. 4), and UV plays a more relevant

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237 role in final survival (Fig. 4E). Nevertheless, even considering the more extensive loss in
238 viability to desiccation and low temperature, *N. friedmannii* and *Exophiala* sp. still survived
239 exposure to full stratospheric conditions in our simulated conditions, more so than *B.*
240 *subtilis spores* (Fig. 3D), probably due to the remarkably high UV resistance of these
241 yeasts.
242
243 **Implications for aerial transportation and astrobiology.**
244 Our results strengthen the recent propositions raised by Schmidt et al. (3, 31) that
245 the species *N. friedmannii* and members of the *Naganishia* clade (formerly of the
246 *Cryptococcus* genus (37)) could be fit to survive atmospheric transportation and aerial
247 dispersion, which could explain its abundance in high-elevation soils and its global
248 dispersion. In fact, members of the *Naganishia* clade have already been found in
249 tropospheric air samples (8) and cloud samples (38), and *N. friedmannii* is constantly
250 reported in volcanic and mountain soils (3, 32, 33, 39, 40). In our first balloon assay,
251 samples traveled for a distance of over 100 km (Fig. S4) and *N. friedmannii* displayed good
252 survival even after UV exposure. However, it is likely that cells could survive traveling
253 even further, especially if partially or totally shielded from UV (if adhered to aerosol
254 particles, for example) or if air transportation occurred at lower altitudes, as for example, at
255 the troposphere. Although no information could be found for airborne cells of the genus
256 *Exophiala* in the literature, our results suggest that these species could also be properly fit
257 for aerial dispersion.
258 It is important, however, to note that probably not all extremophilic yeast groups are
259 equally fit for air transportation. As our data showed, *H. watticus* suffered a great loss of

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260 viability already upon desiccation, which is the most critical step for yeasts for airborne
261 transportation (Fig. 2 and 3). Exposure to low pressure and low temperatures decreased
262 even more cell viability (Fig. 4), and finally, UV exposure reduced survival of *H. watticus*
263 to essentially zero. It is an interesting observation, considering that this yeast, although not
264 as resistant to UV as *N. friedmannii* 16LV1 and *Exophiala* sp. 15LV1 in laboratory
265 conditions, was isolated from a high-elevation volcanic area in the Atacama Desert and
266 nevertheless presented a considerable UV resistance in our previous study (30). These
267 results demonstrate the severity and complexity of the stratosphere, which have already
268 been proposed as Mars analogue environments on Earth (20, 21).

269 Due to the harshness of this environment, microbial survival to the stratosphere is
270 directly conditioned to the time that the cells are exposed (13), and hardly any
271 microorganism can endure prolonged unprotected permanence on such high altitudes.
272 Recently, Khodadad et al. (13) exposed *Bacillus pumilus* spores to full stratospheric
273 conditions at 30 km of altitude for a total of 8 h. Even these extremely resistant *B. pumilus*
274 spores (41) suffered considerable inactivation after two hours of stratospheric exposure and
275 severe inactivation after prolonged exposure (13). In fact, even when considering the best
276 survival of our yeasts, exposure to total stratospheric environment still inactivated about
277 90% of the viable cells of *N. friedmannii* and *Exophiala* sp. 15LV1 (Fig. 4B, at the second
278 and first flight, for each yeast respectively). Therefore, it is likely that prolonged exposure
279 to stratospheric UV would have a greater impact in our yeasts survival, reducing even more
280 its viability. Also, desiccation followed by a low temperature treatment seems to affect
281 more yeasts than bacterial spores (Figure 4). Nevertheless, considering that the
282 stratospheric full exposure assays (Fig. 1 and 2) and the stratosphere simulation (Fig. 3)
283 deeply affected the *B. subtilis* spores to near complete inactivation (5 logs drop in viability),

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284 the survival observed for the yeasts are remarkably high. These results strengthen previous
285 assumptions, that yeasts like *N. friedmannii* and *Exophiala* sp. can be good eukaryotic
286 models for astrobiology (30, 31). In fact, an *Exophiala* species (*Exophiala jeanselmei*) has
287 already been tested in a Mars simulation, where it presented evidences of metabolic activity
288 under Martian environmental conditions (42). In addition, in our previous study, *Exophiala*
289 sp. 15Lv1 scored the highest survival for UV-C and UV-B of our tested yeasts (30) and
290 now we show that it can endure several conditions found in a Mars-like environment.

291 *N. friedmannii* also gathers several interesting characteristics for astrobiology,
292 especially when considering the martian environment. It is expected that on Mars liquid
293 brines are formed temporarily in regions enriched with salts in contact with water ice (43),
294 which could be permissive for microbial life, before water refreezes or evaporates. Also,
295 due to the rarefied martian atmosphere, the UV flux on its surface is more harmful than on
296 Earth, extending from unfiltered UV-B into the shorter wavelengths of the UV-C range
297 (<280 nm) (13). *N. friedmannii* has shown to be capable of growing during freeze and
298 thaw cycles (40) and can endure considerable UV irradiation at different wavelength
299 ranges, including UV-C (30). *N. friedmannii* is able to grow at subzero temperatures (-
300 6.5°C) and at moderate concentration of salts (30). Now we demonstrate that *N.*
301 *friedmannii* can survive even after exposure to all combined stressors found at Mars-like,
302 high-altitude environments: low pressure, desiccation, extremely low temperature, and high
303 solar irradiation. The achieved survival rates were even higher than *B. subtilis* spores,
304 which are commonly studied as potential spacecraft contaminants in the context of
305 planetary protection within Astrobiology (24).

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306 Unfortunately, genomic information is still unavailable for this organism. Besides
307 the absence of pigmentation (in our tested conditions) and the already known ability to
308 perform photorepair (30), no other protection mechanisms of *N. friedmannii* have been
309 studied to date. Therefore, further investigations should include genome sequencing to
310 enable the identification of possible resistance-related genes. Once sequences are available,
311 deeper molecular investigations including classic molecular biology approaches (e.g., gene
312 deletion, overexpression, and heterologous gene expression in other yeasts) will be
313 possible, allowing a better understanding of the mechanisms of *N. friedmannii* to cope with
314 multiple stressing conditions, and also expand our knowledge about the limits of eukaryotic
315 life.

316 **MATERIALS AND METHODS**

317 **Strains, media, and growth conditions.**

318 The yeasts *Exophiala* sp. 15LV1, *Naganishia friedmannii* 16LV2 (formerly
319 *Cryptococcus friedmannii*), and *Holtermaniella watticus* 16LV1, previously isolated from a
320 volcanic region at the Atacama Desert (30) were grown in GYMP broth (glucose, 20 g L^{-1};
321 yeast extract, 5 g L^{-1}; malt extract, 5 g L^{-1}; NaH_2PO_4, 2 g L^{-1}) at 15°C into the late
322 stationary phase, which correspond to roughly 20 days of growth (Fig. S2A). *Bacillus*
323 *subtilis* PY79 spores were obtained by growing the cells at 30°C for 4 days in the
324 sporulation medium DSM (Difco Nutrient Broth, 8 g L^{-1}; KCl, 1 g L^{-1}; $MgSO_4 \cdot 7H_2O$, 0.25
325 g L^{-1}; completed after autoclaving by adding $Ca(NO_3)_2$ to 10^{-3} M, $MnCl_2$ to 10^{-4} M, and
326 $FeSO_4$ to 10^{-6} M). Sporulation efficiency was assayed by phase-contrast microscopy (Fig.
327 S2B) and survival following thermal treatment at 85°C for 15 min. The number of
328 vegetative cells was found to be almost non-existent (data not shown). Table 1 summarizes

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329 the strains characteristics and growth conditions used in this study. Colony forming units
330 (CFU) were quantified on YM plates (malt extract, 3 g L^{-1}; yeast extract, 3 g L^{-1}; peptone,
331 5 g L^{-1}; glucose, 10 g L^{-1}; agar, 20 g L^{-1}) for the yeasts, and on LB plates (tryptone, 10 g
332 L^{-1}; yeast extract, 5 g L^{-1}; NaCl, 10 g L^{-1}; agar, 15 g L^{-1}) for *B. subtilis*. Plates were
333 incubated in the dark.

334 **Sample preparation for the balloon flights.**

335 The cultures were centrifuged, washed twice with NaCl 0.9% (w/v) solution, and
336 diluted, if necessary, to reach a suspension with a final concentration of $\sim 5\times10^7$ CFU ml^{-1}
337 for the yeasts and $\sim 2\times10^8$ CFU ml^{-1} for the *B. subtilis* spores. Multiple replicates of 10 µl
338 droplets of each of these suspensions were placed over PTFE (polytetrafluoroethylene)
339 strips attached to the balloon's sample holder module with carbon tape (Fig. 1A). The
340 droplets were then slowly dried inside an incubator at 15°C and relative humidity of about
341 35% before being stored in a plastic container with silica gel to maintain low humidity until
342 launch. The samples were divided between "Ground Samples (Desiccation)", kept inside
343 the container, "Flight (Non-exposed to UV)", protected from sunlight by a shading cover,
344 and "Flight (Exposed to UV)", receiving the full solar radiation (direct exposure, without
345 any kind of protection) (Fig. 1A and 1B). After the flight, the sample holder recovered from
346 the landed payload was placed back inside the container for transportation back to the
347 laboratory. The dried droplet samples were individually resuspended in NaCl 0.9%
348 solution, diluted, and plated on agar-solidified medium for CFU enumeration. Survival was
349 determined as N/N_0, where N is the number of CFU recovered after each experiment, and
350 N_0 is the initial CFU/concentration of the suspension used for the assays. As an additional

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351 control, the washed cells were kept suspended in saline solution for the duration of some of
352 the assays, at 4°C. No loss of viability was observed for these cells (data not shown).

353 **Balloon flights.**

354 Two independent balloon flights were performed for this work. Instruments housed
355 inside the payload were: Arduino Mega 2560 R3 microcontroller board; MicroSD 8GB
356 memory card; uBLOX MAX-M8 GPS unit; Ds18b20 temperature sensor; Bmp180 Gy-68
357 pressure and temperature sensor; Mpu6050 accelerometer and gyroscope. For tracking the
358 probe, two independent Spot Gen 3 system were used. Images for the launch were acquired
359 using a GoPro Hero 3+ Black. Helium balloons were used, and after the balloon blasted due
360 to high altitude, the probe descent occurred with a parachute system. The first balloon
361 launch (using a Hyowee 1200 g balloon) was performed on 14th May of 2016, carrying
362 several biological and chemical experiments. Launch was done from the city of São Carlos,
363 SP, Brazil (22°00'18.0''S – 47°56'03.6''W) at 11:30 am local time, with the Sun near its
364 zenith, and landing occurred at roughly 105 km in a straight line from the launching point
365 (21.69'02.88''S – 46.83'54.19''W, Fig. S4). Unfortunately, due to avionics malfunction,
366 environmental data were lost for this flight. It is possible to know, however, that the probe
367 remained above 18 km altitude for approximately 40 min (due to the Spot Gen 3 system,
368 that continued to work), but the maximum altitude reached cannot be assured, although we
369 estimate that the probe reached 25-30 km high, based on similar balloon launches
370 performed latter by the group and images acquired (Fig. S1). Also, part of the exposed
371 samples was lost during landing (spots from *B. subtilis*, and complete loss of other exposed
372 microorganisms, not discussed in this work). The second launch (using a Hyowee 800 g
373 balloon) was performed on 25th February of 2018, from the city of Itápolis, SP, Brazil

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374 (21°35'42.59"S – 48°49'59.82''W) at 11:30 am local time. Landing occurred near the
375 launch point (Fig. 1D), which allowed samples to be recovered quickly. Environmental data
376 are shown in Fig. 1C and Fig. S5.

377 **Stratosphere simulation (AstroCam).**

378 Stratospheric environment simulation experiments were performed using a
379 planetary simulation chamber built by our research group (AstroCam, Fig. 3A) capable of
380 recreating most aspects of the high-altitude flight: low pressure, low temperature, and high
381 UV flux. Simulations parameters were adjusted to represent the conditions at 20 km above
382 sea level. UV fluxes were calibrated according to UV parameters used by Smith et al. (12)
383 which simulated the 20 km altitude environment to test *B. subtilis* survival to stratospheric
384 conditions. UV intensities were measured using a Vilber Lourmart RMX-3W radiometer
385 and an Ocean Optics QE65000 UV-Vis fiber-optic coupled spectrometer. Temperature (-
386 56.5°C) and pressure values (58 mbar) were used according to the U.S. Standard
387 Atmosphere.

388 Microbial growth and sample preparation were exactly as performed for the balloon
389 flight experiments (described above). However, silicon chips supports were used instead of
390 PTFE strips (Fig. 3B), since the temperature inside the chamber is controlled at the sample
391 holder and silicon is a more efficient temperature conductor than PTFE. The volume of
392 droplets was also reduced, from 10 µl to 5 µl. The silicon chips were attached to the sample
393 holder by vacuum-compatible adhesive copper tape and positioned inside the simulation
394 chamber (Fig. 3A). The pressure inside the chamber was lowered by an Adixen ACP15 dry
395 mechanical pump to 58 mbar, maintained in flow through a needle valve with ambient air
396 passed through humidity-absorbing silica gel columns. An ARS CS204PB-450 liquid

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397 helium-refrigerated cryostat in contact with the sample holder was used to control the
398 temperature to -56.5°C. The samples were divided between the "Desiccation" controls, not
399 taken to the chamber and kept inside a container with silica gel at low relative humidity,
400 "Desiccation + Stratosphere", exposed to the simulated environment on the underside of the
401 sample holder but protected from the UV by a cover, and "Desiccation + Stratosphere +
402 UV", receiving the full simulated solar radiation (direct UV exposure, without any kind of
403 protection) for a total of 40 minutes. The irradiation was performed with an Oriel Solar
404 Simulator with a 1000 Watt xenon arc lamp equipped with a water filter to attenuate the
405 longer wavelength infrared and an AM1 filter to shape the lamp's emission closer to the
406 solar spectrum with 1atm attenuation path. After the simulation, the samples were brought
407 to room temperature and the chamber was vented with dry air. The samples were processed
408 as described before for the balloon flight experiments. Simulations were performed in
409 triplicates.

410 **Differential decompression rates assay and differential cooling and freezing**
411 **assay**

412 Survival was evaluating for differential decompression rates using a programmable
413 Büchi V-700 vacuum pump with V-855 controller, coupled to a desiccator containing silica
414 gel. Cells were prepared in the same manner as for the simulation chamber assay, pipetting
415 5μl droplets over silicon chips and slowly desiccated. The samples were then positioned
416 inside the desiccator, which was evacuated and, after a 10-minute period, vented at
417 controlled speeds programmed in the pump. Viability was estimated by CFU counting. For
418 a comparative of survival at different cooling rates, cells were grown and washed as
419 described above. Droplets of the same cultures (5μl droplets) were pipetted over silicon

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420 chips and slowly desiccated. A group of silicon chips containing the cells was cooled at the
421 AstroCam simulation chamber at low pressure (maintained in flow with dry air, see
422 methods above), whereas another group was placed inside a desiccator, which was then
423 then cooled down by covering it with dry-ice inside an insulated box. Both cooling
424 treatments were performed for two hours. Viability was estimated by CFU counting and
425 plotted as N/N_0.

426

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438

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568 **Table 1.** Microorganisms, growth conditions, and approximate number of cells tested for

569 each experiment.

Strain	Type	Growth medium	No. of cells per spot (Balloon)*	No. of cells per spot (Simulation)*	Notes
Exophiala sp. 15LV1	Yeast	GYMP	4×10^5	2×10^5	UV-resistant yeasts isolated from a high-elevation area on the Atacama Desert (30)
Naganishia friedmannii 16LV2	Yeast	GYMP	4×10^5	2×10^5	
Holtermaniella. watticus 16LV1	Yeast	GYMP	6×10^5	3×10^5	
Bacillus subtilis PY79	Bacterium	DSM (sporulation medium)	2×10^6	1×10^6	Spore former

570 *** Number of cells estimated by CFU counting**

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576 **Figure 1**. Stratospheric balloon flight experiments. (A) Scheme of the sample holders and
577 all different treatments performed for the microorganisms to be tested. (B) Images of the
578 launches and sample exposure at high altitudes. (C) Altitude and temperature measurements
579 from the second launch (performed at 2018). (D) Travel map of the probe (second launch)
580 during the flight. (Map from Google Earth, 2018.)

581 **Figure 2.** Survival of the tested microorganisms to the stratospheric balloon flights. Tested
582 parameters were: desiccation resistance (Ground sample), desiccation plus exposure to
583 high-altitude environment but without UV exposure (Flight non-exposed), and desiccation
584 plus full exposure to high-altitude environment (Flight exposed). (A) Survival of the
585 microorganisms to the first balloon flight, performed in May, 2016. (B). Survival of the
586 microorganisms to the second balloon flight, performed in February, 2018. Error bars
587 represent the standard deviation between three distinct spots. **= only one spot of *B.*
588 *subtilis* spores was recovered from the exposed assay of the first flight. *= No CFU
589 observed for exposed *H. watticus* on both balloon flights.

590 **Figure 3.** Simulated stratospheric exposure assays. For these assays, cells were exposed for
591 40 min under a UV flux similar to the one found at 20 km of altitude. (A) Images of the
592 simulation chamber (AstroCam). (B) Scheme of the sample holders and all different
593 treatments performed for the microorganisms to be tested. For the "Desiccation +
594 Stratosphere" assay (without UV exposure), an extra protection cover was added over the
595 samples (not shown in the scheme). (C) Simulation parameters and UV spectrum at
596 AstroCam, compared with the spectrum used by Smith et al. (12) (D) Survival of the tested
597 microorganisms in different conditions. Error bars were calculated using triplicates. *= no
598 CFU detected for this treatment.

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600 **Figure 4.** Survival of the tested microorganisms to differential decompression rates and
601 cooling systems (A) Differential decompression rates used for the assay. Decompression
602 assay tested in "A" (40 minutes evacuating – 10 minutes plateau – 40 minutes venting)
603 resembles the decompression rates observed at the balloon flight. (B) Survival of *B. subtilis*
604 spores and tested yeasts to differential decompression rates. No significant differences were
605 observed between treatments. (C) Differential cooling speeds used for the assays, in
606 comparison with the balloon flight. (D) Survival of *B. subtilis* spores and tested yeasts to
607 cooling down at the AstroCam and at the desiccator covered with dry ice.

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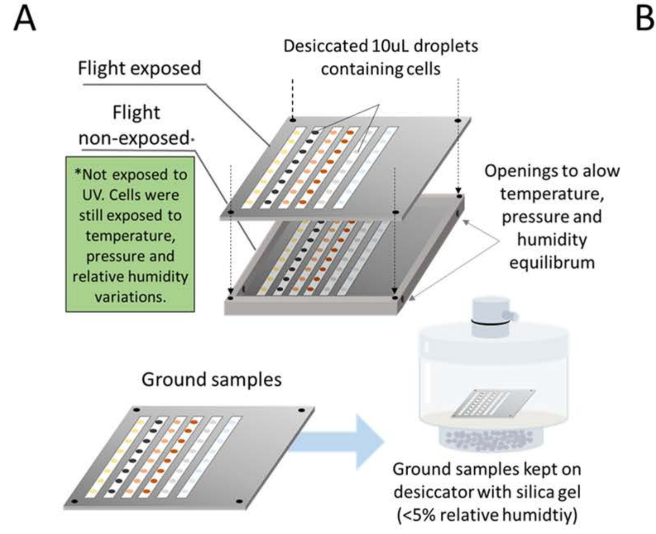

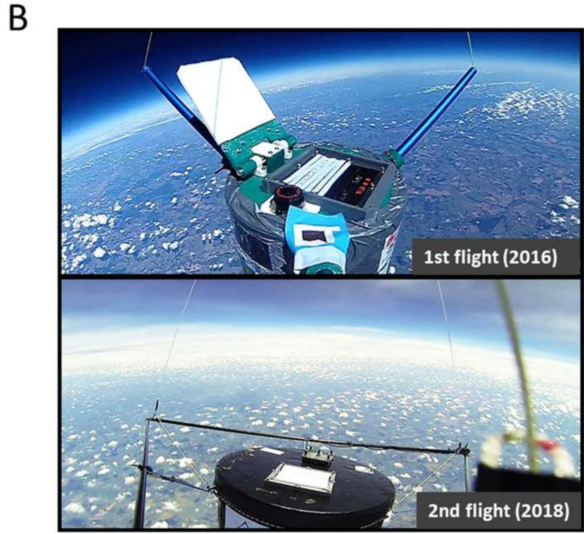

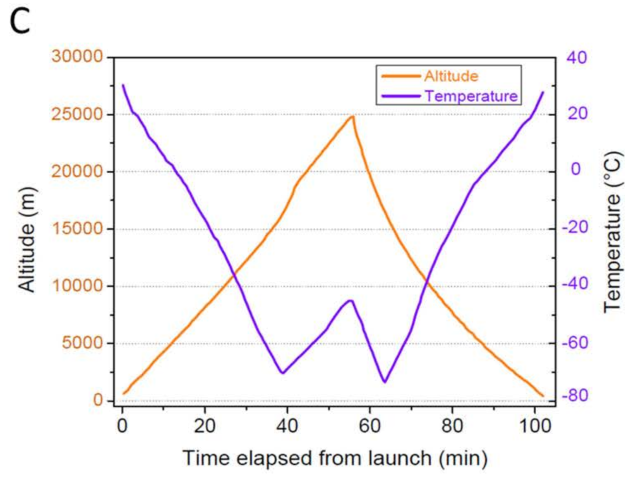

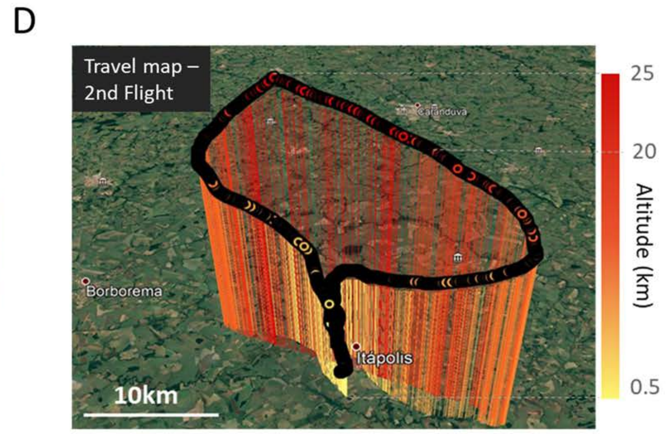

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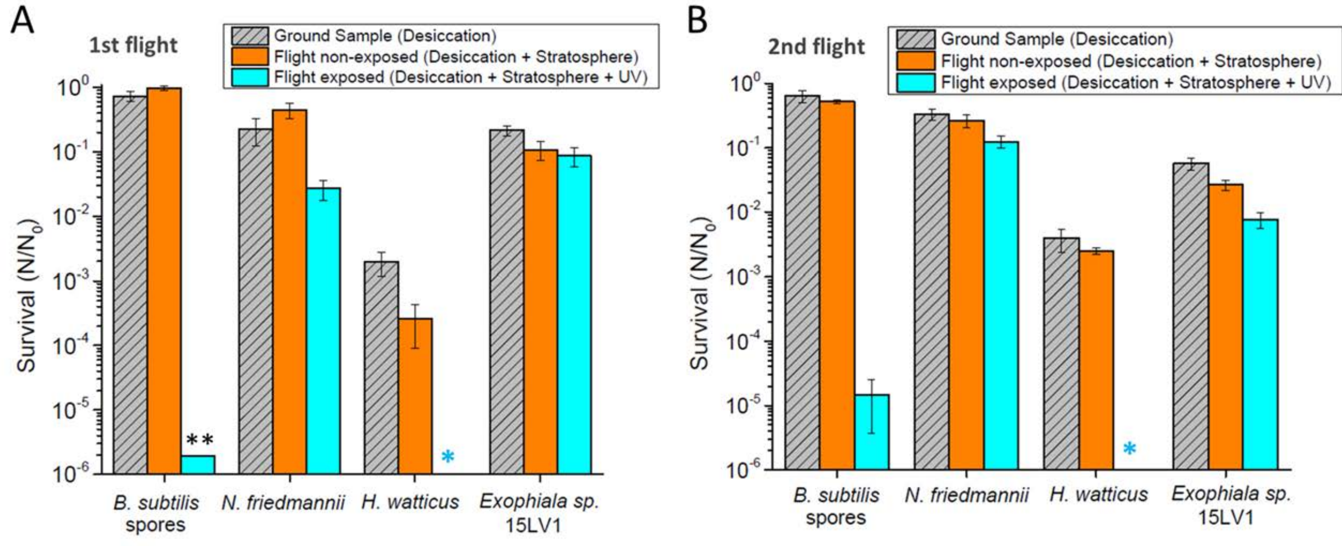

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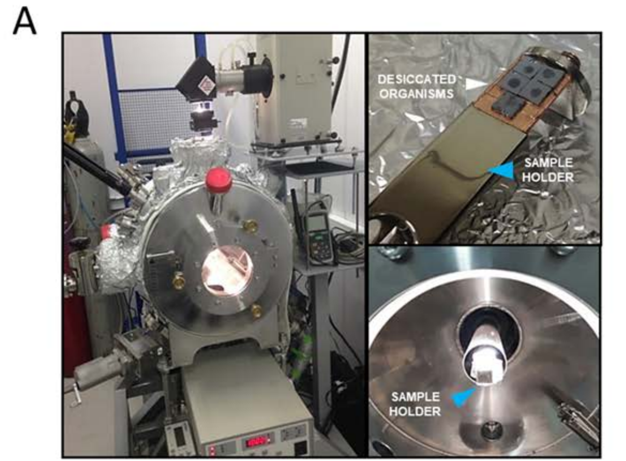

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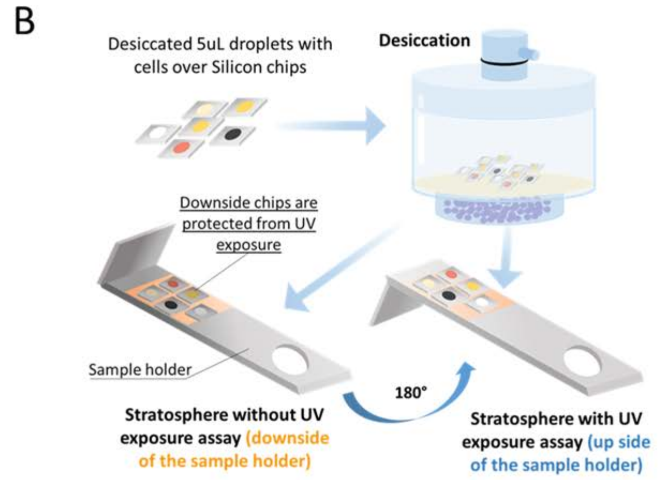

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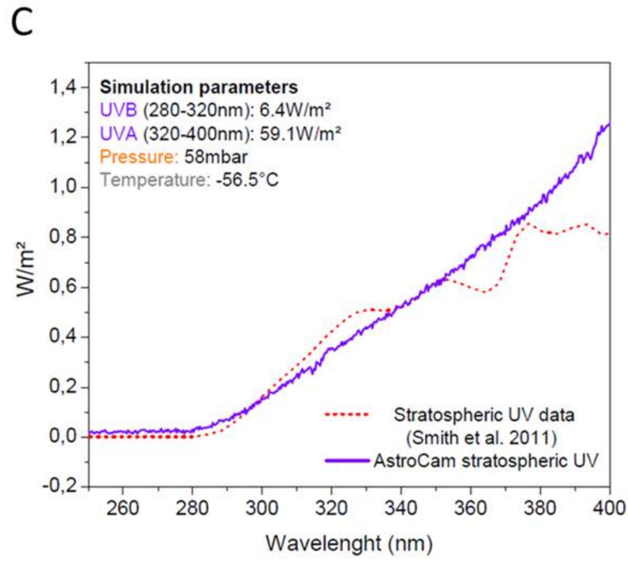

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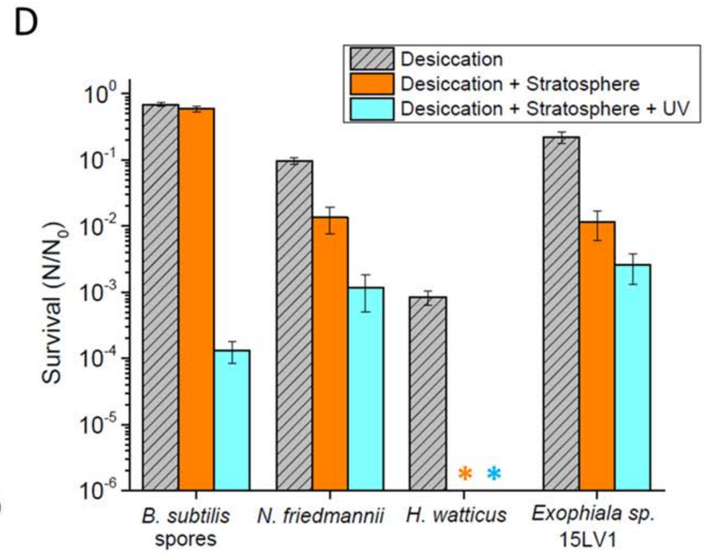

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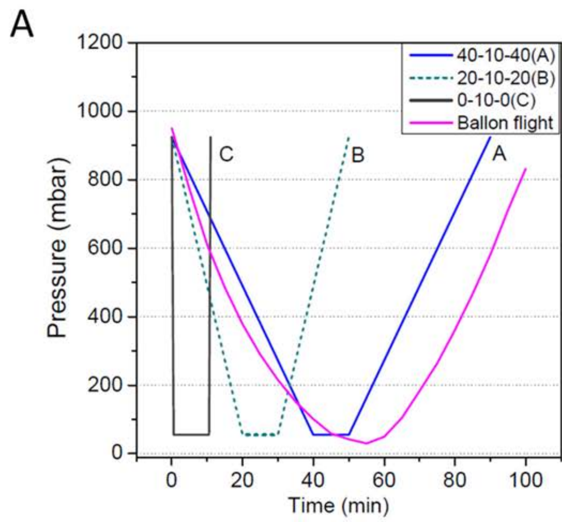

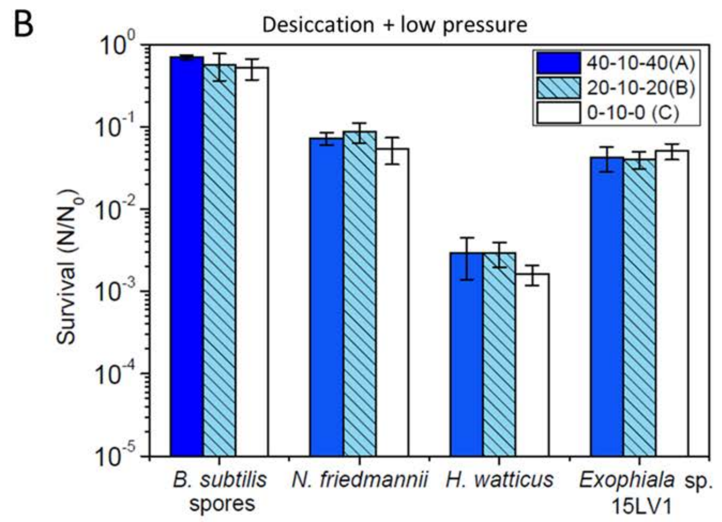

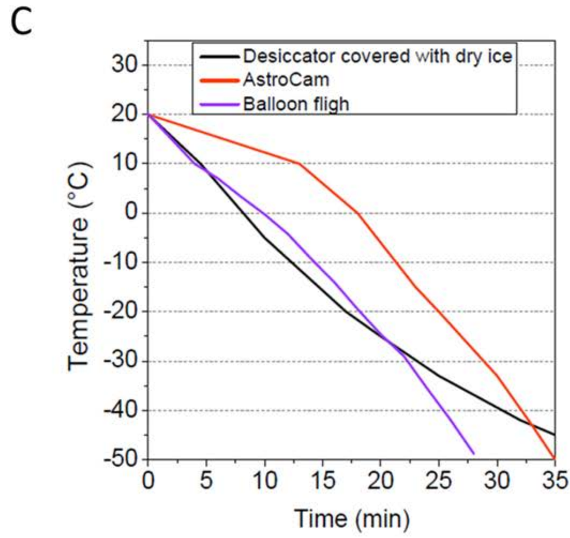

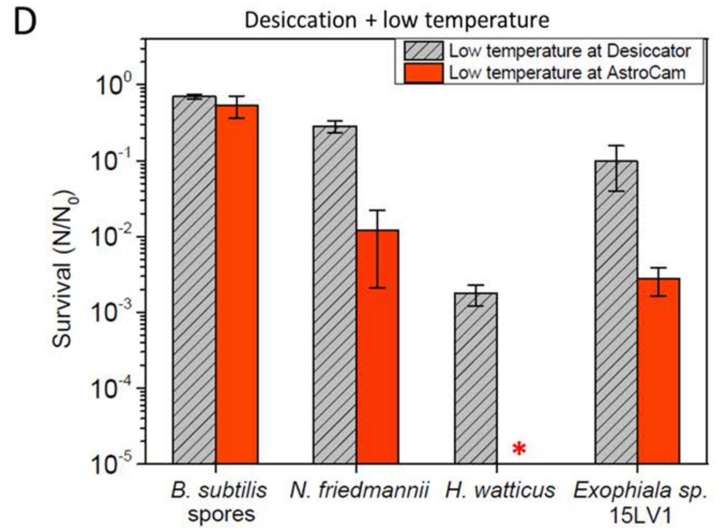

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