

Gravitational and Space Research

Impact of payload shielding on *Enterobacter cloacae* viability and proteomic profile: Insights from a stratospheric weather balloon flight experiment

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Abstract

Enterobacter cloacae, a gram-negative bacterium commonly found in the human gut microbiota, poses potential health risks to astronauts in the unique environment of space flight. This study investigated the effects of payload shielding on *E. cloacae* in a short-duration, student-initiated, weather balloon flight experiment. Faraday fabric-based payload shielding did not impact the viability of the balloon flight samples. However, murine macrophage infection assays showed that shielded balloon flight *E. cloacae* had significantly improved intracellular survival compared to unshielded *E. cloacae*. Proteomic analysis demonstrated distinct profiles in shielded and unshielded samples, with a differential abundance of proteins involved in diverse biological processes. Specifically, decreased abundance of proteins involved in chemotaxis, DNA repair, replication, transcription, peptidoglycan synthesis, and proteolysis were observed in the Faraday fabric-based payload-shielded samples. In contrast, proteins associated with protein translation, transport, tricarboxylic acid cycle, fatty acid biosynthesis, and amino acid metabolism were increased in shielded conditions. This experiment provides a framework for which future long-duration balloon flight experiments can be designed, and the findings provide initial insights into the impact of payload shielding on *E. cloacae* physiology. Understanding the impact of the stratosphere on human gut microbiota is important for preserving human health during future space flight missions.

Keywords

Enterobacter cloacae • stratosphere • balloon flight • proteomics • virulence • bacterial physiology

Introduction

Enterobacter cloacae is a rod-shaped Gram-negative bacterium commonly found as a member of the human gut microbiota. *E. cloacae* is equipped with a Type VI Secretion System (T6SS), a contractile spear-like structure bacteria used to inject effector proteins into target cells ¹. *E. cloacae* is also known for its adherence and biofilm-forming properties that allow it to attach to various surfaces ¹⁻⁴. These virulence properties are particularly significant for its potential opportunistic pathogenicity. *E. cloacae* is a significant opportunistic pathogen that poses a higher risk in hospital environments and is particularly challenging to treat due to its ability to acquire antibiotic resistance. Despite its greater threat to immunocompromised individuals, it can also cause severe infections in healthy people, emphasizing the need for vigilant healthcare practices and further research into effective treatments^{5,6}. *E. cloacae* has been detected on environmental surfaces within the International Space Station (ISS) during three flight missions ⁷, likely originating from the astronauts' microbiome. Consequently, exposure to this bacterium is probable even in such an unusual environment. *E. cloacae*

can potentially pose a health risk to astronauts, particularly in the unique environment of space flight. Space flight affects all organ systems ⁸, and impacts the immune system in space flight and analog environments ⁹. Generally, space flight suppresses innate and adaptive immune cell responses to pathogen-related stimuli ¹⁰⁻¹². Furthermore, the phagocytic function of monocytes isolated from astronauts after space flight was significantly reduced compared to monocytes from non-astronaut control subjects ^{11,13}.

Spaceflight also significantly impacts the host microbiome, as evidenced by NASA's study comparing the gut microbiomes of an astronaut and his Earthbound twin ¹⁴. The astronaut displayed notable changes, including an increased Firmicutes to Bacteroidetes ratio. Another study noted postflight microbiome differences among astronauts, with increased microbial similarity and reduced bacterial taxa ¹⁵. Whereas these studies were primarily concerned with long-term flights, there is a scarcity of work focused on changes to the microbiome upon stratosphere exposure. Only a few studies have been conducted under stratospheric balloon flight conditions, all showing significant

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challenges to bacterial survival. For example, *Salinisphaera shabanensis* and *Staphylococcus capitis* could survive in a UV-shielded Mars-like environment during balloon flight, exposing the microorganisms to the stratosphere conditions for 5 hours¹⁶. However, they exhibited a three-order magnitude decrease in survival without UV shielding¹⁵. In another study involving a shorter, 1 hour 49 minutes stratospheric flight, survival rates of the tested bacterial pathogens were low, with *Pseudomonas aeruginosa* unable to survive and other species showing survival rates ranging from 0.00001% to 0.001%¹⁷. This decreased viability was possibly related to radiation exposure, since increased bacterial survival tended to be observed in shielded flight-control samples¹⁷. However, these studies did not address the impact of stratospheric balloon flight on specific changes in genes, proteins, or specific metabolic adaptations.

Our study investigated the impact of Faraday fabric-based payload shielding on *E. cloacae* during a short-duration weather balloon flight experiment. Faraday fabric-based payload shielding did not affect overall sample viability, but shielded samples showed significantly improved intracellular survival in macrophage cell culture assays compared to unshielded samples. Proteomic analysis revealed distinct protein profiles between shielded and unshielded samples, highlighting changes in various biological processes. These findings offer initial insights into the effects of shielding on *E. cloacae* physiology and provide experimental parameters that can be expanded to a future long-duration balloon flight experiment. This research contributes to understanding potential pathogenic risks posed by gut microbiota under spaceflight conditions.

Methods

Weather Balloon Flight Experiment

Preparation of flight samples:

E. cloacae (ATCC 13047) was streaked from a -80°C frozen stock to a Lysogeny Broth (LB) agar plate and incubated for 24 hours at 37°C. After incubating at 37°C for 24 hours, a single colony was used to inoculate 25 mL sterile LB in a 125 mL flask. After growth for 18 hours at 37°C and 255 rpm (rotations per minute), the optical density at 600 nm (OD_{600}) was measured and used to calculate the volume needed to inoculate 875 mL of sterile LB in a 2 L flask to an OD_{600} = 0.05. This culture was grown for 4 hours (approximately mid-exponential growth phase, average OD_{600} = 0.75) at 37°C and 255 rpm. At this time, 40 mL aliquots of the culture were removed, and cells were collected from each of these by centrifugation. Each cell pellet was resuspended in 10 mL of

sterile LB and used to distribute 1 mL aliquots into 80 cryovial tubes. These cell aliquots were partitioned into two cardboard storage boxes containing 40 tubes, followed by immediate storage at -80°C. Four days before the weather balloon flight, samples were transferred from the -80°C freezer to a dry ice container, where they were maintained until the launch day.

Payload configuration

For the Faraday fabric-based payload-shielded setup, one sample box, initially secured in plastic biological shipping material (to comply with EH&S shipping regulations, aerospace flight standards for additional payload containment, and preventative risk mitigation, given the unpredictability of weather balloon recoveries and the potential risk to the public on unintended landing sites), was further enveloped in a triple-layered Faraday fabric (OUSEXI, a blend of polyester and high-conductivity metals like copper and nickel). The unshielded box was only wrapped in plastic biological shipping material. Within the orange payload box (Length: 33.4 cm, Width: 21.9 cm, and Height: 25.5 cm), there was a temperature and humidity sensor (Adafruit) and Shkalacar Assembled DIY Geiger Counter Kit, DIY Nuclear Radiation Detector Kit with a Miller Tube, Portable Geiger Counter, and an Assembled Radiation Detector System Experimental Module that were securely mounted above each sample box to collect a diverse range of data throughout the flight (columns AH-AR, **Table S1**). A primary flight computer also collected data such as GPS coordinates, an external barometric pressure and altitude sensor (Adafruit), and temperature and humidity sensors (Adafruit) (columns N-AG, **Table S1**). Two UV sensors (Adafruit) located on opposite sides of the primary flight computer collected UV index data (columns AE-AF, **Table S1**). These unprocessed data are provided in **Table S1** and summarized in **Table 1**.

Flight conditions

The balloon was launched from Kennedy Space Center's Educational Center on August 21, 2022, at 11:47 AM EST. The balloon's total travel time was 1 hour and 29 minutes from take-off to landing, and it remained in the stratosphere for 1 hour and 2 minutes prior to its burst. The balloon reached a maximum altitude of 27 km before a parachute was deployed for the descent. Following the GPS tracking of the payload's landing location, the payload was retrieved 1 hour and 28 minutes post-landing and moved to storage in dry ice, followed by long-term storage in a freezer at -80°C.

Ground control

E. cloacae samples were prepared as described in the Section "Preparation of flight samples" to ensure equal exposure. The ground controls remained on the benchtop for 2 hours and 57 minutes to replicate the duration of the balloon flight

Table 1. Summary of Recorded Flight Data. This table presents a summary of the flight data (recorded by the primary flight computer), which lasted for a total of 89 minutes from launch to landing. The table highlights the minimum and maximum values recorded for altitude, external temperature, pressure, and UV index during this flight mission (for the entire dataset see **Table S1**).

Independent parameters:	Minimum	Maximum
Altitude	0 km	27 km
External temperature	-57.8°C	25.04°C
External pressure	1.83 kPa	90.41 kPa
External UV index	1.44	19.13

experiment (1 hour and 29 minutes for the weather balloon flight and 1 hour and 28 minutes for search and recovery, including placement on dry ice). At 2 hours and 57 minutes, the ground controls were removed from the styrofoam box on the benchtop and transferred for subsequent freezing at -80°C to replicate the flight storage conditions.

Macrophage cell culture and infection

Eukaryotic cell culture

RAW 264.7 murine macrophage cells (ATCC# TIB-71, ATCC, USA) were grown and maintained in Dulbecco's Modified Eagle Medium (DMEM) enriched with 10% fetal bovine serum (FBS) and 100 µg/mL Penicillin/Streptomycin (Life Technologies Inc., USA).

Macrophage Infection

At 24 hours prior to infection, RAW 264.7 cells were seeded at approximately 3×10^4 cells/well in a 96-well tissue culture plate. One hour prior to infection, the cell medium was changed to incomplete DMEM (DMEM + 10% FBS). Weather balloon flight samples, both shielded and unshielded, containing *E. cloacae*, were quickly thawed following their removal from -80°C storage by placing them in a 37°C water bath for 2 minutes. After thawing, the samples were centrifuged at 6000 x g for 10 minutes. The bacterial pellets were then washed twice with PBS and resuspended in 1 mL of cold PBS. *E. cloacae* infected the macrophages for 1 hour at different multiplicities of infection (MOIs): 30:1, 50:1, or 80:1. The culture medium was replaced with a medium containing gentamicin (100 µg/mL) for 1 hour and then stored at -80°C for future ELISA processing. For 24-hour time points, the culture medium was replaced with medium containing gentamicin (20 µg/mL) and incubated for 22 hours. Cellular supernatant was removed and stored at -80°C for future ELISA processing. The cells were lysed at 2- and 24-hour timepoints using 0.1% Triton-X for 15 minutes at 37°C. Intracellular bacteria were then plated on LB agar plates for colony-forming units (CFUs) for four biological replicates.

TNF-α enzyme-linked immunosorbent assay (ELISA)

The TNF-α assay measures the release of tumor necrosis factor-α (TNF-α), a key inflammatory cytokine, from immune cells, indicating the cellular response to infection or stress. This assay is of interest because TNF-α plays a crucial role in immune regulation and its levels can provide insights into the potential inflammatory impact of *E. cloacae* under the tested conditions. Cellular supernatant was collected from RAW 264.7 cells infected with *E. cloacae* at 2- and 24-hour time points. Samples were diluted 1:10 with PBS, and TNF-α concentration was determined using a DuoSet ELISA Mouse TNF-α (R&D Systems) according to the manufacturer's protocol for four biological replicates.

Proteomic analysis

An equal amount of 25 µg protein per sample was utilized, and three replicates per sample type were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The entire gel lane corresponding to each sample was excised and diced into 1 mm² cubes, followed by in-gel trypsin digestion as we did previously^{18,19}. The resulting peptide samples were analyzed using a 250-mm Ultrahigh-Performance Liquid Chromatography (UHPLC) system coupled to an Orbitrap Fusion mass spectrometer (Thermo Scientific) similar to what was described previously^{18,19}. After the raw data were collected, tandem mass spectra were extracted, the charge state was deconvoluted, and the charge state was deisotoped using Proteome Discoverer (Thermo Scientific) version 2.1. Mascot (Matrix Science, London, UK; version 2.7.0) which was employed to analyze all MS/MS samples. The Mascot search was performed against the UniProt *E. cloacae* database (5,412 entries), assuming trypsin as the digestion enzyme. Fragment ion mass tolerance was set to 1.00 Da, and parent ion tolerance was set to 10.0 PPM. Pyrrolysine with O+18 and cysteine with carbamidomethyl were specified as fixed modifications, while n-terminal Glu->pyro-Glu, deamidated asparagine and glutamine, and methionine oxidation were set as variable modifications in Mascot. Scaffold (Proteome Software Inc., version 4.11.0) was used to validate peptide and protein identifications based on MS/MS. Peptide identifications were accepted with a greater than 95.0% probability using the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established with a greater than 95.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned using the Protein Prophet algorithm. Proteins that shared similar peptides and could not be distinguished based on MS/MS analysis alone were grouped, and proteins sharing significant peptide evidence were clustered. The reported protein false discovery rate (FDR) was 0.9%, and the peptide FDR was 0.06%. Protein quantification was performed using weighted spectral counts. The fold change was calculated

based on the spectral counts of proteins from Faraday fabric-shielded bacteria compared to unshielded bacteria. A fudge factor of 0.5 was applied to proteins that were not identified to enable calculations. Moreover, only proteins present in 2 out of 3 replicates were reported as identified proteins. Statistical significance was determined using a Fisher's exact test, and proteins with a p-value <0.05 were considered to exhibit statistically significant changes in abundance.

Principal component analysis and hierarchical clustering analysis

To conduct the principal component analysis (PCA) of the differentially abundant proteins, we utilized Clustvis (version 2.00). Row-wise unit variance scaling was applied, and SVD with imputation was used to calculate the principal components. Prediction ellipses were generated to encompass new observations from the same group with a probability of 0.95, ensuring that they fall within the ellipse. Additionally, we performed a hierarchical clustering analysis. Both rows and columns were clustered using correlation distance, and average linkage was employed. Before clustering, rows were centered and unit variance scaling was applied to ensure accurate data representation.

Enrichment analysis

For functional analysis of differentially abundant proteins, we utilized ShinyGO (version 0.80), using *E. cloacae* ATCC 13047 STRINGdb as the selected species. A false discovery rate (FDR) cutoff of 0.05 and a minimum size of 2 proteins per pathway were applied. Redundancies in the enrichment results were removed. For the enrichment of Uniprot terms and InterPro domains, as well as the generation of Local Network Clusters using STRING, we employed the respective tools and databases within ShinyGo.

Results

Summary of *E. cloacae* weather balloon flight experiment

This study was part of the 2022 Florida Space Trek Academy, organized by Atlantis Educational Services and sponsored by NASA's Florida Space Grant Consortium. Undergraduate and graduate university student team members underwent preparatory meetings and submitted written proposals seven weeks before the stratospheric balloon flight. Daily debriefings and support sessions ensured progress and cohesion. The program involved weather balloon deployment discussions, role assignments, and weather analysis for suitable launch sites. Activities included parachute creation, sensor component setup, and students' payload preparation, launch, and retrieval efforts followed by payload release.

A payload of frozen mid-exponential phase *E. cloacae* cells was flown on a small high-altitude weather balloon (**Figure 1**). *E. cloacae* samples were prepared in a frozen state for our stratospheric flight experiment to closely simulate potential microbial conditions within spacecraft environments and to preserve the initial bacterial state, facilitating direct assessment of stratospheric impact on viability and behavior. This approach not only mirrors the states in which microbes may exist onboard, including the cold zones and biological sample storage areas, but also allows for comparative analysis with existing studies employing desiccated samples^{16,20}. In brief, two boxes, each containing 40 replicate aliquots of mid-exponential phase *E. cloacae* cells, were loaded into a styrofoam cooler containing both external and internal sensors that captured a variety of environmental measurements (see **Figures 1-2**). One box of *E. cloacae* aliquots was wrapped in a triple-layered Faraday fabric. This fabric, composed of conductive metals copper and nickel, was designed to shield against a wide range of electromagnetic frequencies, encompassing microwaves, infrared, and UV light²¹. The efficacy of shielding is predicated on the fabric's material properties, which are particularly effective at attenuating electromagnetic radiation across these mentioned spectra. The fabric's effectiveness in attenuating electromagnetic radiation across these spectra is contingent upon its material properties, such as weave density and metal conductivity^{22,23}.

On the flight day, the weather balloon containing the *E. cloacae* shielded and unshielded payloads (**Figure 2**) was launched into the stratosphere. The time from launch to landing spanned 1 hour and 29 minutes, with the payload spending approximately 1 hour and 2 minutes in the stratosphere. The balloon and payload were exposed to various external environmental conditions during the flight (summarized in **Table 1**, **Figure 3**, **Table S1**). The balloon's ascent reached a maximum altitude of 27 kilometers and was exposed to a range of temperatures (-21°C minimum to -5°C in the stratosphere, **Figure 3A**), atmospheric pressures (21.87 kPa to 1.83 kPa, **Figure 3C**), and humidity levels (26% to 7.4%, **Table S1**) during the flight. Internal temperature was measured as well (**Figure 3B**). Although other internal sensor data was also measured for our payload (POD_1 in **Table S1**), water contamination of the POD_1 sensor by an unrelated payload contained in this same stratospheric balloon flight (monitored on POD_3 in **Table S1**) created technical issues and malfunctions with both these sensors. Hence, we did not expand upon these data here. Upon landing and retrieval, the samples were immediately frozen on dry ice and stored at -80°C.

Effect of weather balloon flight conditions on *E. cloacae* viability and intracellular survival in macrophages

First, we evaluated the effect of the flight in the presence or absence of shielding on *E. cloacae* viability. We determined

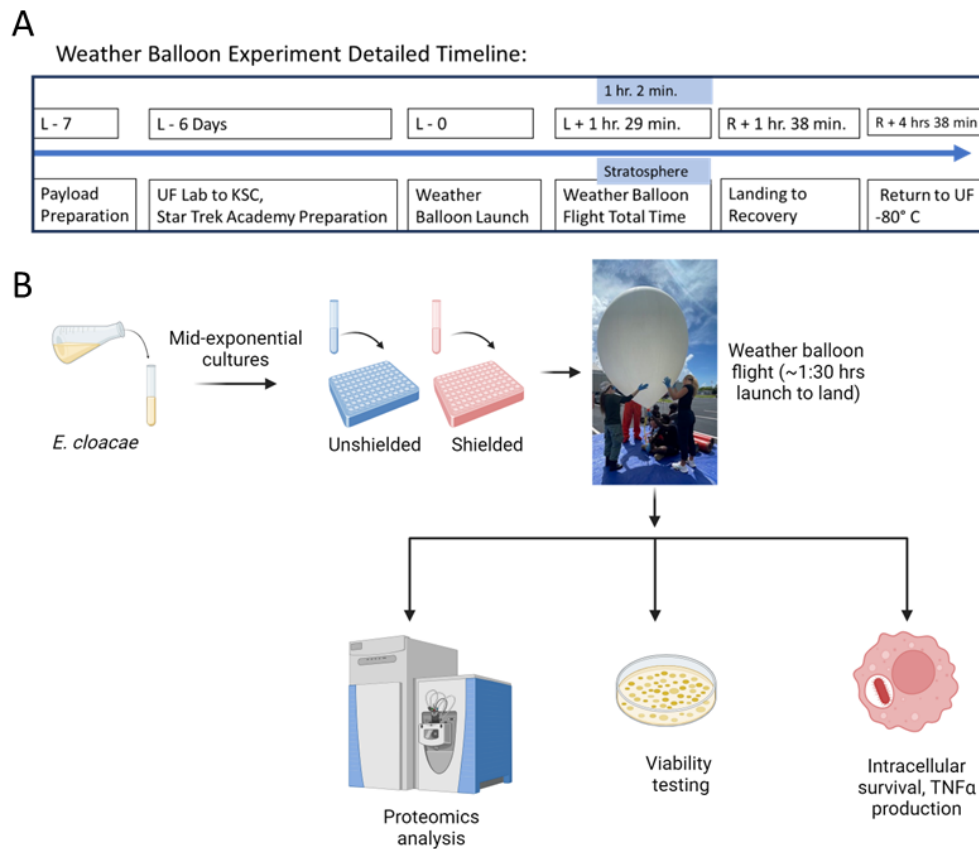


Figure 1. An overview of the experimental design. (A) Timeline of the experiment. (B). Overall experimental plan.

the colony-forming units per milliliter (CFU/mL) to assess bacterial survival after the stratosphere flight. The postflight samples that were shielded or not shielded were compared to each other and to the pre-flight samples (i.e., -80°C stored *E. cloacae* aliquots prepared as described in Materials and Methods, in a manner identical to the balloon flight samples). Interestingly, shielded and unshielded postflight samples had a significant but modest decrease in viability (~0.2 log) compared to pre-flight samples, but the shielding itself did not affect the bacterial viability (**Figure S1**). It is also important to note that long-term storage at -80°C did not affect bacterial viability (**Figure S2**).

Next, we performed a gentamicin protection assay in RAW 264.7 murine macrophages to evaluate whether Faraday fabric-based payload shielding during the weather balloon flight affects the ability of *E. cloacae* to survive within host cells and to assess the potential virulence of this bacterium. We observed that shielding the samples significantly increased the survivability (measured by intracellular CFU/mL) within infected macrophages at 2 hours post-infection (hpi) compared to cells subjected to the flight without shielding (**Figures 4A, C**). A similar trend was also observed at 24 hpi (**Figures 4B, D**).

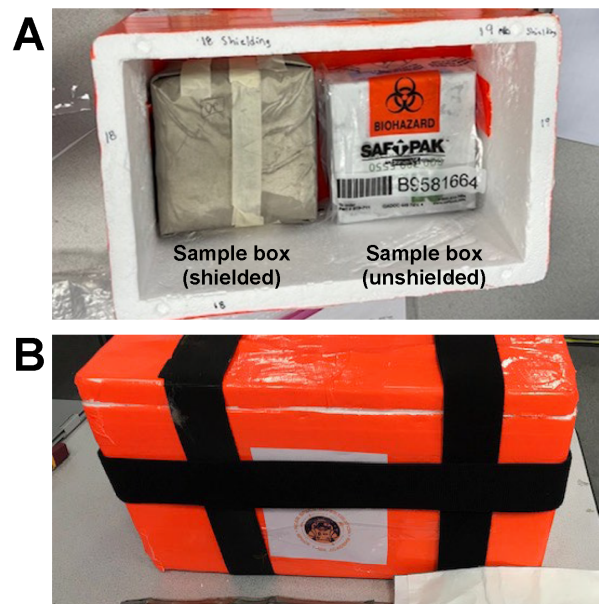


Figure 2. Payload configuration. A: Shielded (left) and unshielded (right) sample boxes. B: Sealed payload box.

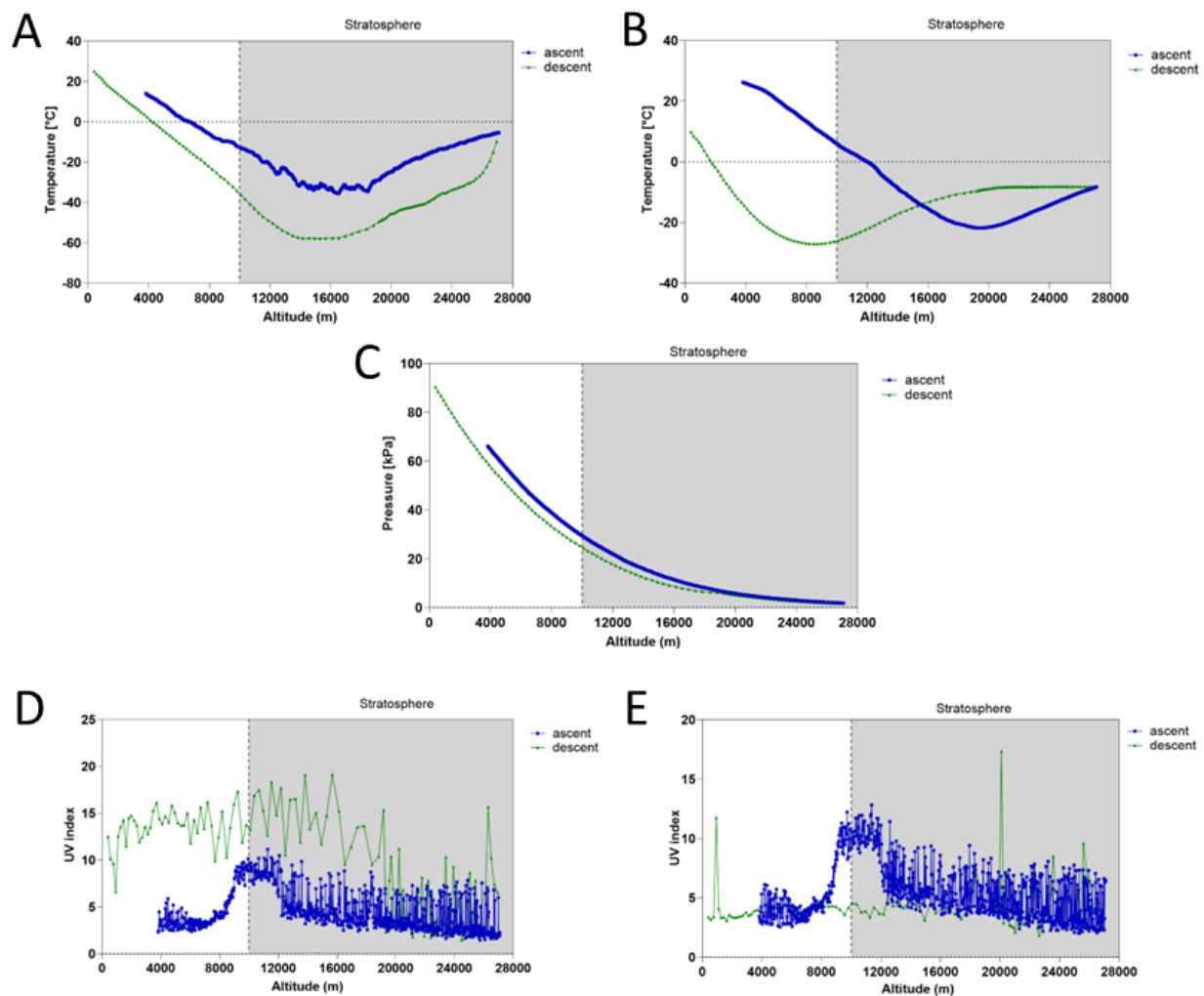


Figure 3. Readings of temperature, pressure, and UV index from during the ascent and descent of the balloon flight. **(A)** External temperature measurements recorded during the flight. **(B)** Internal temperature measurements recorded during the flight. **(C)** Atmospheric pressure measurements taken throughout the flight. External UV index measurements from Sensor 1 **(D)** and Sensor 2 **(E)** during the flight.

To complement these findings, we executed a ground control (GC) experiment designed to replicate the ambient temperature conditions encountered during the flight. Here, frozen bacterial samples were subjected to a 2-hour and 57-minute incubation at room temperature within a styrofoam cooler, mirroring the complete duration of the balloon flight experiment. Post incubation, these samples were swiftly returned to -80°C storage pending further analysis. Analysis revealed that the ground control samples exhibited lower overall viability than the flight samples (**Figure S1**) despite experiencing consistent temperature exposure to the balloon flight. Additionally, in the intracellular survival assay conducted with ground control bacteria, a decrease in bacterial count was observed from 2 to 24 hours post-infection (**Figure S3**, MOI of 43:1). It is worth noting that direct comparison of ground

samples with the flight samples might be somewhat skewed as the MOI for the flight-exposed samples was nearly double that of the ground control bacteria. Despite efforts to standardize, such as maintaining ground controls at room temperature within styrofoam coolers to closely simulate flight conditions, discrepancies between the hypothetical and actual MOIs were evident. These differences were particularly evident in ground controls, where retests confirmed a consistently lower actual MOI. The objective to achieve an infection MOI of 80:1 was specifically challenged by these findings, highlighting the biological variability.

We subsequently assessed the release of tumor necrosis factor- α (TNF- α) from murine macrophages after exposure to *E. cloacae*. A comparative analysis of macrophages infected with shielded and unshielded *E. cloacae* samples exhibited

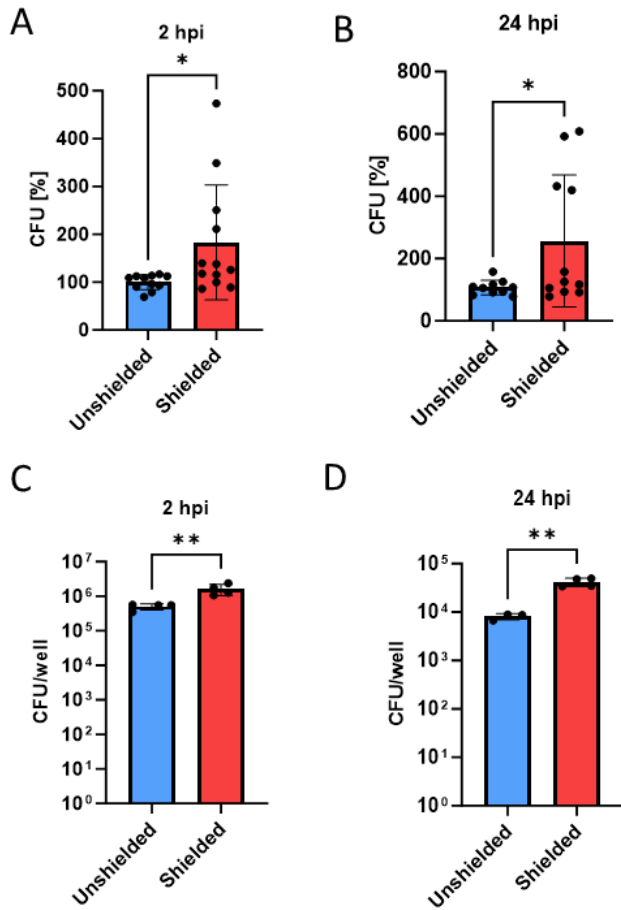


Figure 4. The intracellular survival of *E. cloacae* exposed to weather balloon flight in the payload shielded compared to unshielded conditions. *E. cloacae* exposed to the weather balloon flight in the presence or absence of Faraday fabric-based shielding were used to infect RAW 264.7 murine macrophages at an MOI of 80:1 for 2 hours post-infection (hpi) (A, C) or 24 hpi (B, D). After completion of the infection period, cells were lysed, and lysates were spread on LB plates to count CFUs. The experiment was repeated twice for a biological triplicate. For each individual experiment, average CFUs from infections with unshielded bacteria were considered as 100% (A, B), and one individual experiment is shown as total CFUs/well for each time point (C, D). Statistical significance was determined using a t-test. *, $p < 0.05$, **, $p < 0.005$, n.s. = not significant.

substantial TNF- α release (Figure 5). While no statistically significant differences were observed in the levels of TNF- α releases between macrophages infected with shielded and unshielded *E. cloacae*, a pronounced increase in the pro-inflammatory cytokine was observable when ground control *E. cloacae* was used for infection (Figure 5). This observation is noteworthy, particularly considering the lower Multiplicity of Infection (MOI: ~43:1) applied for ground control *E. cloacae*, in contrast to the higher MOI (~80:1) used for the flight-exposed *E. cloacae*.

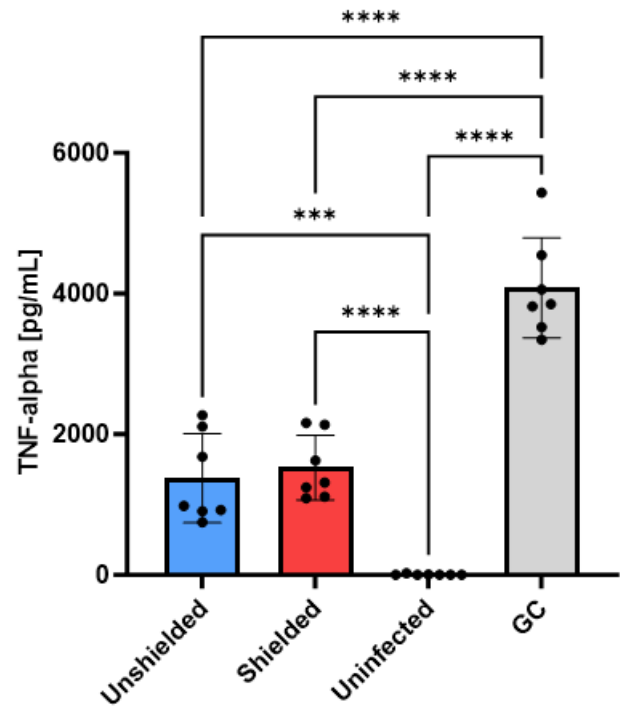


Figure 5. The effect of *E. cloacae* exposure to weather balloon flight in the shielded compared to unshielded conditions on the TNF- α release from infected cells. *E. cloacae* exposed to the weather balloon flight in the presence or absence of Faraday fabric-based shielding as well as ground control were used to infect RAW 264.7 murine macrophages at an MOI of 80:1 for 2 hpi. After the infection time was complete, the cell culture supernatant was collected and the TNF- α was quantified in the media by using ELISA. N=4. For establishing statistical significance, a 1-way ANOVA test was used. *, $p < 0.05$, **, $p < 0.005$, ***, $p < 0.0005$, ****, $p < 0.0001$.

Effect of payload shielding on the *E. cloacae* proteome during the weather balloon flight

Next, the impact of payload shielding on the proteome of *E. cloacae* during the weather balloon flight was investigated. To achieve this, proteome profiling was performed on *E. cloacae* shielded and unshielded postflight samples. Bacteria from each postflight sample were lysed, and cellular proteins were extracted, followed by SDS-PAGE analysis and tryptic digestion of entire gel lanes. The resulting peptides were then analyzed using Orbitrap Fusion Mass Spectrometry.

In our study, 1355 *E. cloacae* proteins were identified, with 1217 proteins detected in both the shielded and unshielded samples during the weather balloon flight (Figure 6A). Additionally, we found 24 unique proteins in the shielded samples and 114 unique proteins in the unshielded samples (Figure 6A). Label-free quantitative mass spectrometry analysis revealed that 97 proteins exhibited altered abundance between the shielded and unshielded conditions (Fisher's test $p < 0.05$, fold change greater than -1.5/+1.5). Among these,

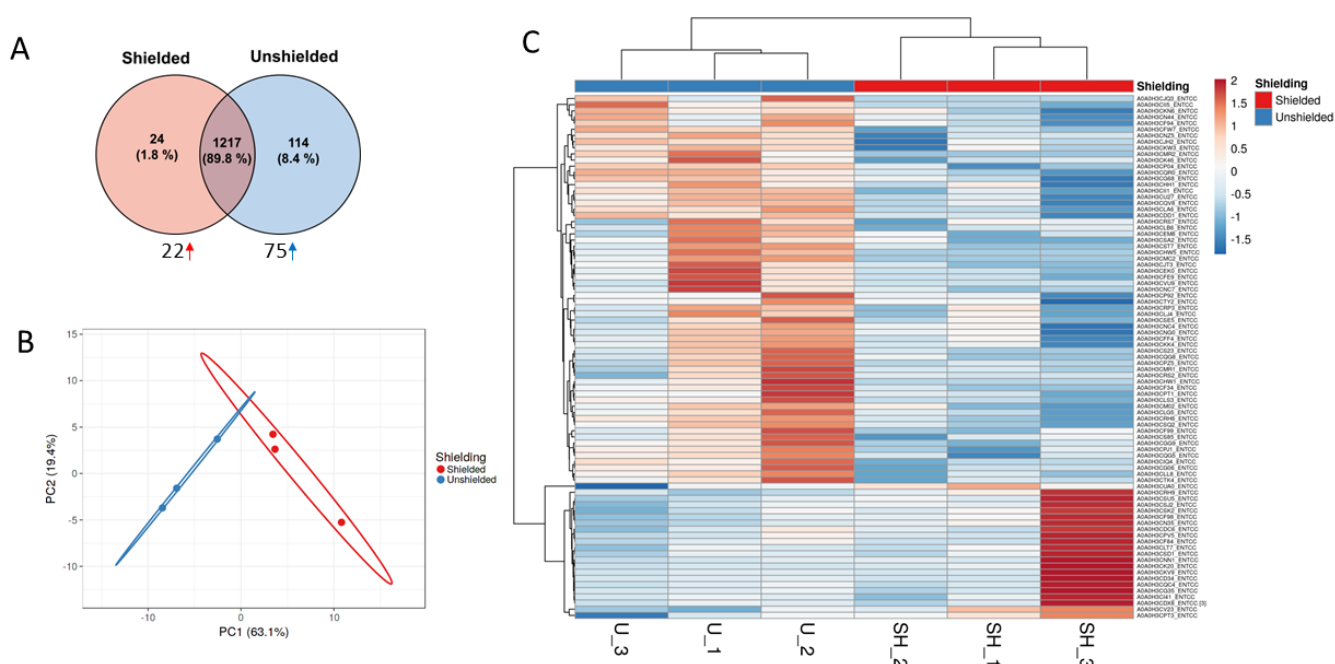


Figure 6. Proteomic analysis of the *E. cloacae* response to payload shielding during weather balloon flight. (A) Comparative proteomic profiling of *E. cloacae* exposed to shielded and unshielded conditions during weather balloon flight. The Venn diagram depicts the distribution of identified proteins, with 1217 proteins detected in both shielded and unshielded samples, 24 proteins unique to shielded conditions, and 114 proteins unique to unshielded conditions. Altered protein abundance analysis highlights 97 proteins with significant changes ($p < 0.05$, fold change $> -1.5/+1.5$); 22 proteins exhibited higher abundance in shielded samples, whereas 75 proteins were more abundant in unshielded samples. (B) Principal Component Analysis (PCA) showed distinct clustering of proteomic profiles between shielded and unshielded *E. cloacae* samples. Each data point represents a sample, with shielded and unshielded conditions forming separate groups based on protein expression patterns. (C) Unsupervised hierarchical clustering analysis of differentially regulated proteins in shielded and unshielded *E. cloacae*. The heat map reveals protein clusters exhibiting specific increases in abundance under each condition, allowing robust differentiation between shielded and unshielded samples.

22 proteins displayed increased abundance in the shielded conditions, while 75 proteins were increased in the unshielded conditions (Figure 6A).

To explore the relationship between the proteins with altered abundance in shielded and unshielded samples, we performed principal component analysis (PCA), which revealed a distinct grouping of the samples based on their proteomic profiles (Figure 6B). Furthermore, unsupervised hierarchical clustering analysis of the proteins in shielded versus unshielded bacteria identified clusters of proteins that specifically showed an increase in abundance in each condition, providing a reliable means of distinguishing between shielded and unshielded samples (Figure 6C).

To gain insights into the biological functions of the differentially abundant proteins in *E. cloacae* under shielded or unshielded conditions during the weather balloon flight, we conducted further bioinformatic analyses (Figure 7, Table S2-S7). We initially investigated the enrichment of specific Uniprot-assigned functions among the differentially abundant proteins. Notable functional categories included transferases, ATP-binding proteins, nucleotide-binding proteins, coiled-coil

proteins, kinases, ribosomal proteins, ribonucleoproteins, helicases, metalloproteases, proteins involved in glycolysis, and other metabolic proteins (Figure 7A). Additionally, InterPro domain mapping revealed that a significant number of the differentially abundant proteins belonged to specific domains, including O-loop containing nucleoside triphosphate hydrolases, pyridoxal phosphate-dependent transferases, tetratricopeptide-like helical domains, CoA-binding proteins, and histone-like DNA-binding proteins (Figure 7B). Moreover, STRING analysis identified various functional associations of the differentially abundant proteins, such as alcohol dehydrogenases, ribosomal proteins, lysine tRNA ligases, proteins involved in glycogen metabolism and glycolysis, DNA-directed DNA polymerases, and others (Figure 7C). Furthermore, through manual inspection of the differentially abundant proteins in shielded versus unshielded conditions, we observed a decrease in chemotaxis proteins in the shielded samples compared to the unshielded samples (Table S2), such as FlgK (A0A0H3CJP9, -10.00) or FliL (A0A0H3CQG8, -2.00). This finding suggests that the mobility of *E. cloacae* may be affected by shielding. Moreover, a notable decrease

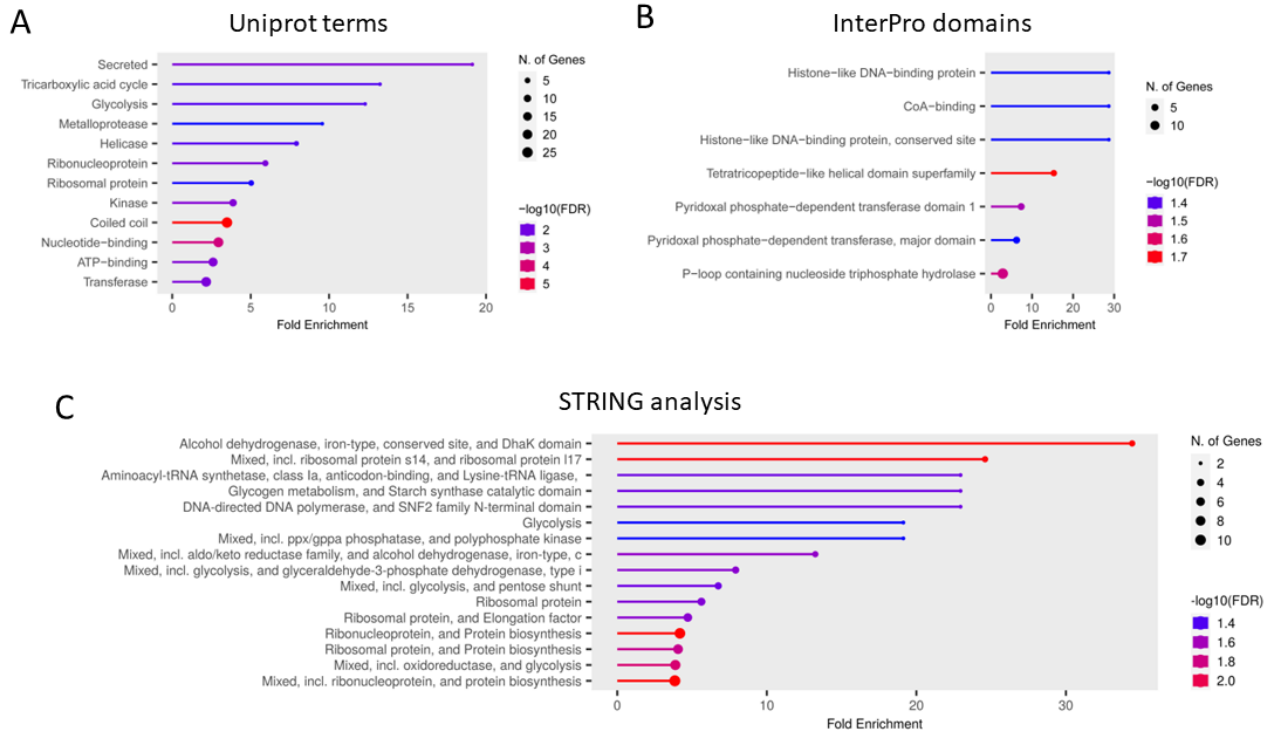


Figure 7. Bioinformatic analysis of differentially abundant proteins in *E. cloacae* under weather balloon flight conditions with and without Faraday fabric-based shielding. (A) Enrichment analysis of Uniprot-assigned functions. (B) InterPro domain mapping identifies enriched protein domains. (C) STRING analysis.

was observed in the levels of proteins involved in DNA repair, replication, and transcription in the shielded samples compared to the unshielded samples (Table S3), such as RapA (A0A0H3CEW2, -10.00), RnK (A0A0H3CMR3, -10.00), RuvA (A0A0H3CIN6, -5.00), MalT (A0A0H3CQR0, -3.33), PolA (A0A0H3CRP3, -2.50), or IhfA (A0A0H3CLA6, -2.00). In addition, decreased abundance of proteins related to peptidoglycan synthesis, turnover, cell division, and other cell wall processes were observed in *E. cloacae* under shielded conditions (Table S4), such as in AnmK (A0A0H3CJQ3, -5.00), ParC (A0A0H3CRH6, -3.33), or Ddl (A0A0H3CIQ4, -3.33). Similarly, proteins involved in proteolysis, such as the chaperone BepA (A0A0H3CNZ5, -2.50), showed primarily a decreased abundance in shielded conditions (Table S5). In terms of proteins involved in protein translation and transport, these molecules exhibited increased abundance in the shielded samples compared to unshielded conditions (Table S6), such as SecB (A0A0H3CD34, +2.22), a protein involved in protein transport, or translation proteins RplV (A0A0H3CUA0, +1.90), RplR (A0A0H3CSK2, +1.80), RplQ (A0A0H3CSJ2, +1.70), RplL (A0A0H3CF84, +1.60), or RplB (A0A0H3CRH9, +1.50). However, other proteins involved in the translation and protein transport showed a

decreased abundance in the shielded samples, including LysS (A0A0H3CP92, -1.67), SrmB (A0A0H3CNG0, -1.67), Ffh (A0A0H3CSE5, -1.67), and ArgS (A0A0H3CGG9, -1.67) (Table S6).

Finally, the largest group of differentially abundant proteins was associated with various metabolic processes. Notably, tricarboxylic acid cycle proteins SucD (A0A0H3CPV5, +1.60) and Mdh (A0A0H3CQC4, +1.60) exhibited increased abundance in shielded conditions, as did several proteins involved in fatty acid biosynthesis and amino acid metabolism (Table S7). Conversely, proteins involved in the metabolism with a decrease in abundance in the shielded versus unshielded conditions were phosphofructokinase (PfkA, A0A0H3CTK4, -1.67), enolase (Eno, A0A0H3CEM8, -1.67), amino acid biosynthetic proteins AroB (A0A0H3CSQ2, -2.00), Asd (A0A0H3CRS7, -2.00), ProB (A0A0H3CG68, -2.00), adenosine deaminase (Add, A0A0H3CKK4, -2.00), Coenzyme A biosynthesis bifunctional protein CoaBC (A0A0H3CEK0, -2.50), PurE (A0A0H3CJT3, -2.50), glycogen synthase (GlgA, A0A0H3CST7, -3.33), GpmB (A0A0H3CII5, -3.33) that is involved in the glycolytic process, and Lgt (A0A0H3CPZ5, -3.33) that regulates the lipoprotein biosynthetic processes.

Discussion

This study was conducted during the summer of 2022 at Florida Space Trek Academy, where university student teams conducted experiments on small, high-altitude weather balloons. Our study demonstrates that student-driven research experiences can be harnessed to generate proof-of-principle feasibility data using short-duration flight experiments (1-4 hours) with small high-altitude weather balloons. Specifically, our study assessed the impact of weather balloon flight conditions and Faraday fabric-based payload shielding of samples of *E. cloacae* exposed to a short-duration stratospheric flight. *E. cloacae* was selected as our model organism due to its relevance as a gram-negative bacterium in human gut microbiota. This bacterium can also pose a pathogenic threat under favorable conditions^{6,24,25}. Understanding the impact of electromagnetic radiation (including UV and infrared radiation) on these bacteria is essential. On Earth, these microorganisms are shielded from most UV radiation by the atmosphere, particularly the ozone layer²⁶. However, during extreme conditions of stratospheric flight, such bacteria can be exposed to UVA, UVB, and UVC radiation. Additionally, the expected radiation levels in the stratosphere are close to those expected on the surface of Mars²⁷. For instance, in a previous study involving a balloon mission to the middle stratosphere, the fluence of UVA-UVB radiation was calculated at 6 mW/cm², resulting in an estimated cumulative radiation dose of 1148 kJ m⁻²¹⁶. Based on previously published research, we expected that *E. cloacae* survival would be significantly compromised under these stratospheric balloon flight conditions. For example, *Salinisphaera shabanensis* and *Staphylococcus capitis* both managed to survive in a UV-shielded Mars-like environment during a balloon flight to the stratosphere, however, their survival rates dropped significantly in the absence of UV shielding¹⁶. Moreover, in that same study, the viability of a Gram-negative *Buttiauxella* sp. was undetectable (both with and without UV exposure)¹⁶. In another stratospheric balloon flight study, which reached a maximum altitude of approximately 31 kilometers, pathogenic bacteria were exposed to high levels of UV light for approximately 1 hour and 49 minutes during the flight, followed by viability and antibiotic resistance assessments²⁰. Interestingly, *Pseudomonas aeruginosa* samples did not survive flight exposure, and survival rates were notably low for *Klebsiella pneumoniae* and *Staphylococcus aureus* flight samples. The radiation exposure appeared to contribute to the diminished survivability of these bacteria since the flight control samples covered with aluminum foil experienced increased survival in that study compared to unshielded samples²⁰. In comparison to these previous studies, our research revealed that the viability of *E. cloacae* was not significantly affected when

comparing flight samples with or without shielding. However, flight samples' overall viability was modestly lower than pre-flight samples. However, we also observed lower viability in ground control samples compared to flight samples, despite similar temperature exposures, which could be attributed to several factors, such as radiation, which may induce stress responses beneficial for bacterial survival. Differences in exposure time of this and past experiments (ranging from 5 hours¹⁶, 1 hour and 49 minutes²⁸ and 1 hour for our study) and payload type (desiccated cells¹⁶, cell pellets²⁸, and frozen suspensions of cells in our study) may contribute to these variations in response to balloon flight conditions. Notably, *E. cloacae* exposed to flight conditions remained viable (possibly due to samples being flown as frozen suspensions of cells) and retained its ability to infect macrophages. Importantly, the continued viability and infectious capability of *E. cloacae* post-flight suggests its adaptability to extreme environments, potentially mirroring conditions on Mars or within spacecraft. We also examined the intracellular survival of Faraday fabric-shielded and unshielded *E. cloacae* flight samples within macrophages. This cell type is a key innate immune cell responsible for the phagocytosis of bacteria. *E. cloacae* ATCC 13047 has previously shown the highest rates of macrophage cell attachment and macrophage invasion among several tested *E. cloacae* strains, and its invasiveness was significantly greater than that of *Salmonella* Typhi²⁹. Our study showed that while Faraday fabric shielding used during the stratospheric flight did not impact bacterial viability, it significantly enhanced the intracellular survival of *E. cloacae* within infected RAW 264.7 macrophages over time compared to infection of bacteria subjected to flight without shielding. This result suggests that the improved intracellular survival of these bacteria in macrophages results from exposure to shielding during the weather balloon flight, despite the absence of any impact on bacterial viability during the flight. Moreover, the release of TNF- α from macrophages was significantly decreased when *E. cloacae* cells exposed to flight were used for infection, compared to infection with the ground control *E. cloacae* cells. This observed phenomenon does not correlate with increased bacterial loads within the infected macrophage, as ground control infection MOIs were almost half of the flight sample infection MOIs yet had significantly elevated levels of TNF- α release. This result might indicate that the ground control cells are eliciting more pro-inflammatory responses. Elevated TNF- α levels are often associated with adverse outcomes, which can lead to fulminant neonatal septic shock caused by *E. cloacae*³⁰. However, the underlying reasons for the decreased TNF- α levels mediated by exposure of macrophages to flight-exposed *E. cloacae* compared to ground control remain unknown.

We performed a proteomic investigation of changes in bacterial protein abundance between these two weather

balloon flight samples to gain more insight into the reason behind improved intracellular survival of Faraday fabric-shielded *E. cloacae* flight samples compared to unshielded flight samples. We identified differentially abundant proteins, with functional enrichment analysis revealing their involvement in various biological processes. These processes included transferases, ATP-binding, ribosomal, and metabolic proteins. Further analysis unveiled decreased chemotaxis proteins and proteins associated with DNA repair, replication, transcription, peptidoglycan synthesis, cell division, and proteolysis in the shielded samples. Proteins related to protein translation, transport, the tricarboxylic acid cycle, fatty acid biosynthesis, and amino acid metabolism exhibited increased abundance under shielded conditions, while specific metabolic proteins experienced a decrease. These findings highlight adaptive strategies employed by *E. cloacae* in extreme environments. None of the previous studies testing exposure of microorganisms to stratospheric flight provided information on the changes to genes/proteins in bacteria exposed to flight^{16, 20}. However, it is crucial to acknowledge several limitations of our study. These include the singular exposure timepoint in the stratosphere, the timing of sample retrieval post-landing, and the lack of a more robust ground control group for a comprehensive comparative analysis across all assays. Moreover, further investigations are necessary to fully understand the long-term effects of stratospheric exposure on bacterial viability and functionality. Building on the foundational data and insights from this preliminary investigation, we underline the need for replication and further experimentation. The nature of high-altitude balloon experiments, accentuated by the interaction of diverse environmental factors, calls for repeated trials under a variety of conditions to deepen our understanding. For instance, one of the aspects of future studies will be to examine the differential impacts of diurnal variations on microbial survival and behavior through flights conducted during both day and night. Such research will shed light on the adaptability and resilience of microorganisms to these extreme environments.

Conclusions

Our study offers insights into the resilience of *E. cloacae* under extreme stratospheric flight conditions and the effects of Faraday fabric-based payload shielding. We have revealed notable changes in bacterial viability, intracellular survival, and proteomic profiles, revealing how stratospheric radiation can affect bacteria in challenging environments, even during a short-duration exposure. This finding is crucial for understanding how extremophiles^{31 32 33 34} and bacteria survive in harsh environments such as space flight and Martian terrain³⁵. Moreover, our work highlights the practicality of using stratospheric balloon flights for controlled

biological experiments, especially in studying radiation's impact on organisms^{16 36 37}. Finally, understanding the effects of radiation on gut microbiota and opportunistic pathogens is important for learning more about astronaut health during spaceflight.

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