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Survival strategies of *Rhinocladiella similis* in perchlorate-rich Mars like environments

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Studying the survival of terrestrial microorganisms under Martian conditions, particularly in the presence of perchlorates, provides crucial insights for astrobiology. This research investigates the resilience of the extremophile black fungus *Rhinocladiella similis* to magnesium perchlorate and UV-C radiation. Results show *R. similis*, known for its tolerance to acidic conditions, exhibits remarkable resistance to UV-C radiation combined with perchlorate, as well as to high concentrations of magnesium perchlorate, surpassing *Exophiala* sp. strain 15Lv1, a eukaryotic model organism for Mars-like conditions. Growth curve analyses revealed both strains can thrive in perchlorate concentrations mimicking Martian perchlorate-rich environments, with *R. similis* adapting better to higher concentrations. Morphological and protein production changes were investigated, and mass spectrometry identified perchlorate-induced proteins, advancing molecular understanding of potential microbial life on Mars. These findings advance knowledge of extremophile capabilities, contributing to the search for life beyond Earth and informing the design of future Martian rovers equipped for biosignature detection.

The quest to understand habitability on extraterrestrial planets, particularly Mars, is an intriguing journey that expands the frontiers of our knowledge. The discovery of perchlorates in Martian soil, confirmed by several NASA missions, such as the Phoenix lander, Mars Reconnaissance Orbiter, and the Curiosity rover, underscores the importance of investigating the interaction between terrestrial microorganisms and these highly challenging chemical and environmental conditions[1–3]. Specifically, in the context of Mars, these challenges involve the high concentrations of salts, such as perchlorates, coupled with environmental factors that exacerbate their severity: the absence of liquid water, low atmospheric pressure, and intense radiation. These conditions impose significant osmotic stresses and oxidative challenges on potential microbial life, as perchlorates can act as strong oxidants, particularly when exposed to ionizing radiation[4]. Currently, there is evidence that perchlorate salts, possibly magnesium perchlorate, are predominant compounds in Martian soil, with concentrations ranging between 0.4% and 0.6% by weight. However, the specific counter ion associated with these perchlorate ions has not yet been definitively determined, as some studies also suggest calcium as a potential counter ion[5,6]. The propensity of these salts to form brines by absorbing atmospheric water vapor, and subsequent deliquescence proposes a hypothetical medium for the existence of liquid water at sub-zero temperatures, creating potential niches for microbial life. However, it is important to note that liquid perchlorate brines have not been definitively detected on Mars; instead, some studies have proposed exciting interpretations based on rover and satellite data[7–9].

Discovering terrestrial microorganisms that can tolerate perchlorates has been a challenge. Perchlorate is a potent oxidant, and toxic to most of terrestrial life by promoting denaturation of macromolecules, oxidative stress, and DNA damage[10]. However, recent research has shown that some archaea (i.e., *Haloferax mediterranei* and *Halorubrum lacusprofundi*), extremophilic bacteria (i.e., *Halomonas venusta* and *Planococcus halocryophilus)*, and fungi

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(i.e., *Purpureocillium lilacinumcan, Debaryomyces hansenii*, and *Cryomyces antarcticus*) can resist to surprisingly high concentrations of perchlorates. For example, *Planococcus halocryophilus* can tolerates 1.1 M sodium perchlorate ($NaClO_4$) and the fungal species *Debaryomyces hansenii* tolerates 2.4 M ($NaClO_4$); but usually, bacteria can tolerate up to 0.1 M, and fungi may tolerate up to 0.15 M[1,7,11–14]. These can provide insights for future research to develop more effective and useful biological systems on Mars to understand better how life could have been established on the Red Planet and for in situ resource utilization. The tolerance of eukaryotic organisms, such as yeasts and other fungi, to magnesium perchlorate remains poorly explored, especially at high concentrations. While some studies have investigated the potential of the fungus *Debaryomyces hansenii* to survive at high concentrations of magnesium perchlorates, most research has focused on sodium perchlorate and other salts[15,16]. Therefore, there is a significant gap in the literature that calls for more comprehensive investigations, including other types of fungi and magnesium perchlorates. Since sodium ions have an osmotic effect at high concentrations and are not chaotropic similar to magnesium, sodium perchlorate is only useful for certain studies, such as understanding the effect of the perchlorate anion[13]; therefore, they may not fully reflect the challenges posed by Martian soil, which is predominantly composed of perchlorates associated with divalent cations, such as magnesium[5]. Hence, investigating the effects of magnesium perchlorate is crucial, as it provides a more accurate simulation of Martian soil conditions.

Another factor that remains underexplored is how microorganisms survive simultaneous exposure to perchlorate salts and ultraviolet (UV)-C radiation. While the harmful effects of UV-C radiation on bacterial cells—primarily targeting DNA through the formation of reactive oxygen species (ROS) and inducing mutagenesis—are well documented and characterized[17], the combined impact of perchlorate and UV-C radiation is particularly relevant to Mars. This is because Mars' extremely thin atmosphere permits UV-C radiation to reach the surface, where it can interact with potential perchlorate brines. The coexistence of both environmental stressors has shown significant bacteriocidal effect on bacterial cells[18]; however, further investigation is needed for the understanding of the effects on extremophile eukaryotic microorganisms.

The black yeast *R. similis*, belonging to a class of fungi known for their robustness in extreme terrestrial environments, has emerged as an intriguing model to investigate microbial resistance and adaptability in simulated Martian conditions, given that this class of microorganisms has previously been used as an astrobiological model[19]. Faced with the aforementioned challenges, it is essential to report unique microbial-origin chemical entities (i.e., molecules and biominerals) produced in the presence of perchlorate. Such molecules may serve as indicators of biological activity, assisting in distinguishing between abiotic and biotic processes. Although the stability of perchlorate brines on the Martian surface is debated, with analyses suggesting they can only be temporarily stable at very high concentrations (e.g., 44 wt% in the case of magnesium perchlorate eutectic brines[8,13]), more diluted brines in the subsurface could be stable for longer periods[9]. Determining how extremophile microorganisms could survive under these conditions, even at different concentrations of perchlorate, at the molecular level, is essential for understanding potential life forms on Mars, especially at different concentrations of perchlorate. Additionally, understanding the chemical signatures and molecules produced by microbial life in perchlorate-rich environments can significantly contribute to ongoing efforts in astrobiology, assisting in the formulation of life detection strategies for future missions to Mars[15,20,21].

This study investigates the tolerance of *Rhinocladiella similis* to magnesium perchlorate and its survival under UV-C radiation, harsh conditions found on Mars. Aiming to understand the resistance of black yeasts to environmental stresses (UV-C and magnesium perchlorate), we compared the survival of *Rhinocladiella similis* strain LaBioMMi 1217 and *Exophiala* sp. 15Lv1 to UV-C only, and UV-C combined with magnesium perchlorate (0.9 wt% $Mg(ClO_4)_2$). Addicionally, by analyzing the metabolic and proteomic responses of *R. similis* to high concentrations of magnesium perchlorate, we seek to uncover this organism's molecular profile and survival mechanisms in extreme environments. The results from this study may enhance our understanding of how the survival of these black yeast would occur in perchlorate brines, which are hypothesized to be present in certain Martian regions. These fungal species have demonstrated notable adaptability to Martian-like conditions and inform the design of future Mars rovers for biosignature detection, indicating which molecules could be antecipated on the Martian surface.

Results

Resistance to UV-C radiation in magnesium perchlorate conditions

In a 0.9 wt% magnesium perchlorate aqueous solution (~40.3 mmol L^{-1}), the eukaryotic model *Exophiala* sp. 15Lv1 showed a lower survival rate at all tested UV-C radiation doses compared to the control experiment performed with a 0.9 wt% sodium chloride solution (no magnesium perchlorate). While the 0.9 wt% concentration was chosen for its widespread use in biological studies, it is important to note that the molar concentrations of these salts differ, leading to variations in actual ion availability in solution. This ensured that the observed effects were specifically attributable to magnesium perchlorate, eliminating the potential influence of salinity variations. In contrast, the isolated *Rhinocladiella similis* strain LaBioMMi 1217 demonstrated significant resistance to high doses of UV-C radiation in the perchlorate matrix compared to the standard *Exophiala* sp. 15Lv1 strain. Moreover, *R. similis* exhibited a higher survival rate under the oxidative stress induced by the combination of UV-C and magnesium perchlorate (Fig. 1). For example, at the highest UV-C fluence of 0.4 kJ/m^2, *R. similis* demonstrated ~80% survival, while *Exophiala* sp. 15Lv1 survival was reduced to around 50%. The cell viability of *R. similis* LaBioMMi 1217 was reduced only once, whereas *Exophiala* sp. 15Lv1 suffered a much greater decrease in viability under the same conditions. These results suggest that combining UV-C radiation with perchlorate salts can be particularly harmful, even for microorganisms well-known to resist UV-C irradiation; however, *R. similis* can cope better with such a harsh environment.

Survival under different magnesium perchlorate concentrations

Considering the significance of perchlorate-rich environments on Mars as promising locations for the existence of potential extraterrestrial life, this study focused on monitoring the growth of two species of black yeasts from the *Herpotrichiellaceae* family. These species have demonstrated notable

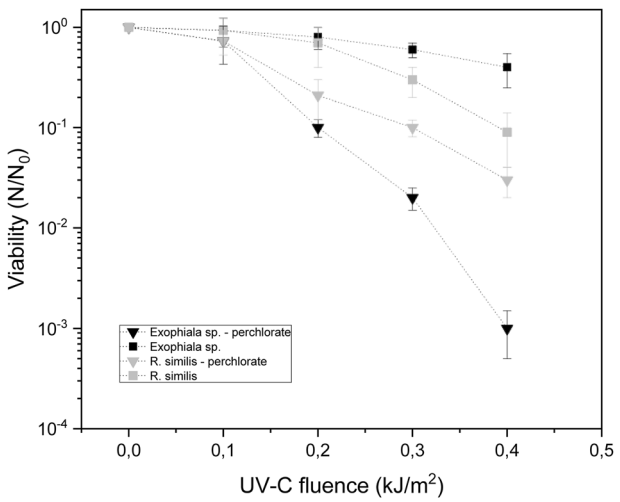

Fig. 1 | Effects of UV-C-irradiated $Mg(ClO_4)_2$ on the growth of two different species of black fungus (*Exophiala* sp. 15Lv1 and *Rhinocladiella similis* LaBioMMi 1217). ▪ = Control experiment (only UV-C), black yeast UV-C with 0.9 wt% NaCl. ▼ = black yeast with perchlorate 0.9 wt% $Mg(ClO_4)_2$. The viability of the fungal strains is represented in the ratio N/N_0, where N is the current fungal density at each sampling point (dose of UV-C) and N_0 is the initial count. Error bars indicate standard deviations across the five replicates.

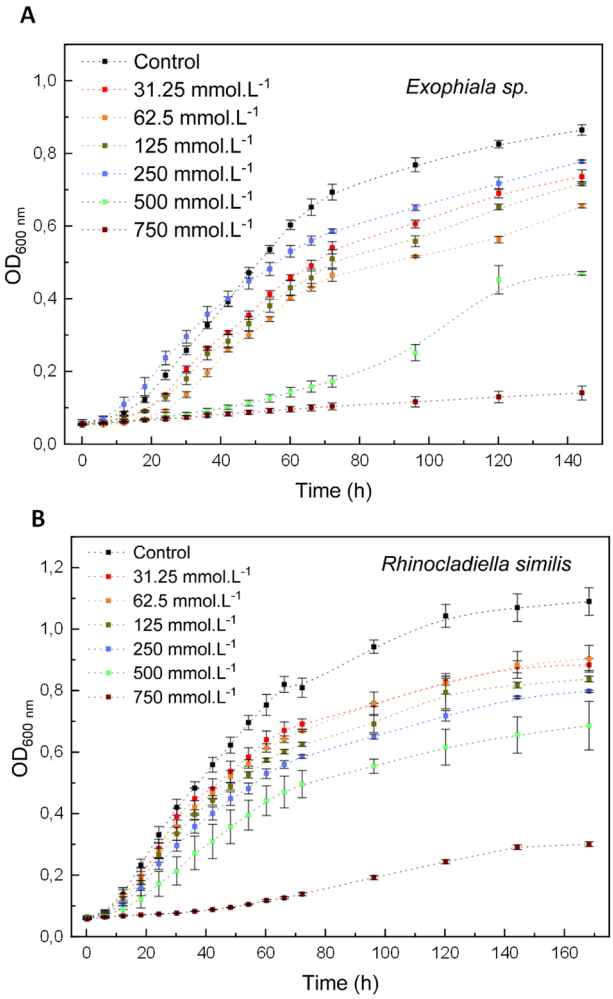

Fig. 2 | Growth response of two black fungi to perchlorate stress. Growth curves of Exophiala sp. 15Lv1 (**A**) and Rhinocladiella similis LaBioMMi 1217 (**B**) cultured in PDB under various concentrations of $Mg(ClO_4)_2$ and control (perchlorate-free medium). Cultures were incubated at 25 °C in 96-well plates with an initial OD_{600} of 0.125. Growth was monitored by measuring OD_{600} over time to assess the impact of perchlorate exposure.

adaptability to Martian-like conditions[22,23]. Experiments were conducted across a gradient of magnesium perchlorate concentrations, ranging from 31.25 mmol L^{-1} to 1 mol L^{-1}. The choice of this specific salt was strategic, considering the lack of data on the effects of magnesium perchlorate salts on microorganisms, coupled with its higher toxicity compared to sodium perchlorate and its prevalence on the Martian surface.

Overall, a common trend in fungal growth was detected: A decrease when the perchlorate concentration increased. The growth curve analyses (Fig. 2) revealed that both tested strains of black yeast sustained great growth at magnesium perchlorate concentrations of 250 mmol L^{-1}, with *Exophiala* sp. and *Rhinocladiella similis* both reaching an $OD6_{00}$ of 0.70 at 120 h. This indicates substantial tolerance to this electrolyte. However, increasing the concentration to 500 mmol L^{-1} resulted in a significant delay in the growth of *Exophiala* sp. (Fig. 2A) compared to *R. similis* (Fig. 2B). For instance, at 500 mmol L^{-1}, *Exophiala* sp. reached an OD_{600} of 0.25 by 100 h, while *R. similis* maintained a higher OD_{600} of 0.50. This contrast becomes even more pronounced at a concentration of 750 mmol L^{-1}, where *Exophiala* sp. failed to exhibit an effective growth profile, with an OD_{600} of bellow 0.20, while *R. similis*, despite a reduction in growth rate and a decrease in cell density, still managed to increase its OD_{600} to 0.30 by 140 h. These findings indicate the differential resilience of these species to high concentrations of perchlorate, a critical aspect for understanding the viability of life on Mars brines.

Fig. 3 | Microscopic (SEM) and macroscopic responses of *Rhinocladiella similis* LaBioMMi 1217 to magnesium perchlorate conditions. A Left: magnification of cells in PDB culture medium, showing pseudohypha behavior. Right: cells dispersed in PDB culture medium with a gray coloration. **B** left: magnification of the fungus cells in PDB culture medium at a 500 mmol L^{-1} $Mg(ClO_4)_2$ concentration, highlighting the yeast form. Right: cells are dispersed in a PDB culture medium with a darker color due to the intensified melanin production through the formation of darker cells.

Morphology analysis of *Rhinocladiella similis* strain LaBioMMi 1217 in perchlorate brines

Given the notable resistance of *R. similis* to magnesium perchlorate, additional experiments were conducted to investigate its morphological changes. It was observed that *R. similis* exhibited predominantly filamentous growth under magnesium perchlorate-free conditions (Fig. 3A), suggesting that the presence of magnesium perchlorate favors the phenotypic change in yeast cell morphology. In addition, it was noted that the fermentation broth acquired a greenish-brown coloration after cultivation. In contrast, in the presence of magnesium perchlorate (Fig. 3B), yeast-like cells predominated, indicating that the dimorphism of *R. similis* can be influenced by the magnesium perchlorate. Furthermore, the fermentation broth appeared darker under this condition, suggesting an increase in melanin production by the fungus in response to the osmotic stress caused by the perchlorate.

Proteomic analysis of the fungus *R. similis* at a high concentration of magnesium perchlorate

To better understand the response of *R. similis* strain LaBioMMi 1217 to magnesium perchlorate-induced stress at the protein level, the proteome of cell cultures with $Mg(ClO_4)_2$ and without additional salts in the growth medium was analyzed. The microorganism was cultivated under the same conditions used for morphological analyses, with a concentration of 500 mmol L^{-1} of $Mg(ClO_4)_2$. This concentration was selected as it represents a relevant range of perchlorate exposure while balancing measurable biological effects with cell stability. To ensure greater reliability in the data, all samples were prepared as biological quintuplicates ($n = 5$). The cells were harvested in the stationary phase, as this phase is more representative of the microorganism's long-term response to stress, providing a protein profile that closely resembles the survival conditions that might be encountered in Martian brines, where environmental stress would likely induce a metabolic shift towards stationary growth.

In total, 1321 proteins were quantified in the proteomic assays, whereby it was observed that 169 proteins were upregulated and 227 were downregulated, considering the p-value $\leq 0.05 + \log_2(FC) \pm 1$

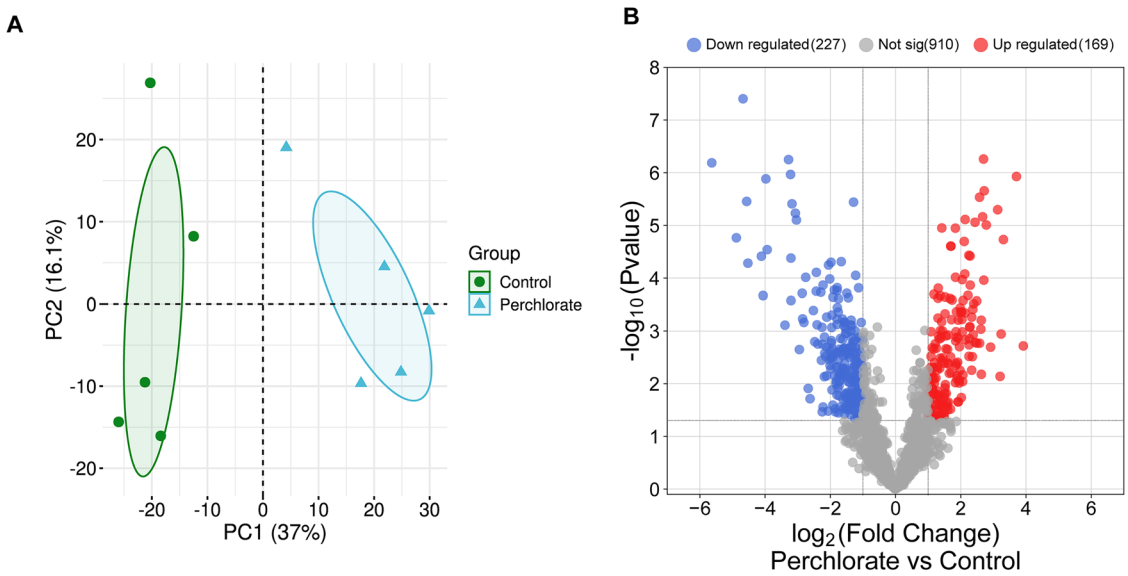

Fig. 4 | Proteomic shifts in *R. similis* under perchlorate exposure. A Principal component analysis (PCA) reveals clustering patterns among proteomic samples of *R. similis* exposed to 500 mmol L^{-1} $Mg(ClO_4)_2$ versus control (untreated). **B** Volcano plot shows significantly up- and downregulated proteins ($log_2FC \pm 1$, $p < 0.05$) in the perchlorate-treated group. Red and blue dots indicate upregulated and downregulated proteins, respectively.

(Supplementary Data 1). The principal component analysis (PCA) graph (Fig. 4A) shows two principal components, accounting for 53% of the total data variance (37% for PC1 and 16% for PC2), revealing a separation trend between the two groups: The fungus in the presence of magnesium perchlorate (Perchlorate) and the fungus grown in the absence of magnesium perchlorate (Control). This indicates that magnesium perchlorate played a significant role in changing the protein profiling of *R. similis*. Differentially regulated proteins can be observed in the volcano plot analysis (Fig. 4B). These analyses demonstrate that magnesium perchlorate significantly impacts the proteome of *R. similis*. The proteome differentiation identified by PCA, which is a robust unsupervised analysis, indicates that the perchlorate-rich environment is not the most suitable for *R. similis* cells to inhabit. The proteins responsible for this difference were further explored in terms of Gene Ontology (GO) annotations.

Gene Ontology (GO) analysis was used to investigate the biological processes altered in the fungus due to the high perchlorate concentrations (Fig. 5). The downregulated biological processes (BPs) were significantly enriched for categories such as "carboxylic acid metabolic process" (Fold Enrichment: 2.998, FDR: 4.44E−08, nGenes: 38), "nucleobase-containing small molecule metabolic process" (Fold Enrichment: 5.020, FDR: 1.36E−07, nGenes: 20), "nucleotide metabolic process" (Fold Enrichment: 6.181, FDR: 1.46E−08, nGenes: 19), and "cellular respiration" (Fold Enrichment: 1.352, FDR: 1.01E−09, nGenes: 13). Additionally, metabolic pathways such as "tricarboxylic acid cycle" (Fold Enrichment: 1.941, FDR: 3.37E−09, nGenes: 10), "*aerobic* respiration" (Fold Enrichment: 1.601, FDR: 3.39E−09, nGenes: 11), and "ATP metabolic process" (Fold Enrichment: 1.409, FDR: 2.12E−11, nGenes: 15) were significantly affected. These results suggest a metabolic shift likely driven by the stress induced by high perchlorate levels, reflecting a downregulation in key energy-generating processes.

Conversely, the enriched biological processes associated with the upregulated proteins included "cellular response to chemical stimulus" (Fold Enrichment: 3.221, FDR: 4.532, nGenes: 10), "organic substance catabolic process" (Fold Enrichment: 2.049, FDR: 43.846, nGenes: 22), "regulation of protein phosphorylation activity" (Fold Enrichment: 1.494, FDR: 43.748, nGenes: 3), and "small molecule metabolic process" (Fold Enrichment: 3.161, FDR: 38.701, nGenes: 11). Other processes such as "lactate metabolic process" (Fold Enrichment: 1.358, FDR: 16.271, nGenes: 4), "response to toxic substance" (Fold Enrichment: 6.378, FDR: 12.343, nGenes: 7), "dephosphorylation" (Fold Enrichment: 5.899, FDR: 8.588, nGenes: 9), and "cellular detoxification" (Fold Enrichment: 769.145, FDR: 8.588, nGenes: 7) were highly enriched. The increase in these proteins reflects a robust cellular adaptation mechanism to counteract the oxidative and chemical stress induced by perchlorate exposure.

Altogether, these data indicate a shift in fungal metabolism towards stress response and detoxification, characterized by the downregulation of energy generation pathways and the upregulation of processes associated with stress tolerance. This metabolic reorganization potentially underpins the survival of *R. similis* under extreme perchlorate conditions.

A more comprehensive analysis of the enriched biological processes, specifically for the upregulated proteins, indicates that the majority of the expressed proteins are involved in mechanisms aimed at mitigating stress induced by reactive chemical species (Fig. 5). Among the most prominently expressed biological processes, seven are associated with defense responses or chemical detoxification pathways, suggesting that the perchlorate-rich environment presents significant challenges to *R. similis* cell survival. Despite this, the cells are able to proliferate and endure, which underscores their tolerant nature rather than an extremophilic one. Additionally, one negatively regulated biological process stands out among the analyses. Proteins associated with aerobic respiration exhibited decreased expression, indicating that cells prefer to undergo fermentation rather than cellular respiration under high perchlorate concentration conditions. This finding is consistent with the morphology of the fungus under these conditions, which appears predominantly in the yeast form, considering that it has dimorphic characteristics and can alternate between filamentous and yeast forms depending on the conditions in which it is found. Furthermore, several processes related to cell division and multiplication were downregulated, indicating stagnation in population growth. However, the decision to conduct measurements after 20 days of cultivation eliminates the impact of differences in growth phases, as both samples were in the stationary phase. It is worth noting that most black yeasts, including the organism under study, typically reach the stationary phase between 7 and 14 days of cultivation in various culture media[24,25]. This suggests that the observed effects are more likely influenced by the specific treatment conditions rather than inherent growth dynamic.

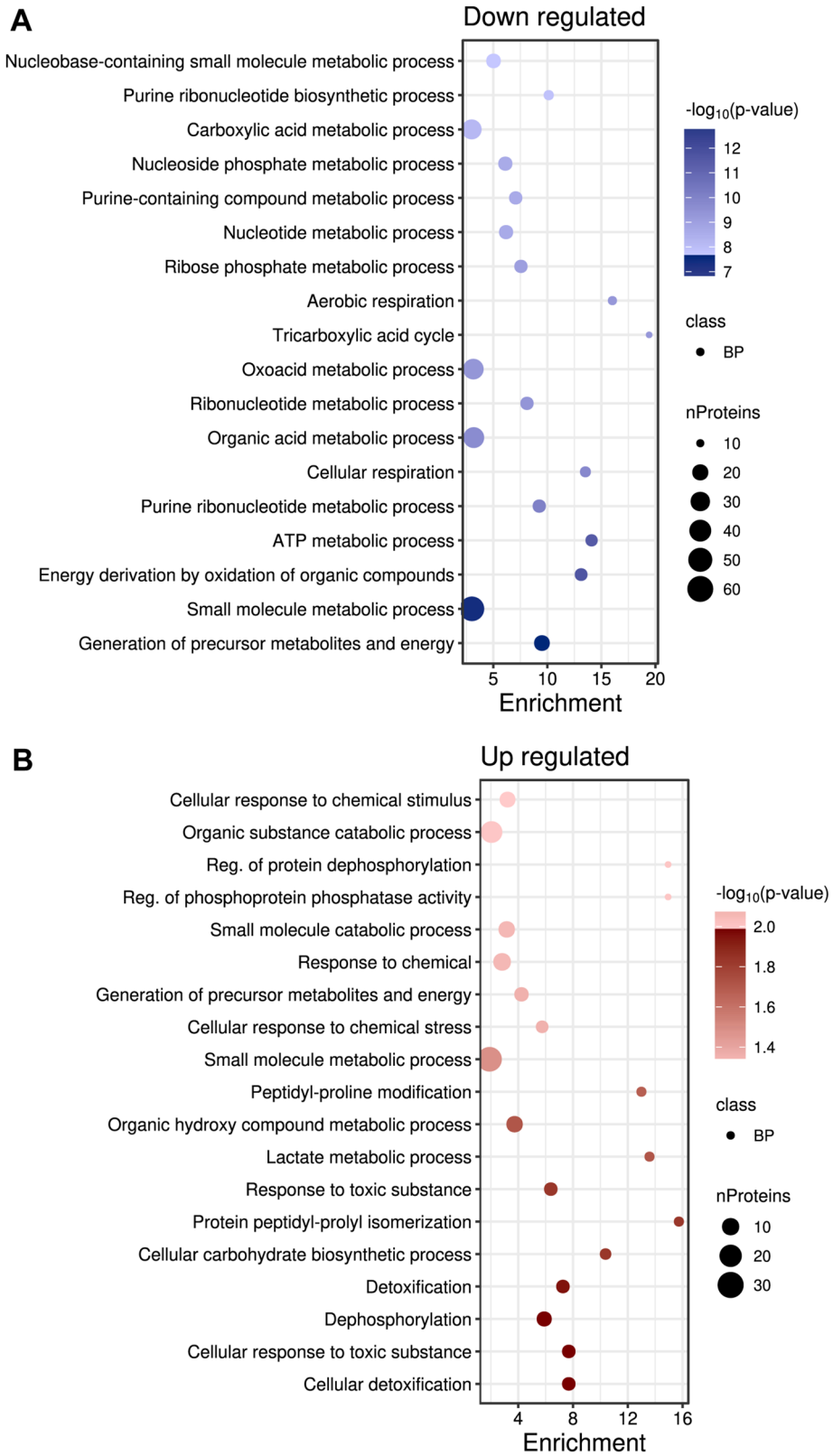

Fig. 5 | Assessing the Gene Ontology (GO) biological processes associated with up- and down-regulated proteins. A Enriched biological processes related to downregulated proteins. Data indicate the processes that are potentially reduced in the presence of perchlorate. Each node represents a different biological process; darker blue means more significant statistics (lower *p*-value). **B** Enriched biological processes associated with upregulated proteins. Each node represents a different biological process; darker red means more significant statistics (lower *p*-value). Data indicate the processes that are potentially increased in the presence of magnesium perchlorate salt.

Molecular biosignature production in high concentrations of magnesium perchlorate

To investigate the effect of magnesium perchlorate on the production of extracellular biomolecules (i.e., metabolites) of the black yeast *R. similis*, crude extracts of the fungus in the presence of the salt were evaluated by UHPLC-MS/MS. A principal component analysis was conducted to provide an overview of the data (Supplementary Fig. 3).

The first two principal components, accounting for 60.6% of the overall data variance (36.4% for PC1 and 24.2% for PC2), showed a separation trend between the two groups: The fungus in the presence of perchlorate salt

(Perchlorate group) and the fungus grown solely in the PD culture medium (Control group), both highlighted in the PCA plot (Supplementary Fig. 3). The separation between the groups was much clearer through the unsupervised method, making a supervised analysis unnecessary. The reproducibility of the instrumental system, both chromatographic and mass spectrometric parts, was assessed by jointly analyzing the QC samples. The tight clustering of the QCs in the center of the PCA score plot demonstrated the instrument's stable performance. The statistical treatment result table from MetaboAnalyst 4.0 can be found in Supplementary Data 2.

To discover the chemical identity of features that exhibited an increase in production, features were selected with $\log_2(FC) > -2$ and p-values < 0.05 for a precise mass analysis (with error margins ranging from 0 to 2 ppm) and tandem mass spectrometry (MS/MS) fragmentation profiles, summing 324 features of interest, which were submitted to the Global Natural Products Social Molecular Networking (GNPS) and Sirius platforms for their annotation.

The molecular network generated by the GNPS platform is depicted in Supplmentary Fig. 4. Using this approach, it was possible to annotate four compounds (Fig. 6), along with the plots illustrating the increase in production. In the molecular network analysis, various molecular clusters are observed, totaling 12 clusters, each with more than three nodes. This suggests that magnesium perchlorate may play a role in activating previously silenced genes belonging to different molecular families. Among these clusters, one had a chemical entity identified, while another exhibited four nodes ranked among the top 10 most upregulated.

The molecules annotated based on their fragmentation profiles belong to the class of peptides. Among these are decanoyl arginine (**1**), with $[M + H]^+$ at *m/z* 236.1852; L-phenylalanyl-L-proline (**2**), with $[M + H]^+$ at *m/z* 288.2015; ***N,N***-dimethylarginine (**3**), with $[M + H]^+$ at *m/z* 329.2535; 5-oxopyrrolidine-2-carbonyl-L-isoleucine (**4**), with $[M + H]^+$ at *m/z* 243.1335. The error variation in parts per million (ppm) for the annotated molecules was <2. All the ions highlighted in the molecular network are listed in Table 1, along with their *m/z*, RT, log2(FC), and *p*-values.

Due to the scarcity of specialized libraries on extremophile fungi, none of the features among the top 10 areas of increased ions had a spectral match in the databases; however, based on the fragmentation profile, the cluster that concentrates the ion features **5**, **6**, **7**, **8**, and **9** could be a peptide. The mass spectra (Fig. 7) revealed that this molecule is doubly charged due to *m/z* values being greater than the chosen precursor ion for fragmentation. This indicates that the mass of these molecules is greater than 1000 Da. The charge state for the respective peptides could be verified by the mass difference of 0.5 units in the isotopic envelope, corresponding to 2 charges $[M + 2H]^{2+}$ for the analytes in question. The fragmentation profile also displays typical peptide fragments, as shown in Fig. 7. For the precursor ion *m/z* 978.939 (peptide 5), it is possible to observe the fragment ions (*m/z* 838.427; 709.387; 610.318 and 497.235) formed from characteristic peptide cleavages in its MS/MS spectrum; generating fragments of the type *y*- and *b*-series ions based on the difference in mass between these fragments (delta *m/z*), it is possible to infer which amino acids are connected to the same peptide backbone.

The peptides detected by mass spectrometry in the experiment with magnesium perchlorate are in the same cluster of the molecular network generated by the GNPS platform due to their similar fragmentation pattern, suggesting that they are part of the same chemical class or even that they are structurally very similar. All these ions relating to peptides (**5**, **6**, **7**, **8**, and **9**), when subjected to fragmentation by CID (collision-induced dissociation), generated a fragment ion of the immonium type *m/z* 86.096, which is diagnostic of the presence of the amino acid residues I/L[26]. By utilizing the difference between the fragmented ions, many amino acids could be identified by de novo sequencing, for example, in Fig. 7. However, other spectroscopic techniques are required to elucidate the peptide completely.

Discussion

Perchlorates have been found on the surface soil of Mars, and due to their characteristics of absorbing water from the thin Martian atmosphere, stable brines can (temporarily) exist and provide water for the potential existing life[3,27]. Thus, it is necessary to test, under laboratory conditions, the ability of the microorganisms to adapt and survive in perchlorate brines, as they could be a habitat for putative life on Mars[11]. However, at high concentrations, perchlorate can have detrimental effects on cells, primarily due to its ability to interfere with essential metabolic processes, such as ion transport and osmotic regulation. Although its stability in solution makes it less reactive under normal conditions, this stability can be disrupted in specific environments, such as in the presence of ionizing radiation or other energy sources[28,29].

Wadsworth and Cockell (2017) observed a pronounced bactericidal effect of short-wave UV-C radiation (up to 60 s) on *Bacillus subtilis* cells growing in magnesium perchlorate culture medium[18]. The increased germicidal power is most likely linked to the formation of highly oxidizing species. UV-C radiation, a high-energy component of sunlight reaching the Martian surface, can break down perchlorate ions (ClO_4^-) into reactive chlorine species (RCS). This initial decomposition may produce lower oxychlorine species, such as chlorate (ClO_3^-) and chlorite (ClO_2^-). Chlorite (ClO_2^-), a product of perchlorate decomposition, can further undergo photolysis under UV-C radiation. This process generates hypochlorite (ClO^-) and other reactive species, which may be the chemical species responsible for increasing the germicidal power[4,30]. In contrast, the data reported here showed that *R. similis* LaBioMMi 1217 exhibited substantial resistance to the tested UV-C radiation dose in the presence of magnesium perchlorate, as evidenced by the survival curves. When compared to the other tested black fungus, *Exophiala* sp. strain 15Lv1, which had previously been noted for its high resistance to UV-C, *R. similis* demonstrated superior resilience under this combination of conditions.

The fungal strain *R. similis* LaBioMMi 1217 showed surprising tolerance and grew in magnesium perchlorate conditions up to 0.75 M during the 7 days (168 h) of cultivation. Similarly, the *Exophiala* sp. 15Lv1 was able to grow at the same maximum tested concentration (0.75 M), however, not as proeminet as LaBioMMi 1217. Furthermore, resistance to the toxic effects of irradiated perchlorate was observed at the highest radiation dose used, 0.4 kJ/m^2. Black yeast specializes in extremotolerance and is among the most stress-resistant eukaryotes on Earth[19]. This group of melanized fungi has been shown as a good candidate for astrobiology studies through ground-based facilities and space missions. For example, we now know the ability of *Cryomyces antarcticus* and *C. minteri* to survive long-term space travel. After 1.5 years of exposure at the International Space Station (ISS), remarkably, the cells remained viable[31]. In terms of salt tolerance, several microorganisms have been reported as tolerant to perchlorates, including halotolerant archaeal and bacterial strains, yeast *Debaryomyces hansenii*[7], and fungi, such as *Purpureocillium lilacinum* and *Cryomyces antarcticus*[2,7]; however, most can grow in conditions up to 0.4 mol L^{-1} of perchlorate concentration. Currently, the most tolerant microorganism reported is the halotolerant yeast *Debaryomyces hansenii*, being able to grow in a liquid medium containing 2.4 M $NaClO_4$[7], more than twice the record of the prior microbe, the bacterium *Planococcus halocryophilus*[13]. Follow-up studies to reveal the mechanisms of resistance of the black fungus *R. similis* and *Exophiala* strains to the deleterious properties of these combined factors would constitute interesting lines of inquiry for astrobiological implications (i.e., extraterrestrial life, planetary protection, and Bioregenerative Life Support Systems based on in situ resource utilization).

Changes at a structural level were detected using SEM, in addition to macroscopic observations, highlighting the stress responses to perchlorates, whereas we observed that the *R. similis* changed from a multicellular filamentous growth form to unicellular yeast. The morphogenic shift is a common strategy among fungi to adapt to the new prevailing conditions[32]. Melanin production, as we observed, plays a critical role in the growth of black yeast, as demonstrated in *Hortaea werneckii* under high NaCl concentrations[33] and in *Cryomyces antarcticus* exposed to magnesium perchlorate[2]. Several studies have highlighted that pigment accumulation serves as a protective strategy to mitigate oxidative stress[34], particularly when organisms are exposed to perchlorate salts[6]. Melanin possesses the ability to

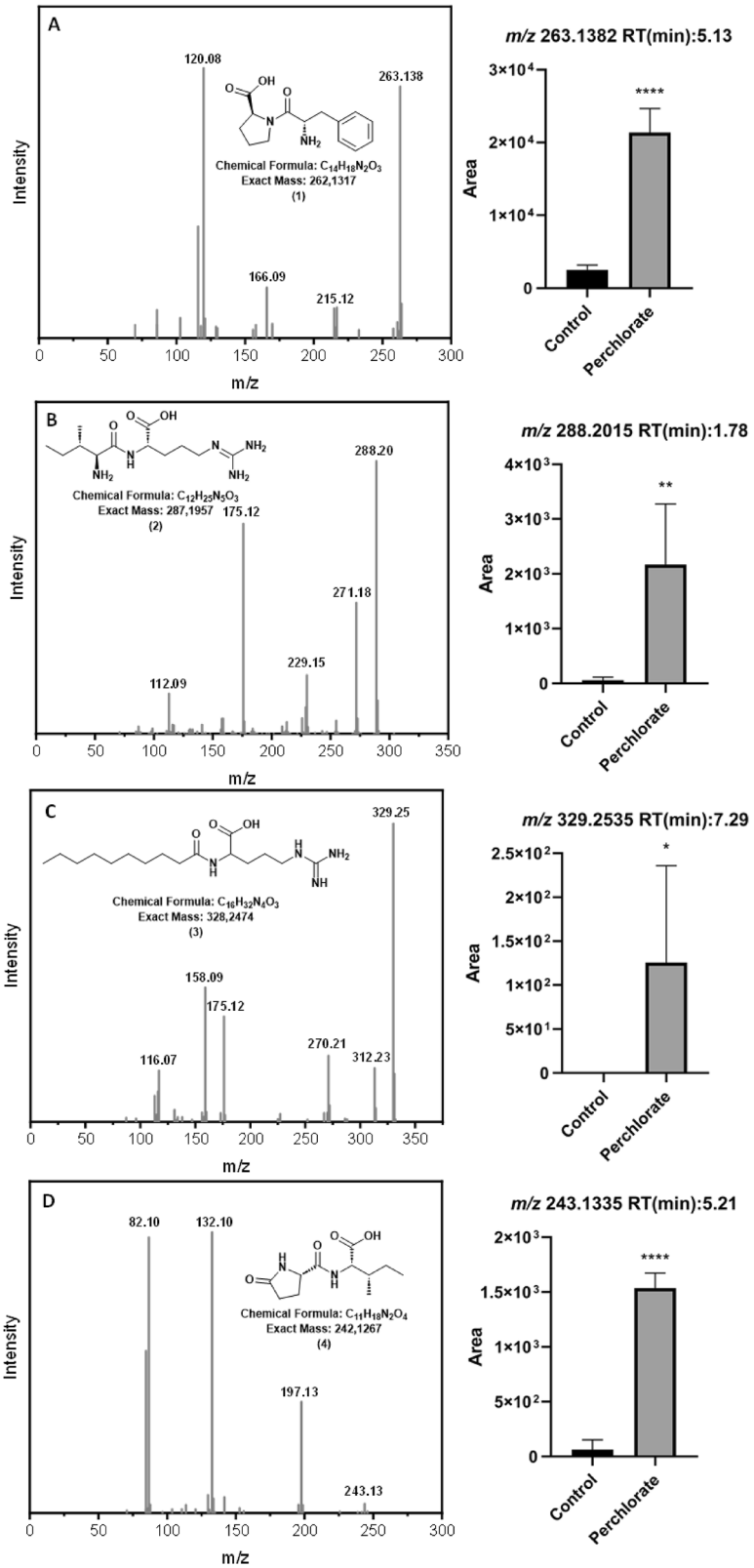

Fig. 6 | Selected differential metabolites between the control experiment and the magnesium perchlorate experiment were annotated using the library of the GNPS platform. The '*' symbol indicates the summary of the statistical significance level (*p*-value).

neutralize free radicals, functioning as a protective shield against oxidative damage[33,35]). Furthermore, evidence suggests that melanin also safeguards cells from UV-induced damage and osmotic stress, as observed in *Hortaea werneckii*[36].

The investigation of the protein machinery to understand the effects of perchlorate stress on fungi is in its early stages, with only one study identified so far. Heinz et al. (2020) developed a study using proteomics to investigate the fungus *Debaryomyces hansenii* in high concentrations of sodium perchlorate and observed that salt stress-resistant yeasts undergo significant changes when cultured in this salt[16]. In bacteria, recent studies have revealed protein expression patterns in response to perchlorate. Model organisms such as *Escherichia coli* and the extremophile *Colwellia psychrerythraea*

activate adaptive pathways to counteract its toxic effects. Identified mechanisms include DNA repair, RNA methylation, de novo nucleotide biosynthesis, and modulation of proteins related to oxidative and chaotropic stress[37,38]. However, these responses may vary depending on the species and perchlorate counterion.

Despite these advances, no studies have investigated proteomic changes in black yeasts under magnesium perchlorate-induced stress. Addressing this knowledge gap, the present work provides preliminary insights into how these fungal cells respond to high concentrations of this salt, contributing to our understanding of microbial adaptation to extreme conditions.

Table 1 | Relationship of the highlighted ions in the molecular network with log_2 (FC information) and *p*-value

Entry	Name	Precursor ion (*m/z*)	RT (min)	log_2 (FC)	*p*-value
1	Decanoyl arginine	263.1382	5.13	2.307	5.37E−06
2	L-phenylalanyl-L-proline	288.2015	1.78	4.3232	3.11E−03
3	N,N-dimethylarginine	329.2535	7.92	2.0753	1.34E−03
4	5-oxopyrrolidine-2-carbonyl-L-isoleucine	243.1335	5.21	3.2194	2.18E−06
5	Unknown peptide	978.9351	5.88	8.409	2.00E−03
6	Unknown peptide	947.4641	6.25	9.3673	6.03E−04
7	Unknown peptide	929.9464	6.2	9.041	5.34E−05
8	Unknown peptide	939.452	6.89	7.1738	2.52E−03
9	Unknown peptide	938.9517	6.26	12.064	1.10E−03
10	Unknown	838.442	7.7	8.267	7.42E−07
11	Unknown	736.4276	6.4	7.0625	1.31E−07
12	Unknown	1019.977	6.18	7.4388	4.39E−03
13	Unknown	375.121	5.76	8.0418	4.04E−06
14	Unknown	739.3218	3.72	7.2852	7.67E−07

In filamentous fungi and yeasts, environmental stress often initiates communication from the cell surface to the nucleus via specific signaling pathways, such as those involving mitogen-activated protein kinases, alongside changes in cytoskeletal structures. Perchlorate exposure has been shown to positively activate several proteins within these pathways[16]. For instance, activated serine-threonine phosphatases (RBB50_004446) and histidine phosphatases (RBB50_005453) are known to regulate genes involved in glycerol production[39,40], which helps reduce intracellular water activity more effectively than other solutes[41].

Osmotic stress commonly induces oxidative stress, such as through the generation of reactive oxygen species (ROS) in mitochondria[34]. Consequently, proteins that respond to oxidative stress are expected to play a role in managing cellular responses under saline conditions. However, it should be considered that the observed responses may be general to salt stress and not specifically related to perchlorate. Indeed, we found that the antioxidant activity and metabolic processes of glutathione are positively regulated in $Mg(ClO_4)_2$ stressed samples, including the enzymes catalase (RBB50_004981) and superoxide dismutase (RBB50_012728), allowing cellular protection against oxidative stress. The increased abundance of these proteins was also found in the fungus *Debaryomyces hansenii* under conditions of high perchlorate anion concentration, indicating that different species of fungi have the same response to this stress[16]. Additionally, a proteomic analysis of *Debaryomyces hansenii* under various salt stresses, including exposure to sodium perchlorate and sodium chloride, revealed that fungi adapt by modulating pathways related to energy metabolism and antioxidant defense[16]. Our findings with *Rhinocladiella similis* exposed to magnesium perchlorate reveal similar adaptive mechanisms, particularly the increased expression of proteins involved in the osmotic stress response, as shown in the enrichment graphs. This suggests that, despite differences in stressors (magnesium perchlorate vs. sodium chloride), fungi likely activate similar pathways to cope with osmotic stress.

We also observed that a family of enzymes had their expression increased in the presence of high concentrations of magnesium perchlorate, where three peptidyl–prolyl cis–trans isomerases (RBB50_004565; RBB50_003292; RBB50_007691; RBB50_011626) were positively regulated. These enzymes have been previously investigated in halotolerant strains of *P. oxalicum*, with increased expression reported under salt stress conditions,

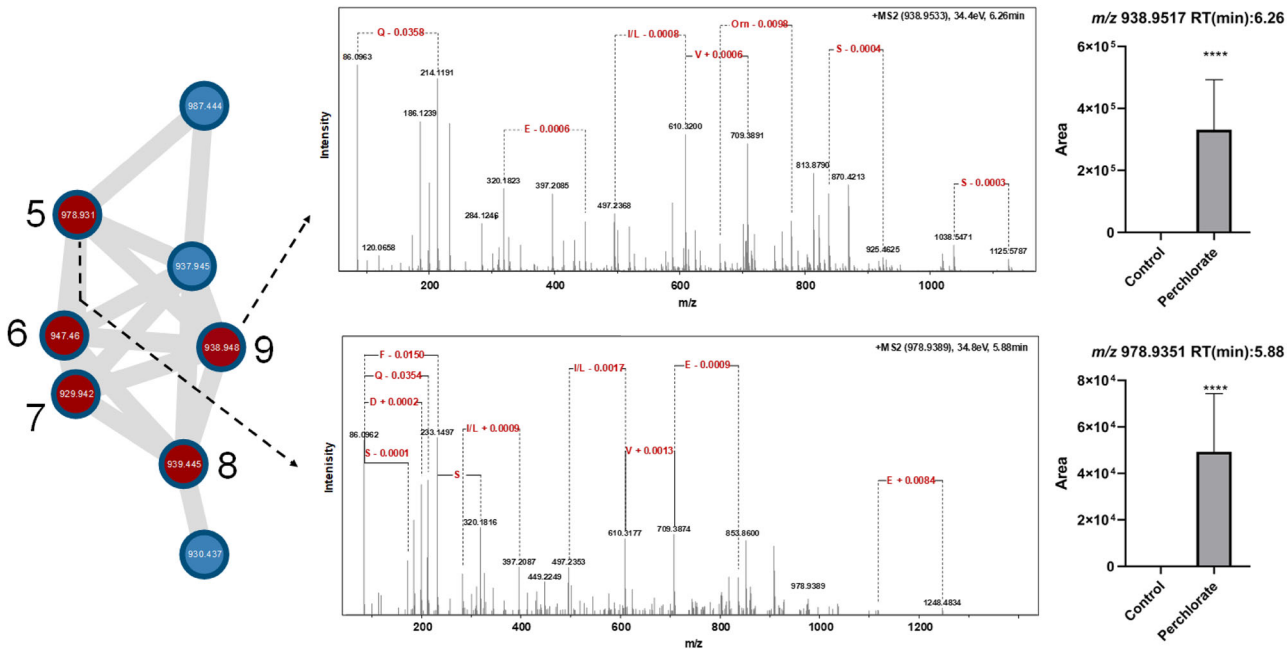

Fig. 7 | Fragmentation patterns of upregulated peptide-like metabolites. Molecular clusters showing five highly upregulated nodes, along with MS/MS spectra for ions at *m/z* 938.9517 and *m/z* 978.9351. The fragmentation profiles display strong similarity and characteristic peptide cleavage patterns, supporting their classification as peptide-like secondary metabolites.

which was attributed to their role in protein folding. Given the chaotropic properties of perchlorate, which can destabilize macromolecules and disrupt protein structure[42], it is possible that its presence may promote the expression of these enzymes as part of a cellular response to aid in the proper folding of proteins. This suggests that the increased expression of peptidyl–prolyl cis–trans isomerases could not only be a general adaptation to salt stress but also a specific response to the destabilizing effects of perchlorate. Peptidyl–prolyl cis–trans isomerases (PPIases) facilitate the proper folding of proteins by catalyzing the cis–trans isomerization of prolyl peptide bonds, a process crucial for maintaining protein structure and function[43].

Black yeast, which was used in this study, contains melanin in the cell wall in addition to the classical biomolecules chitin and glucan[35]. Therefore, the darkening of the fungal biomass in the presence of perchlorate salt may reflect melanin synthesis in response to stress. The expression of the enzyme laccase (RBB50_005731), belonging to melanin biosynthesis, was increased, indicating that melanin production is indeed occurring in the cell as a way to make the cell wall more resistant to the chaotropic effects of magnesium perchlorate[44]. Additionally, the enrichment of the biological process organic hydroxy compound metabolic process indicates that hydroxylated molecules, such as fungal melanin, are being produced. Melanin production is extremely interesting in this context because it protects against chaotic and oxidative effects generated by chemical species or UV-C radiation, situations also found on Mars.

An enzyme that has garnered considerable attention due to its increased abundance in marine environments with elevated concentrations of halogenated compounds is the haloacid dehalogenase-like hydrolase (RBB50_003417). This enzyme plays a vital role in mitigating toxicity by catalyzing the dehalogenation of harmful molecules, thereby contributing to environmental detoxification[45]. Considering that perchlorate derivatives (i.e., hypochlorites) can act as efficient halogenators of organic molecules, a plausible explanation for the observed increase of this enzyme is that perchlorate may be promoting the halogenation of organic compounds[46], thereby exacerbating the toxicity of the environment. In this context, the fungus could produce this enzyme as part of a defense mechanism against toxic stress. In the Martian surface scenario, such a mechanism could be particularly relevant for hypothetical microbial life, as it would allow adaptation to extreme environmental condition.

The search for life beyond Earth, especially on celestial bodies within our solar system, remains a significant challenge due to limitations in human resources for collecting samples and conducting microbiological analyses. However, various space agencies have been investing in technologies for detecting molecules in situ, known as bio-signatures[47]. Advances in mass spectrometry applied to space exploration actively contribute to the development of more robust equipment in terms of sensitivity, resolution, and the ability to analyze molecules of different sizes, making the use of complex organic molecules as bio-signatures a powerful tool for detecting evidence of extraterrestrial life[48,49]. The search for complex molecules as evidence of life beyond Earth turns out to be a very promising approach, as many of these molecules are produced exclusively by biotic processes, such as long-chain unsaturated lipids, polypeptides, and other molecules resulting from secondary metabolism[47]. In this sense, it becomes necessary to understand which molecules could be produced in astrobiologically relevant environments.

The molecules produced by the fungus *R. similis* in our simulated magnesium perchlorate brine experiment provide extremely interesting insights into what microorganisms could produce under these conditions. The presence of various molecules with a proline group, such as L-phenylalanyl-L-proline (**2**) and 5-oxopyrrolidine-2-carbonyl-L-isoleucine (**4**), observed in the directed annotated metabolomics experiment, indicates that the fungus under study used molecules as osmolytes to mitigate the saline stress it was being subjected to. This type of molecule production has already been observed in studies with halophilic bacteria[46]. Proline and derivatives are crucial in maintaining cellular osmotic balance and ensuring proper function under saline stress conditions[50]. Furthermore, proline is vital in determining protein and membrane structures and eliminating ROS in complex organisms during water stress[50]. While proline can also participate in other metabolic pathways, its detection in experiments involving perchlorate suggests a potential association with saline stress, as it functions as an osmolyte under such conditions. The detection of these molecules on Mars, combined with evidence of other complex organic molecules associated with similar functions, could be associated with a potential biosignature exhibiting osmolyte properties, using terrestrial life as a model.

Decanoylarginine (**1**) and *N,N*-dimethylarginine (**3**) were also observed and are two molecules derived from arginine, also considered polyamines. These molecules may result from the degradation of proteins related to the signaling process from stress caused by perchlorate. The methylation of arginine post-translationally is a method for some cells to signal the stress caused by an oxidative agent[51,52]. The presence of these metabolites may be associated with the degradation of proteins that undergo this post-translational modification[51], given that in this experiment, the expression of peptidases increased in the presence of perchlorate. Another possibility is that polyamines are important in response to stress and that their metabolic pathway has been conserved in all living organisms, suggesting that the main role of polyamines is to promote the restoration of cellular homeostasis, allowing survival under stressful conditions. In *S. cerevisiae*, the expression of the main permease for high-affinity polyamine import coincided with the osmotic stress imposed by high concentrations of NaCl, KCl, or sorbitol[53], thus generating an increase in the production of these molecules.

Indeed, the production of peptides with molecular masses between 1800 and 2000 Da was significantly increased in the presence of perchlorate. Peptides in this size range have been reported for other fungi as a result of secondary metabolism, in which some cyclic peptides are produced in the presence of chemical stress by fungi in the genus *Penicillium*; in this case, the fungus started to produce these peptides as chelators to deal with high concentrations of manganese and copper cations[54]. Considering the high magnesium concentration in the tested samples, the fungus *R. similis* may also use peptide chelators to deal with these cations. However, the increase in the production of peptides in fungi in the presence of perchlorate ions was reported only in our study. Another possible explanation, considering that plants and fungi have similar biological processes, is the production of these molecules as salinity stress signaling agents. Plants produce linear peptides of this size as peptide hormones, which signal cascades to mitigate salinity stress caused by sodium chloride[55].

Many other feature molecules had their production increased (Supplementary Data 2), although the annotation was limited due to the lack of MS/MS spectra of microbial metabolites deposited in dedicated mass spectrometry databases. However, it is worth noting that the reported annotated molecules showed increased production only in our experiments under perchlorate salt conditions. However, further investigations considering other salts are necessary, as this preliminary study indicates that these molecules serve as a starting point for selecting which compounds might be relevant to identify in perchlorate brines on Mars.

Perchlorate resistance is essential for the survival of potential microbial life in Mars's regolith and brine environments, making it a critical factor for evaluating the habitability of present-day Mars. Thus, this study provides, for the first time, insight into the growth and resistance of the black fungus *Rhinocladiella similis* to magnesium perchlorate and the combined effect of ionizing radiation. Defensive mechanisms to cope with the chaotropic action were also observed, citing the production of antioxidant enzymes and peptidyl–prolyl cis–trans isomerases, that are upregulated to manage oxidative damage and protein folding issues induced by perchlorate. Additionally, the upregulation of melanin-associated laccase supports the role of melanin in protecting the cell wall from chaotropic and oxidative stress, a defense mechanism potentially beneficial in extreme environments like Mars. The detection of haloacid dehalogenase-like hydrolase also suggests an adaptive response to the toxic effects of halogenated molecules resulting from perchlorate breakdown, enhancing the microorganism's survival under high perchlorate conditions. The metabolites found in the simulated

experiment with magnesium perchlorate, such as proline-derived osmolites and peptides, may aid in the identification of molecules to be sought by the new generation of rovers and mass spectrometers that will possibly be sent to Mars in the future for life detection.

This study advances the frontier of knowledge in astrobiology and sets a precedent for the continuous exploration of life in extreme environments, whether on Earth or beyond. The findings presented herein promote a better understanding of the extremophile capabilities of terrestrial microorganisms, paving the way for more studies integrating omics (proteomics, transcriptomics and genomics) to better elucidate the molecular pathways activated in black yeasts under magnesium perchlorate stress for their tolerance and survival. These investigations will enhance our understanding of extremophile adaptations and contribute valuable knowledge for astrobiological applications, on the exploration of life beyond the boundaries of our planet, but also on Earth, with potential application in bioremediation technologies.

Methods

Microbial material

The fungi strains used in this study include two species of black yeasts isolated from distinct environments. *Rhinocladiella similis* strain LaBioMMi 1217 was identified as a contaminant in a hydrochloric acid solution with a pH of 1.5[56]. The *Exophiala* sp. strain 15Lv1 was isolated from the desertic soil of the Atacama Desert by Puschen et al. (2015)[57] to use as a comparative control in the conducted experiments due to its promising application for astrobiology research. Both strains were characterized and taxonomically identified using molecular biology approaches[56–58].

Resistance to UV-C radiation in a perchlorate magnesium condition

Following a 78-h incubation at 25 °C in potato dextrose broth (PDB), the fungi cells of both cultured strains were rinsed twice with 1 mL of a 0.9 wt% NaCl and $Mg(ClO_4)_2$ solution, facilitated by centrifugation. This saline solution was chosen to match the standard conditions used in other UV-C resistance studies[57,59]. The resulting pellet was resuspended in 5 mL of sterile NaCl and $Mg(ClO_4)_2$ solutions, and the concentration was adjusted to 10^6–10^7 cells L^{-1} using a spectrophotometer.

This cell suspension was then transferred to a sterile 10 cm diameter glass Petri dish and subjected to UV-C irradiation (Model G30T8 from LightTech Lamp Technology, Hungary) within a biosafety cabinet, emitting at a wavelength of 254 nm. The irradiation process was meticulously monitored throughout the experiment via a radiometer (model VLX-3W) equipped with a UV photocell (model CX254). The precise distance between the UV-C lamp and the sample was adjusted to ensure accurate UV-C flux. The Petri dish, housing the cell suspension in saline solution, was exposed to escalating doses of UV-C radiation, starting with zero (serving as control), followed by doses of 100, 200, 300, and 400 J m^2 s^{-1}. From the control, a 100 µL aliquot of the cell solution was carefully removed from the Petri dish, and a serial ten-fold dilution (10^{-2}–10^{-6}) was performed. Subsequently, 10 µL from each dilution was inoculated onto square Petri dishes laden with potato dextrose agar. The aforementioned process was replicated for other radiation doses, with the Petri dish containing the cell suspension positioned within the UV-C radiation field.

Following irradiation, the plates were incubated for 72 h, and the colony-forming units (CFUs) were assessed by counting the colonies in the dilution range of 30–300 CFUs. The acquired data were plotted to generate survival curves (N/N_0), illustrating the resilience and survivability of the cells under varying levels of UV-C radiation in conjunction with magnesium perchlorate exposure.

The *Exophiala* sp. 15Lv1 strain was chosen as a comparative model based on the understanding of the responses induced against UV-C radiation in previous studies, where it demonstrated strong resistance to UV-C radiation, establishing this yeast as an astrobiological model for understanding how eukaryotic microorganisms survive in Martian surface conditions[59]. Given these observations, we expanded our studies to the *Rhinocladiella similis* strain LaBioMMi 1217, another black yeast from the same Herpotrichiellaceae family.

Screening of survival under different magnesium perchlorate concentrations

Both strains of black yeast were cultured in triplicate in PDB, under agitation at 150 rpm, at 26 °C for 72 h. An aliquot from each triplicate was removed and diluted with PDB medium to achieve an OD_{600} of 0.125 for the cell suspension, corresponding to a final OD_{600} of ~0.05 in each well of the 96-well plate. The experiment was conducted using a SpectraMax Paradigm Multi-Mode Microplate Reader (Molecular Devices), with the absorbance measured at 600 nm. In each well, 80 µL of the fungal suspension (OD_{600} of 0.125) and 120 µL of growth medium with varying concentrations of magnesium perchlorate were added, resulting in a final volume of 200 µL in each well. The magnesium perchlorate concentrations evaluated included 31.25, 62.5, 125, 250, 500, 750, and 1000 mmol L^{-1} Absorbance spectra were obtained every 6 h during the first few days and every 24 h after 4 days, over a period of 7 days.

Cultivation of *Rhinocladiella similis* strain LaBioMMi 1217 on a magnesium perchlorate substrate for microscopy, proteomics, and metabolomics

The *R. similis* strain was cultivated in 50 mL Erlenmeyer flasks containing 20 mL of PDB (Sigma Aldrich) with 0.5 mol L^{-1} of magnesium perchlorate. This concentration of magnesium perchlorate was selected as an intermediate point, aiming to balance a high salt concentration with relatively satisfactory cell growth. The fungus was also grown in a pure PDB medium for use as the biological control. Both treatments had their respective negative controls (culture medium without microbial cells) and were conducted in five replicates. The microorganism was grown in an orbital shaker at 25 °C and 150 rpm for 20 days. After the cultivation period, the content was transferred to Falcon tubes, and centrifugation was performed to separate the broth from the fungal biomass. Biomasses were washed using saline solution, and the broth was frozen at −80 °C before being lyophilized.

Scanning electron microscopy (SEM) analysis

For SEM samples, 10 µL of each treatment (fungus exposed to 0.5 mol L^{-1} magnesium perchlorate and absence of magnesium perchlorate) were transferred to a silicon glass support, followed by fixation in a 3% glutaraldehyde solution for 3 h. The fixed samples were dehydrated in a series of isopropanol gradients (35%, 50%, 75%, 90%, and 100%), subjected to critical point drying for 5 h, and finally coated with metallic iridium particles. Images were captured using a Zeiss Merlin Gemini field emission SEM instrument.

Sample preparation for proteomics

Proteins from the strain LaBioMMi 1217 were extracted to analyze the proteome. After washing and lyophilizing, fungal cell lysis was performed using the mechanical lysis method through maceration with liquid nitrogen. The triturated material was resuspended in phosphate-buffered saline (PBS) and centrifuged at 5000×*g* to remove cellular residues. In the protein extract, precipitation was carried out with cold acetonitrile (ACN), followed by pelleting of the proteins through centrifugation at 16,000×*g* for 15 min. This process was repeated three times to ensure the protein material was adequately cleaned. The proteins in the pellet were resuspended in a digestion buffer (50 mmol L^{-1} ammonium bicarbonate), and protein concentrations were determined using the Bradford protein assay. Using 200 µg of the sample, the stages of denaturation and reduction/alkylation (8 M urea, 50 mM DTT, 250 mM iodoacetamide) were performed, incubating the samples at 95 °C for 5 min. Then, modified trypsin (Promega) was added in an enzyme-to-protein ratio of 1:50, and the samples were incubated at 37 °C for 16 h. The solution containing the digested proteins was centrifuged at 5000×*g* for 2 min. The peptides were desalted using OASIS® HLB (Waters) desalting columns, according to the manufacturer's protocol. The desalted samples were dried in a vacuum concentrator. Dried peptides were dissolved

in 0.1% (v/v) formic acid and quantified by measuring absorbance at 280 nm using a NanoDrop-type spectrophotometer.

Mass spectrometry (LC–MS/MS) proteomics analysis

The resuspended samples containing 330 ng of digested peptides were analyzed using the Ultimate 3000 nano-RSLC system coupled to an Orbitrap Fusion Lumos mass spectrometer (both from Thermo Scientific). A 100 µm × 2 cm C18 trapping column was used for the preconcentration of peptides for 10 min using 0.1% TFA (v/v) with a flow rate of 20 µL/min followed by separation on a 75 µm × 50 cm C18 analytical column (both PepMap RSLC, Thermo Scientific) with a 90 min LC gradient ranging from 3% to 35% of buffer B: 84% ACN, 0.1% formic acid at a flow rate of 250 µL/min. The Orbitrap Fusion Lumos MS was operated in the data-dependent acquisition (DDA) mode, and MS survey scans were acquired from *m/z* 300 to 1500 at a resolution of 120,000 using the polysiloxane ion at *m/z* 445.12002 as lock mass. The quadrupole isolated the precursor ions with a window of 0.4*m/z*. The acquisition was performed in the top speed mode, selecting the most intense signals within a cycle time of 3 s between survey scans and subjecting them to higher energy collisional dissociation (HCD) with a normalized collision energy of 32%. Fragment ions were analyzed in the orbitrap using a resolution of 15.000 (MS/MS). Selected parent ions were included in a dynamic exclusion for 15 s. Automatic gain control (AGC) target values were set to 2×10^5 for MS and 5×10^4 for MS/MS, for which the maximum injection times were 120 and 250 ms, respectively. Precursor ions with charge states of +1, >+7, and unassigned were excluded from the MS/MS analysis, and monoisotopic peak determination was set to 'peptide'.

Proteomics data analysis

The raw spectra (.raw) were deconvoluted using the MaxQuant software (version 1.6.17.0). Spectra were matched against the FASTA file with all CDS of *Rhinocladiella similis* LaBioMMi 1217 derived from genome sequencing (13,921 entries). The search parameters included a tolerance of 20 ppm for the first search and the main search with a tolerance of 4.5 ppm. Trypsin was chosen as the enzyme, allowing a maximum of two missed cleavage sites. The carbamidomethyl (Cys) modification was selected as a fixed modification, and oxidation (Met) and acetylation (protein N-terminal) were set as variable modifications. For peptide identification, a minimum of 1 unique peptide was used. A reverse decoy database determined that the false discovery rate (FDR) was <1%.

The data statistical analysis was performed in the R environment using the packages "tidyverse", "dplyr", "missForest", and "genefilter". Briefly, the potential contaminants and reverse IDs were filtered, the intensity values were transformed to Log_2, and the proteins containing >50% of the missing values were removed. The random forest (RF) method was used for data imputation[60]. Following, the values were normalized by the column median (protein intensities for each experimental data) and submitted to the Student *t*-test. Upregulated proteins were defined using a p-value < 0.05 and log_2-transformed fold change (FC) > 1. Downregulated proteins were defined using a p-value < 0.05 and log_2-transformed FC < 1. The enrichment of biological processes with up- and downregulated proteins was performed using the ShinyGO 0.80 platform (http://bioinformatics.sdstate.edu/go/). The PCA, volcano plot, and enrichment graphs were generated using the online platform (https://www.bioinformatics.com.cn/en).

Extraction of extracellular microbial metabolites

After 20 days of cultivation, 20 mL of fermentation broth was collected and centrifuged in 50 mL Falcon tubes. The same amount of magnesium perchlorate was added to all control samples to standardize the procedure. Subsequently, the samples were frozen at −80 °C for 24 h. After complete freezing, samples were lyophilized until the total sublimation of the fermentation broths. The resulting dry extracts were rehydrated with 5 mL of ultra-pure water. These aqueous extracts were then subjected to a clean-up process using Shop Sep-Pak® C18 Cartridges, 500 mg (Waters Corporation), to remove excess magnesium perchlorate. The elution of metabolites was conducted with ACN, and the eluted fractions were concentrated and transferred to vial bottles for subsequent analysis by LC–MS.

LC–MS/MS analysis and processing of metabolomic data from metabolic extracts

The fungi extracts were characterized by UHPLC (Shimadzu, Nexera X2, Japan) equipped with a Bruker BRHSC18022050 column. All solvents were HPLC grade (>99%, J.T. Baker, Phillipsburg, USA, or Sigma-Aldrich, St. Louis, USA). The separation gradient comprised solutions A (0.1% v:v formic acid) and B (ACN with 0.1% v:v formic acid). The gradient was set using 5% B (0–1 min), 70% B (1–10 min), 98% B (12–20 min), and 5% B (20–25 min). Temperature and flow rate were set at 40 °C and 0.250 mL min^{-1}, respectively.

The eluted compounds were analyzed by a quadrupole/time of flight (QTOF) high-resolution mass spectrometer (MS, Impact II, Bruker Daltonics Corporation, Germany) equipped with an electrospray ionization source (ESI) in positive mode. MS parameters were as follows: capillary voltage of 4500 V (potential plate end of −500 V), gas flow of 8 L min^{-1} at 180 °C, nebulization gas pressure of 4 bar. Data acquisition was monitored at mass/charge (*m/z*) ranges between 50 and 1300 at 5 Hz. The five most intense ions were selected for automatic tandem mass spectrometry (Auto MS/MS). Data acquisition and the feature table process were performed using Hystar Application software version 3.2 and MetaboScape®. The multivariate and univariate statistical analyses, including principal component analysis (PCA), were carried out using the MetaboAnalyst 4.0 platform by removing features with at least 50% missing values and estimating the remaining missing values using the KNN algorithm. Additionally, feature filtering with RSD (relative standard deviation) >30% in the QC samples, normalization by the median, and data scaling by the autoscaling method were performed. To screen out significant differential metabolites, it was considered variable importance in the projection Log_2 (FC) ≥ 2.0 and p-value < 0.05 using the *t*-test.

A molecular network of the metabolites of interest was created using the online workflow (https://ccms-ucsd.github.io/GNPSDocumentation/) on the GNPS website (http://gnps.ucsd.edu). The data were filtered by removing all MS/MS fragment ions within ±17 Da of the precursor *m/z*. MS/MS spectra were window-filtered by choosing only the top 6 fragment ions in the ±50 Da window throughout the spectrum. The precursor ion mass tolerance was set to 0.02 Da, and an MS/MS fragment ion tolerance of 0.02 Da. Then, a network was created where edges were filtered to have a cosine score above 0.60 and more than 4 matched peaks. Further, edges between two nodes were kept in the network if, and only if, each node appeared in each other's respective top 10 most similar nodes. Finally, the maximum size of a molecular family was set to 20, and the lowest-scoring edges were removed from molecular families until the molecular family size was below this threshold. The spectra in the network were then searched against GNPS' spectral libraries. The library spectra were filtered in the same manner as the input data. All matches kept between network spectra and library spectra were required to have a score above 0.55 and at least 4 matched peaks. The annotation process conforms to Level 2 identification standards outlined by the Metabolomics Standards Initiative (MSI)[61].

Data availability

The WGS, genome annotation and raw data of the analyzed fungal strain (*Rhinocladiella similis* LaBioMMi 1217) have been deposited in GenBank under BioProject PRJNA1005689 and accession number JBBMOB0 00000000. For metabolomic data acquired using the ESI + MS technique, interested parties can access them through the Global Natural Products Social Molecular Networking (GNPS) using the identifier ID = 31 f75023299848cbb0c7539b1fed5b58. Proteomics mass spectra and associated data have been uploaded into PRIDE (ProteomeXchange Consortium) under the dataset identifier PXD050600.

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Author contributions

A.S., J.S., A.S.R., and E.R.F. designed the study and wrote the manuscript. A.S. and J.S. performed the microbiological experiments and created figures. A.S., J.S., L.R.R., and T.V.B. conducted the proteomic analysis. A.S., F.O.S., E.J.P., and E.R.F. analyzed and interpreted the data from chemical profiles and generated figures. E.R.F., E.J.P., and A.S.R. funded the project. All authors read, revised, and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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