RESEARCH PAPER

Effects of a novel bioprocess for the cultivation *Synechococcus nidulans* on Mars on its biochemical composition: focus on the lipidome

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Abstract

In the present work, the possibility to grow the strain *Synechococcus nidulans* CCALA 188 on Mars using a medium mimicking a one obtainable using in situ available resources, i.e. the so-called Martian medium, under an atmosphere obtainable by pressurization of Mars CO_2, is investigated. The goal is to obtain a biomass with high-value products to sustain a crewed mission to Mars. The results show that the replacement of 40% vol of Z-medium with the same volume of Martian medium does not affect the cultivation and leads to a slight improvement of biomass productivity. Under an atmosphere consisting of pure CO_2 the growth rate was reduced but the strain managed to adapt by modifying its metabolism. Total proteins and carbohydrates were significantly reduced under Mars-like conditions, while lipids increased when using CO_2. A balanced diet rich in antioxidants is crucial for the wealth of astronauts, and in our case, radical scavenging capacities range from 15 to 20 $mmol_{TEAC}$/kg were observed. Under CO_2, a reduction in antioxidant power is observed likely due to a decrease in photosynthetic activity. The lipidome consisted of sulfoquinovosyldiacylglycerol, monogalactosyldiacylglycerol, digalactosyldiacylglycerol, phosphatidylcholine, phosphatidylglycerol, and triacylglycerol. A significant increase in the latter ones was observed under Mars simulated atmosphere.

1 Introduction

There is a high probability that life on Earth will become hard in the future due to factors such as natural disasters, global warming, population growth, the use of weapons of mass destruction but also simply due to astronomical phenomena [1]. To address this issue, it could be important to consider plans for long-term survival of humanity. Space exploration and colonization are potential options that could allow to establish human settlements on other planets or celestial bodies. Among the candidate planets to host the first human settlements, Mars represents nowadays the most feasible option, due to its closeness to Earth, its thermobaric conditions, the incident radiation, and the gravity levels [2]. To ensure the sustainability and cost-effectiveness of the mission, it is necessary to exploit natural resources available on Mars according to a paradigm known as in situ resource utilization (ISRU). Indeed, transporting all the needed materials from Earth would be quite expensive and impractical [2, 3].

Main ISRU technologies so far available are meant to produce oxygen, water, and propellants by using Mars regolith and atmosphere as feedstocks but they cannot produce biological compounds such as food, supplements or drugs for the sustainment of the crew on Mars [4, 5]. To overcome these limitations, researchers are proposing new bioprocesses capable to integrate or operate in synergy with ISRU technologies for the production of biological materials for the crew. In a classical configuration, such bioprocesses rely on metabolic wastes of astronauts, such

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as carbon dioxide (CO_2), urine, and faeces, to grow photosynthetic organisms like crops, macroalgae, microalgae or cyanobacteria, which produce oxygen and could serve as a source of biological products for the astronauts [6, 7]. Moreover, cyanobacteria could be biological link between on-site resources and life support system [8]. Cyanobacteria possess diazotrophic capabilities, enabling them to utilize nitrogen (N_2) from Mars for growth and are being conceptualized as a mean to convert raw mineral resources, locally extracted water, atmospheric gases, and human-generated waste into essential resources, including oxygen, food, fuel, and substrates [8–10]. These substrates, in turn, can support the growth of other organisms, creating a sustainable production cycle for diverse supplies [8–10]. In addition, cyanobacteria but also microalgae can synthesize a wide range of bioactive compounds, including proteins, lipids, carbohydrates, vitamins, and pigments such as phycobiliproteins, carotenoids, and chlorophyll [11, 12] which can be used as supplements with antioxidant and anti-inflammatory functions to enhance their diet and their protection against the oxidative stress determined by the exposition to radiations during long-term missions on Mars [8, 13, 14].

Nevertheless, it is important to highlight that the extreme conditions of Mars, i.e. very low temperatures and pressures, atmosphere composition and high ionizing radiations, may pose a significant hurdle to the growth of photosynthetic microorganisms in situ [8, 15–20]. Moreover, the reliance of the proposed approach on the only metabolic wastes as fertilizers to cultivate microalgae and cyanobacteria would not permit to completely sustain the crew needs since the former ones would be produced in very low amounts [13]. Therefore, there is a call for novel bio-ISRU technologies capable to also exploit regolith and atmospheric CO_2, beyond astronauts' metabolic wastes, to produce fertilizers for microalgae and cyanobacteria cultivation. Such technologies should be also capable to minimize the effects of harsh conditions on Mars on the cultivated microalgae and/or cyanobacteria.

In this regard, only a limited number of scientific studies have so far explored the potential of cyanobacteria to convert atmospheric N_2 and CO_2 along with nutrients in Martian soil or regolith, for producing, food, supplements, other biocompounds and oxygen [8, 13, 16–23].

Moreover, to the best of our knowledge, no study on the change of cyanobacteria biochemical composition and lipidome triggered by the harsh conditions on Mars is so far available in the literature albeit such analysis would be crucial to ascertain whether cyanobacteria produced in situ might be utilized as a source of food or other useful bioactive compounds for astronauts.

To address this lack, we conducted an experimental investigation with *Synechococcus nidulans (*or *Anacystis nidulans)* CCALA 188 which is a cyanobacterium that can adapt to extreme environments and is therefore potentially exploitable for space applications [13]. The goal was to evaluate the possibility to exploit in situ available resources, i.e. regolith and atmospheric CO_2, along with astronauts' metabolic wastes, as fertilizers to grow it on Mars. Another target was to investigate the changes induced on the biochemical composition and the lipidome of *S. nidulans*, induced by the operating conditions taking place during its cultivation on Mars.

It should be pointed out that apart from gravity, the operating conditions adopted in our experiments were the same which theoretically would occur in a pressurized and heated dome on Mars, wherein the bio-ISRU process recently patented by Cao et al. [22] would be run. This process is composed of two interacting sections, i.e. the physicochemical and the biological one as shown in Fig. 1.

In the physicochemical section, different modules work under Martian conditions to produce water, oxygen, and propellants [4, 6]. The biological section uses in situ resources (CO_2 from atmosphere and regolith), astronauts' urine and the outputs from the physicochemical section to produce edible biomass and photosynthetic oxygen by growing microalgae and or cyanobacteria in photobioreactors hosted by pressurized and heated domes [24].

This process was artificially reproduced in our laboratory-scale experiments using suitable simulants of Martian regolith and pure CO_2 atmosphere along with synthetic urine as source of macro- and micronutrients for the cultivation of *S. nidulans*. This test organism was chosen since in 1994 *Synechococcus* sp. was the first cyanobacterium used in space experiments by the European Space Agency [25]. Moreover, during the EXPOSE-R mission (2009–2011), *Chroococcidiopsis* and *Synechococcus* cells demonstrated to be able to withstand high radiation levels taking place in space and Mars [26, 27].

The membrane lipid composition of *Synechococcus* sp. was for the first time described by Bishop et al. [28] and includes glycolipids and phospholipids. These lipids can be extracted and processed into a high-energy food source for astronauts. For astronauts, these compounds are particularly beneficial for several reasons. Firstly, they provide a concentrated source of energy that is necessary for the physically demanding and high-stress environment of space. Secondly, fats are essential for the absorption of fat-soluble vitamins such as vitamins A, D, E, and K, which are vital for maintaining good health [29]. Thirdly, they help regulating hormones and inflammation, which is particularly important in a high-stress environment [4].

For all these reasons, it is very important to study if and how the biochemical composition and the lipidic profile of *S. nidulans* would be affected by the cultivation conditions potentially taking place on Mars according to the bio-ISRU process by Cao et al. [19].
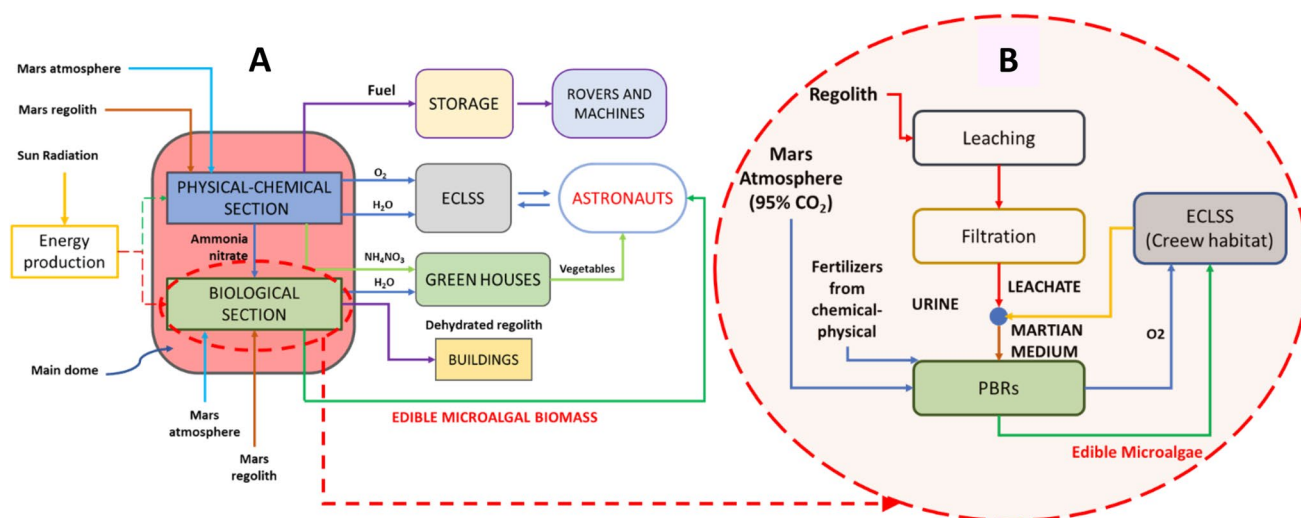
Fig. 1 Scheme of the ISRU process to produce useful materials on Mars (**A**) and focus on the production of microalgae or cyanobacteria in the biological section (**B**). Adapted from the article of Brughitta et al. [24]. ISRU: in situ resource utilization, PBR: photobioreactor

Such investigation could be also useful to identify key enzymes and pathways involved in the biosynthesis and degradation of specific bio-compounds and in particular of lipids. This information might be in turn exploited to suitably tune the cultivation conditions so to maximize or minimize the production of specific lipid categories or other bio-compounds needed by astronauts on Mars.

2 Materials and methods

2.1 Strains and culture conditions

This study is focused on the cyanobacterium *S. nidulans* (CCALA 188, holotype Nageli), which was obtained from the Culture Collection of Autotrophic Organisms (CCALA) located in Třeboň, Czech Republic [30]. The cyanobacterium was kept under axenic conditions at the Interdepartmental Center of Environmental Sciences and Engineering (CINSA) in Cagliari, Italy, and was grown in phototrophic conditions at a temperature of 20 ± 1 °C with 12 h of light and 12 h of darkness, using 100 µmol photons m^{-2} s^{-1} of white light as measured by a Delta light meter (Delta OHM, GHM group). Its cultivation was performed using the Z-medium (Table S1), and continuous agitation was maintained at 100 rpm by means of a Stuart SSM1 orbital shaker (Biosigma).

2.2 Growth experiments

The study aims to investigate the possibility of cultivating *S. nidulans* in a system simulating the best the possible the integrated bio-ISRU process to be implemented on Mars [19]. According to this process the cultivation should be carried out in a dome wherein CO_2 obtained from Mars atmosphere, is introduced. The process also foresees the use of a medium obtained by mixing regolith leachate (RL) with urine from the cabin crew, i.e. the so-called Martian medium (MM). The light source can be the sun or lamps suitably disposed within the dome. To simulate the process above on Earth, the experimental setup shown in Fig. 2 was adopted.

Briefly, it consisted of a transparent sealed jar illuminated by artificial light, wherein an atmosphere of pure CO_2 is continuously blown in. Within the dome laid, the laboratory-scale photobioreactors containing the cultures of *S. nidulans* in the growth medium obtained by mixing specific amounts of Z-medium (the natural best medium for this stain) and MM to verify which was the highest percentage of MM that could be employed.

It should be noted that in the framework of a previous work, carried out in air, the maximum tolerable amount of MM was verified to be 40% vol. For this reason, experiments were carried out by using a volume percentage of MM in the growth medium equal to 0% vol (pure Z-medium) or 40%.

As for the atmosphere, two different conditions were tested, i.e. the use of air or an atmosphere of pure CO_2 within the jar. The experiments were performed in triplicate in laboratory-scale photobioreactors consisting of transparent vented cap flasks with a maximum capacity of 50 mL, filled up to 40 mL. At the beginning of the experiment, the optical density of the culture was approximately 0.2 at a wavelength of 750 nm. The growth was monitored by measuring the optical density of chlorophyll-a using a Genesys 20 spectrophotometer (Thermo Scientific), at the wavelength of 750 nm and using a suitable calibration line, reported
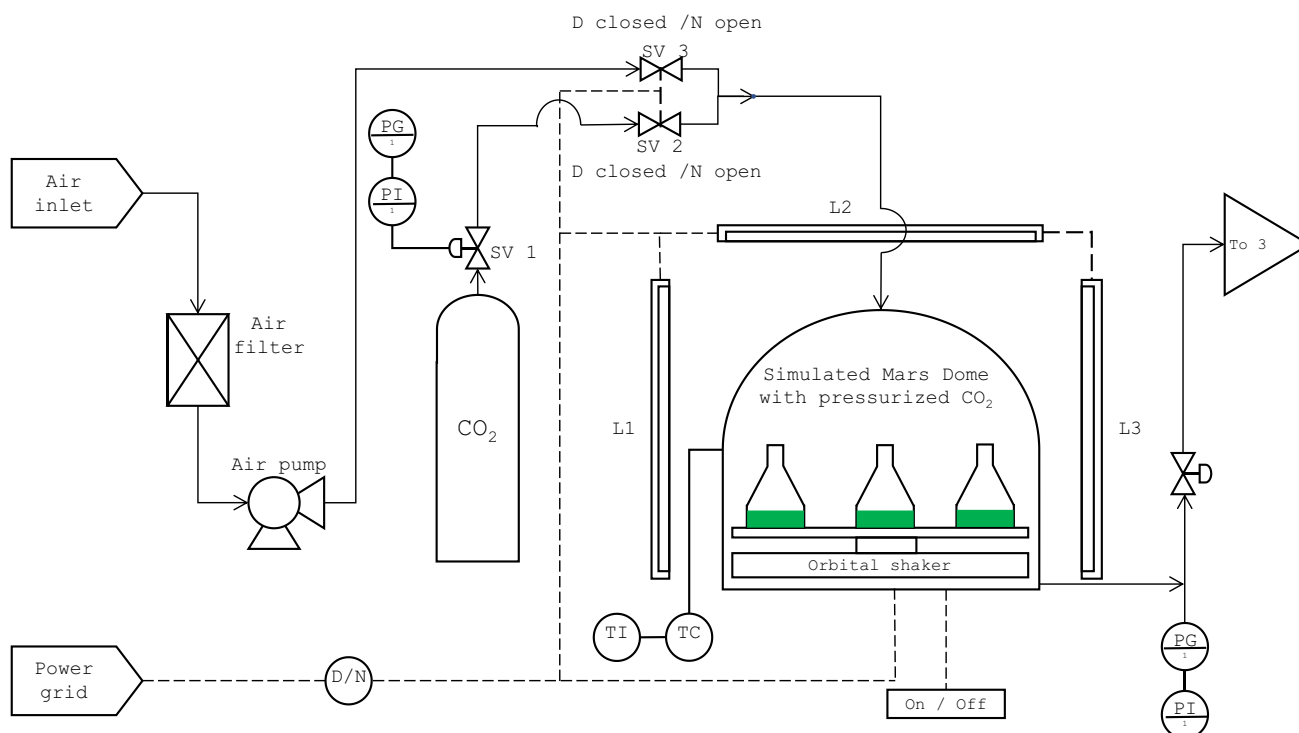
Fig. 2 Scheme of the experimental apparatus adopted to simulate the pressurized Martian domes hosting the process by Cao et al. [22] on Mars

elsewhere to convert the optical measures into biomass concentration [6].

2.3 Preparation and composition of the MM

To create the synthetic MM, a mixture of a leachate of Martian regolith simulant (JSC MARS-1) and synthetic human urine (MP-AU) was used.

2.3.1 Preparation of the RL

The components of the JSC MARS-1 simulant, in terms of oxides (% by weight), are reported in Table S2. To prepare the RL, the procedure proposed by Fais et al. [13] was employed. In brief, the RL was obtained by leaching 50 g of regolith simulant (< 1 mm diameter size) with 500 mL of ultrapure water at a pH of 6.80. The resulting mixture was stirred for 24 h at 25 °C within a 1 L Erlenmeyer flask with a cap and then filtered with bibulous paper.

2.3.2 Preparation of synthetic urine

Synthetic human urine (MP-AU) was then produced according to the literature and then diluted with ultrapure water at a ratio of 1:10 v/v [31].

2.3.3 Composition of the MM

Finally, the Martian RL and diluted urine were mixed in a 1:1 v/v ratio to produce MM. The medium has been sterilized at 121 °C for 15 min prior to use. Table 1 provides information on the composition characteristics of MM.

2.4 Chemicals

Analytical LC grade isopropanol, methanol, acetonitrile, acetic acid, formic acid, ammonium formate, ammonium acetate, 2,2-diphenyl-1-picrylhydrazyl and Trolox were purchased from Sigma-Aldrich. Sulphuric acid 96%, orthophosphoric acid 85%, sodium nitrate, potassium chloride, phenol, copper sulphate, sodium hydroxide, and sodium potassium tartrate were analytical grade and were purchased from Carlo Erba. Sodium carbonate and Folin–Ciocalteu reagent were acquired by Sigma-Aldrich Inc. Glucose, bovine serum albumin (BSA) and vanillin standards were acquired by Sigma-Aldrich. Bi-distilled

Table 1 Concentration of macro-nutrients and metals acting as micronutrients in the Martian medium

Macronutrients		Micronutrients	
Component	(g/L)		(mg/L)
Na_2SO_4	0.085	Al	2.4
$C_5H_4N_4O_3$	0.012	Ca	4.06
$Na_3C_6H_5O_7 \times 2H_2O$	0.036	Fe	3.205
$C_4H_7N_3O$	0.044	K	4.16
CH_4N_2O	0.750	Mg	0.74
KCl	0.115	Mn	0.095
NaCl	0.087	Na	2.33
$CaCl_2$	0.009	P	0.125
NH_4Cl	0.063	Si	5.14
$K_2C_2O_4 \times H_2O$	0.002	Ti	0.635
$MgSO_4 \times 7H_2O$	0.054		
$NaH_2PO_4 \times 7H_2O$	0.146		
$Na_2HPO_4 \times 2H_2O$	0.041		

water was obtained by a Milli-Q purification system (Millipore). A SPLASH® LIPIDOMIX® standard lipid component mixture was purchased from Sigma-Aldrich.

2.5 Sample preparation

Synechococcus cultures were centrifuged at 4000 rpm for 10 min at 20 °C. The supernatant was eliminated, the pellet was resuspended in Milli-Q water, and the washing procedure was repeated for three times. The cellular pellet was frozen at -80 °C, lyophilized with LIO-5PDGT freeze-dryer (5 Pa) and pulverized with mortar and pestle.

2.6 Total carbohydrates, lipids, and soluble proteins

Total carbohydrates determination was carried out using the modified method proposed in the literature [32]. Briefly, 2 mg of powder biomass were weighted into an Eppendorf tube and suspended in 1000 μL of phosphate-buffered saline (PBS) (20 mM, pH 7.4). The samples were vortexed for 10 min and sonicated in an ultrasonic bath for 10 min at 20 °C for three times. 200 μL of the extract was transferred to a glass tube, while 200 μL of phenol 5% (w/v) and 1,000 μL of concentrated sulfuric acid were added. The samples were filtered with 0.45 μm PTFE membrane filter and analysed at $\lambda = 490$ nm using a Varian Cary 50 spectrophotometer. The external standard method using glucose as a reference standard was adopted to quantify the samples. A calibration line was built with 5-points standard solution by correlating the absorbance with the glucose concentration. The results were reported as average value $\pm$ standard deviation and expressed in g/100 g of glucose. All samples were analysed in triplicate.

Total lipids were carried out using the protocol indicated in the literature, albeit with small changes [33]. The 100 μL of PBS, 1.5 mL of 25% methanol, and 1 N sodium hydroxide solution were added to 2 mg of lyophilized sample. The suspension was sonicated for 10 min in an ultrasonic bath and then heated to 100 °C for 30 min. Lipids were extracted according to the method proposed in the literature [34]. The colorimetric reaction was obtained with the method proposed by Mishra et al. [35]. . All samples were analysed in triplicate, and the results were expressed in g/100 g $\pm$ SD.

Total protein content was determined using the method proposed by Lowry et al. [36]. 1,000 μL of PBS, 20 mM, pH 7.4, was added to 2 mg of lyophilized sample. Approximately, 150 mg of 1–1.3 mm glass balls were added and vortexed for 10 min and sonicated in an ultrasonic bath for 10 min at 20 °C for three times. 250 μL of this solution reacted with 250 μL of 1 N sodium hydroxide for 5 min at 100 °C. After cooling at room temperature for 10 min, 2.5 mL of a 5% sodium carbonate (w/v), 0.5% (w/v) cupric sulphate and 1% (w/v) sodium potassium tartrate solution were added. After 10 min, 0.5 mL of 1 N Folin–Ciocalteu reagent was added. Samples were analysed at $\lambda = 750$ nm with a spectrophotometer (Varian Cary 50). BSA was used as a reference standard to quantify the samples with the external standard method. All samples were analysed in triplicate and the results were expressed in g/100 g $\pm$ SD of BSA.

2.7 Antioxidant activity

The free radical, 2,2-diphenyl-1-picrylhydrazyl (DDPH) spectrophotometric test was performed using the method proposed in the literature [37]. 250 μL of methanol was added to 2 mg of lyophilized *Synechococcus* powder. To maximize the extraction, approximately 100 mg of 1–1.3-mm glass balls were added, and the solution was vortexed for 10 min and sonicated in an ultrasonic bath for 10 min at 10 °C three times. The sample was centrifuged for 5 min at 4000 rpm. Then, 50 μL of the methanol extract was added to 2 mL of a methanol solution of DDPH (50 μmol) for the determination of total polyphenols (Trolox). The solutions were analysed at $\lambda = 517$ nm after 60 min of incubation. The external standard method (Trolox) was used to quantify the samples, correlating the absorbance with the concentration. The results were expressed in mmol/kg TEAC (Trolox equivalent antioxidant capacity).

2.8 Chlorophyll-a and total carotenoids content

The method proposed by Zavřel et al. [38] was adopted to estimate chlorophyll-a and total carotenoid content. Briefly, 1000 μL of culture were placed inside Eppendorf tubes and centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatants were eliminated, and 1 mL of neutralized

methanol was added to the pellets. The solutions were left overnight in the fridge, and after 24 h, the samples were homogenized using three cycles of 10 min of a vortex and ultrasonic bath. The solutions were centrifuged for 10 min at 10.000 rpm. The content of chlorophyll-a and total carotenoids was estimated with a spectrophotometric analysis at $\lambda = 720$ nm, $\lambda = 665$ nm, respectively, while $\lambda = 470$ nm was used for methanol as a blank. The following correlations proposed by Ritchie [39] and Wellburn [40] were used to estimate the total carotenoids and chlorophyll-a concentrations, respectively:

$$\text{Carotenoids } \left[\mu g/mL\right] = \frac{1000\left(A_{470} - A_{720}\right) - 2.86\left(\text{Chl-}a\left[\mu \text{ gmL}^{-1}\right]\right)}{221}$$

2.9 Lipidomic analysis

The total lipids of twelve samples of lyophilized biomass were extracted using a modified Folch method [41]. Briefly, 10 mg of each lyophilized biomass sample was transferred to an Eppendorf tube containing 10 μL of the internal mixture of standards (Splash, Lipidomics, Sigma-Aldrich), and then a mixture of chloroform/methanol (2:1 v/v) was added. The solution was sonicated for three times using a sonicator (GM solution). Finally, 90 μL of aqueous 0.2 M potassium chloride were added. The suspension was centrifuged at 14.000×g for 10 min. After centrifugation, the lipophilic layer was transferred to a glass vial and dried by a gentle N_2 stream.

The dried phase of the cyanobacteria extracts was reconstituted using 20 μL of a mixture of methanol/chloroform (1:1 *v/v*) and diluted with 980 μL of a mixture of 2-propanol/acetonitrile/water (2:1:1 *v/v/v*). Then, the samples were analysed with a UHPLC-QTOF/MS coupled with an Agilent 1290 Infinity II LC system, injecting 1 μL and 5 μL in the positive and negative ionization mode, respectively. Chromatographic separation of lipids was obtained with a Kinetex 5 μm EVO C18 100 A, 150 mm × 2.1 μm column (Agilent Technologies).

The column was maintained at 50 °C at a flow rate of 0.4 mL/min. The mobile phase for positive ionization mode consisted of (A) 10 mM ammonium formate solution in 60% of Milli-Q water and 40% of acetonitrile and (B) 10 mM ammonium formate solution containing 90% of isopropanol, 10% of acetonitrile. In positive ionization mode, the chromatographic separation was obtained with the following gradient: initially 60% of A, then a linear decrease from 60 to 50% of A in 2 min, then at 1% in 5 min staying at this percentage for 1.9 min and then brought back to the initial conditions in 1 min. The mobile phase for negative ionization mode differed only for the use of 10 mM ammonium acetate instead of ammonium formate.

The Agilent jet stream source was operated with the following parameters: gas temperature, 200 °C; gas flow (N_2) 10 L/min; nebulizer gas (N_2), 50 psig; sheath gas temperature, 300 °C; sheath gas flow, 12 L/min; capillary voltage 3500 V for positive and 3000 V for negative; nozzle voltage 0 V; fragmentor 150 V; skimmer 65 V, octapole RF 7550 V; mass range, 50–1700 m/z; capillary voltage, 3.5 kV; collision energy 20 eV in positive and 25 eV in negative mode, mass precursor per cycle = 3; threshold for MS/MS 5000 counts.

Before the analysis, the instrument was calibrated using an Agilent tuning solution at the mass range of m/z 50–1700. Samples were acquired in an auto MS/MS method using the iterative mode with a mass error tolerance of 20 ppm with a retention exclusion tolerance of 0.2 min.

The Agilent MassHunter LC/MS Acquisition console (revision B.09.00) and lipid annotator from the MassHunter suite was used for data acquisition and data processing. Lipid levels were normalized using the following internal standard PE 33:1 (d7) for positive and negative ionization modes, respectively. The resulting relative abundances were imported into GraphPad Prism software (version 8.3.0; Dotmatics) to build the graphics and to perform a *t* test analysis.

3 Results and discussion

In this study, we investigated the chemical and biochemical composition of *S. nidulans* CCALA 188 cultivated using a combination of Martian RL and synthetic urine, i.e. the MM under atmosphere of air or pure CO_2 to simulate the Martian one after pressurization. The main goal was to determine whether this cyanobacterium could endure these conditions, with the aim of utilizing its biomass for bio-ISRU applications, such as producing biomass enriched with high-value products for astronauts during crewed space missions on Mars.

Two groups of experiments were conducted, each involving different operating conditions. In the first set of experiments, air at a pressure of 1 atm was used, while in the second set of experiments, operating conditions theoretically taking place on Mars in the ISRU process proposed by Cao et al. [19] were simulated by exposing the cells to a continuous flow of CO_2 in a closed dome.

Each experimental group involved two different growth media consisting of different percentages of MM, i.e. 0% v/v (that is pure Z-medium) and 40% v/v. Each experiment was identified by an acronym obtained by considering the medium adopted, i.e. ZM or MM40, followed by the atmospheric composition, i.e. air or CO_2.
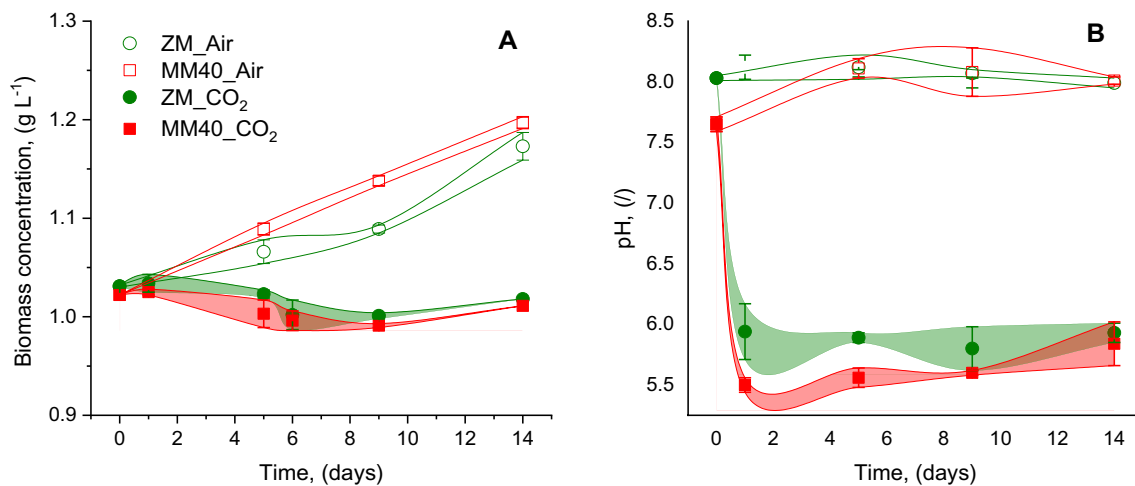
Fig. 3 Time evolution of growth (**A**) and pH (**B**) of *Synechococcus nidulans* cultivated in different media and atmospheric conditions. MM: Martian medium

3.1 Effect of continuous CO_2 bubbling and MM content on the growth of *S. nidulans*

The time profile of the biomass concentration and pH during these experiments is shown in Fig. 3.

The growth in air was consistent with the one observed in the framework of a previous work with a maximum specific growth rate of around $1.8 \cdot 10^{-2}$ (1/h) [6]. It is interesting to note that the replacement of 40% vol of ZM with the same volume of MM (culture M40_Air) did not affect the growth and, on the contrary, resulted in a slight improvement of the growth. A higher biomass concentration at the end of the experiment was correspondingly detected after 14 days of cultivation. Figure 3 shows also the growth under an atmosphere consisting of pure CO_2 like the one that should be in a Martian dome according to the ISRU process patented by Cao et al. [19]. The graphic shows that the growth rate was significantly reduced under pure CO_2 probably because of the very low pH achieved in the culture which could have inhibited specific enzyme of *Synechococcus*. In particular, up to the sixth day of cultivation, a slight decrease in the biomass concentration was observed but after this adaptation phase, the culture restarted growing with positive rate and re-achieved the initial biomass concentration. The re-activation of photosynthesis is confirmed by the pH evolution which, after an initial decrease, due to the dissolution of CO_2, started increasing after the sixth day. Such behaviour was the same for the cultures grown in both ZM and MM40 without a statistically meaningful difference between the two series of data. Ultimately, it can be stated that the strain managed to adapt and survive to the very harsh conditions determined by the continuous inflow of CO_2 and the consequent low pH achieved in the culture. Thomas et al.

[42] have observed a similar pH reduction in their investigation of the impact of elevated CO_2 on the growth of various cyanobacteria, including the moderately CO_2-tolerant *Synechococcus* sp. PCC7942. Photosynthetic microorganisms possess the ability to modulate gene transcription, translation, or protein stability in response to stress [43]. Prolonged cultivation under diverse stress conditions may induce gene mutations and foster evolutionary adaptations [43]. For instance, Uchiyama et al. [43] conducted experiments and genome sequence analyses to identify genes and proteins involved in the acid tolerance mechanism of the cyanobacterium *Synechocystis* sp. PCC 6803. Following their experiments, they obtained acid-tolerant strains with distinct mutations. Notably, a mutation in the Fo F1-ATPase operon, responsible for the expression of the Fo F1-ATPase proton pump, was detected. The authors hypothesized that this protein plays a role in one of the acid stress tolerance mechanisms of *Synechocystis* 6803 [43].

These results underline that it would be possible to cultivate *S. nidulans* using the bio-ISRU method by Cao et al. [19]. It should be noted in fact that rather than continuously pumping CO_2 within the dome a different strategy might be used in the in situ application of this technology. Such strategy would involve the pumping of CO_2 only when strictly necessary to the algae, i.e. when the total pressure within the dome would drop down to a specific value. This way the achievement of too low pH within the culture, and its subsequent inhibition, would be avoided. To overcome this limit in new experiment, we supply CO_2 to the simulated dome in the form of periodic (daily) pulses to avoid a too high pH reduction and the too high payloads of the temperature adsorbers (zeolites) needed on Mars to pressurize CO_2.
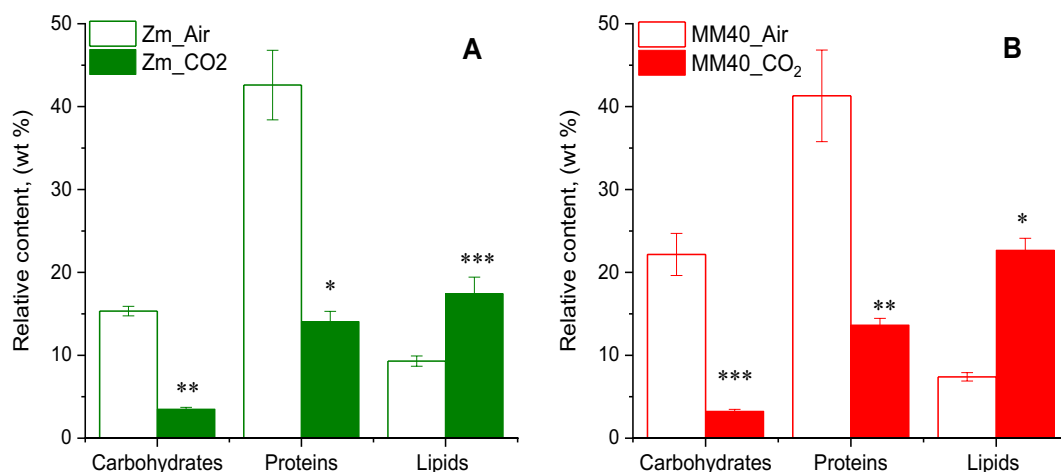
Fig. 4 Carbohydrates, lipid, and protein content of the *Synechococcus nidulans* biomass grown ZM (**A**) and MM_40 (**B**) and different atmospheric conditions ($n = 3$). MM: Martian medium. (*p value < 0.05; **p value < 0.01; ***p value < 0.001)

3.2 Total carbohydrates, lipids, and soluble proteins

The possibility of using *S. nidulans* biomass as a source of bioactive compounds for astronauts has been further investigated in terms of total lipids, proteins, and carbohydrate content (Fig. 4).

The biochemical macro-composition of the obtained biomass showed a prevalence of proteins (~ 40% wt) followed by carbohydrates and lipids (~ 15–20% wt and 7–8% wt, respectively). Figure 4 clearly shows that the replacement of a specific volume ZM with an equal one of MM did not cause any significant change in the biomass composition of *S. nidulans*. On the contrary, the latter one, was not significantly affected by the use of MM40 as a growth medium rather than pure ZM. When the cyanobacterium was grown in an atmosphere of CO_2 a significant decrease in carbohydrates and proteins was registered. However, it is interesting to note that the production of lipids was increased under CO_2 atmosphere indicating that the biomass potentially produced on Mars might be characterized by a high energetic and nutritional value and thus particularly suited to feed astronauts.

3.3 Antioxidant activity

The biomass of *S. nidulans* has been investigated in terms of antioxidant capacity through a TEAC assay. The space factors as exposure to ionizing radiation and reduced gravity during deep space missions can result in the excessive generation of radical oxygen species (ROS) that could cause cellular stress and consequently damage in astronauts [44]. Hence, a balanced diet rich in antioxidants is an attractive option for astronauts' wealth. The antioxidant capacity of *S. nidulans* is shown in Fig. 5.
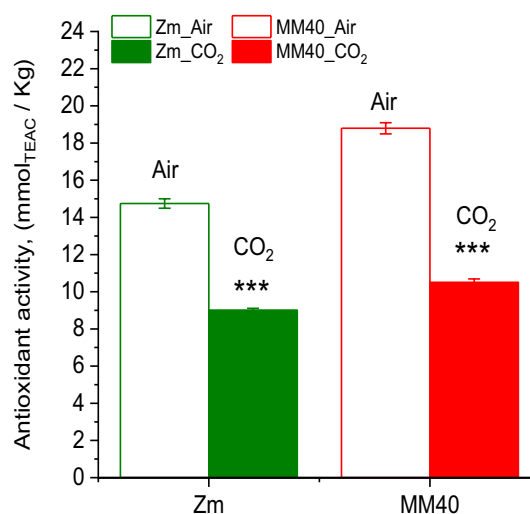

Fig. 5 Antioxidant capacity of *Synechococcus nidulans* biomass grown in different media and atmospheric conditions ($n = 3$). MM: Martian medium. (***p value < 0.001)

Radical scavenging capacities ranging from 15 to 20 mmol$_{TEAC}$/kg were obtained for the biomass grown under air in ZM and MM40, respectively. Such results were comparable to those (~ 20 mmol$_{TEAC}$/kg) reported by Goiris et al. [45], for different microalgal strains.

The cultivation in MM40 resulted in an antioxidant power 33% higher than that one obtained with the ZM. Instead, under high CO_2 atmosphere the antioxidant activity is decreased and varied from 8 to 11 mmol$_{TEAC}$/kg.
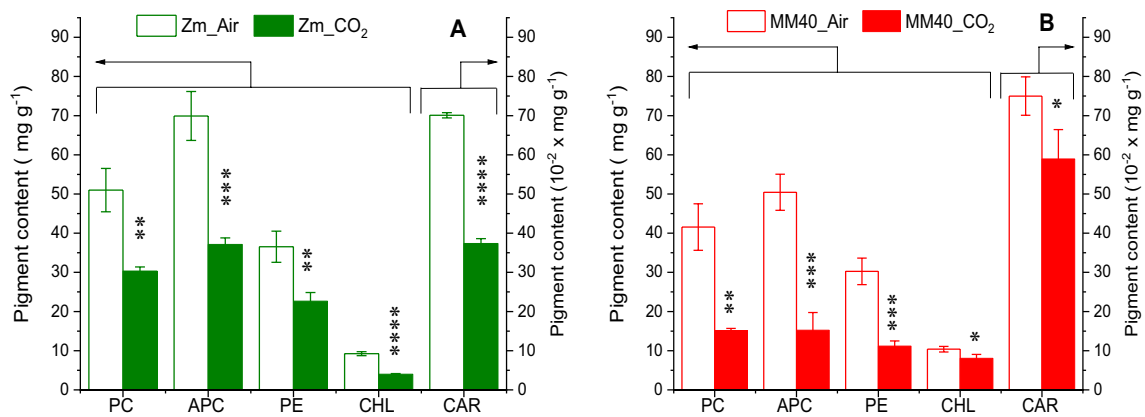
Fig. 6 Contents of chlorophyll-a, carotenoids, PC, APC and PE in the Synechococcus biomass grown in different media and atmospheric conditions ($n = 3$). MM: Martian medium, PC: phycocyanin, APC: allophycocyanin, PE: phycoerythrin, CHL: chlorophyll-a, CAR: total carotenoids. (*p value < 0.05; **p value < 0.01; ***p value < 0.001; ****p value < 0.0001)

3.4 Pigment content

Carotenoids and phycocyanin play a key role in the diet of astronauts because they have health benefits such as anti-inflammatory, immunomodulatory, neuroprotective, and hepatoprotective effects [46, 47]. For this reason, the pigment content of the microalgal biomass has been quantified and shown in Fig. 6.

The use of MM40 caused a decrease in the pigment content except for the case of chlorophyll and carotenoids. The increase in carotenoid content using MM40 represent an attractive opportunity because these compounds possess significant antioxidant and anti-inflammatory properties [48]. However, the differences between the results obtained in ZM and in MM40 are not statistically relevant. In general, the application of CO_2 resulted in a statistically significant decline in pigment production compared to the air content. These results are consistent with the ones obtained by other authors [47].

3.5 The effect of Martian simulated conditions on lipids biosynthesis

To further investigate the total lipids extracted from *S. nidulans* biomass, the lipidomic analysis was performed with the goal of identifying lipids with high nutritional power for astronauts and how their synthesis could be affected by the in situ cultivation conditions.

The lipidome investigated in this study consists of three classes of glycolipids (sulfoquinovosyldiacylglycerol [SQDG], monogalactosyldiacylglycerol [MGDG], and digalactosyldiacylglycerol [DGDG]), two phospholipids (phosphatidylcholine [PC] and phosphatidylglycerol [PG]) and one neutral lipid (triacylglycerol, TG). These results are consistent with other studies of cyanobacterial lipidic composition [49–52].

An example of positive and negative electrospray ionization (ESI) extracted ion chromatogram (EIC) of lipid compound found in *S. nidulans* growth at different percentage of MM and air/CO_2 atmosphere is shown in Fig. S1. The normalized abundances of lipids detected in the biomass grown under different conditions are reported in Fig. 7A in the form of heatmap with dendrogram and aggregated on the basis of the specific lipid class in Fig. 7B, G. The specific composition of each lipid class and the characteristics of the high-resolution mass spectrometry for all lipid species are listed in Table S3 and graphically reported in Fig. 8 to further highlight the difference between each experimental condition.

The heatmap in Fig. 7 provides a global overview of the effect of CO_2 and MM on the synthesis of specific lipids by clearly showing that the atmosphere wherein the strain is grown (air or CO_2) had the greater effect on the lipidome of *S. nidulans* rather than the growth medium.

A general reduction in triglyceride (TAG) content was registered when the cyanobacterium was grown using MM40 under CO_2. From the heatmap in Fig. 7 and from Fig. 8, it can be observed that such a generalized reduction in TAGs can be ascribed mainly to TAG 46:0, 46:1, 49:0, 52:2 and 54:3, while the other TAGS remained substantially unchanged.

SQDG are lipid molecules carrying negative charge. In general, the content SQDGs decreased when using CO_2 (Fig. 7C) even if the simultaneous use of MM40 and CO_2 led to the significant increase in SQDG 34:1 SQDG 32:1 (16:0/16:1) which is probably a result of the most stressful growth conditions (Figs. 7A, 8).

MGDGs increased mainly when using MM40 and, in a lower extent due to the CO_2 (Fig. 7D). In particular, a strong
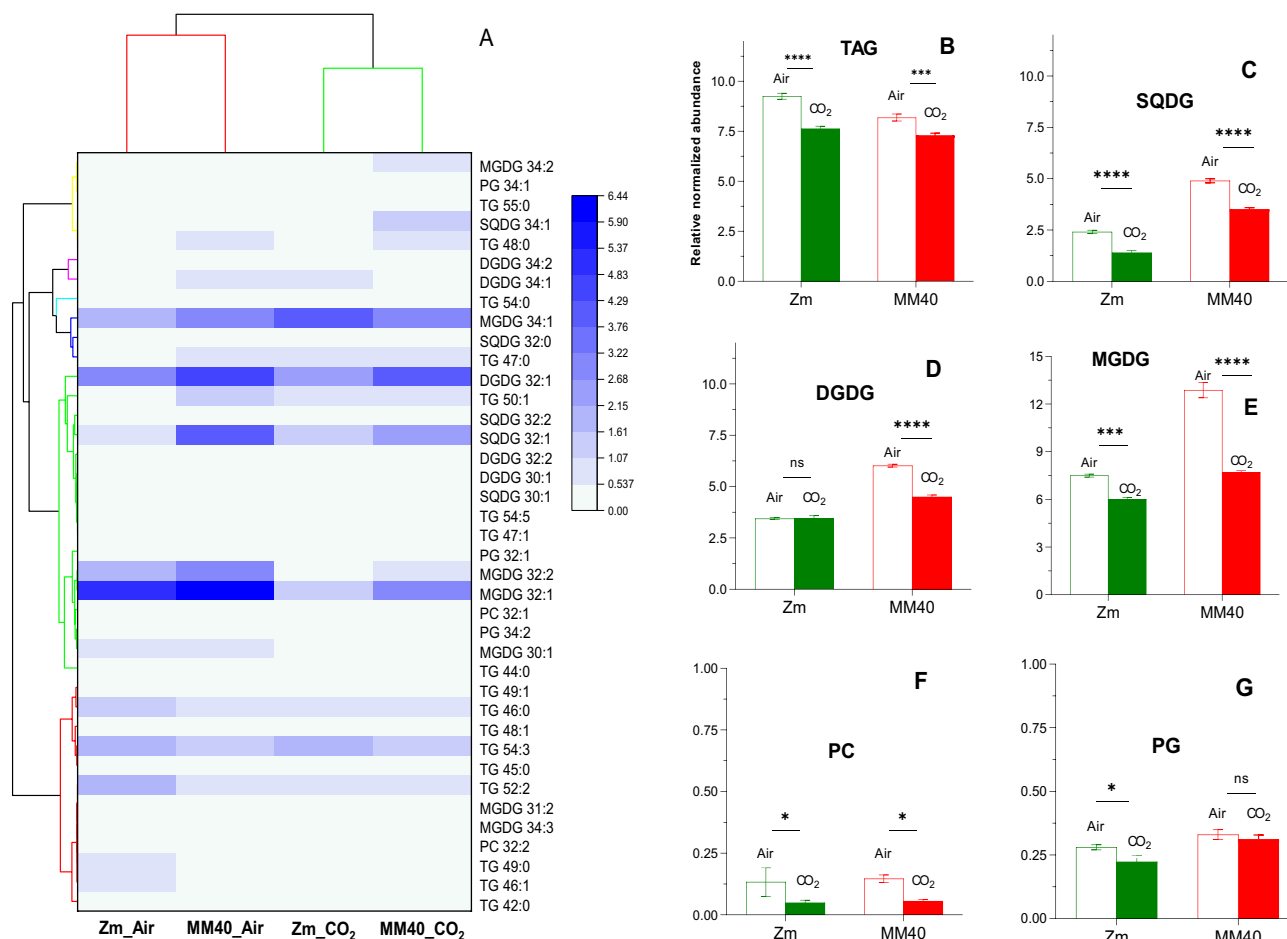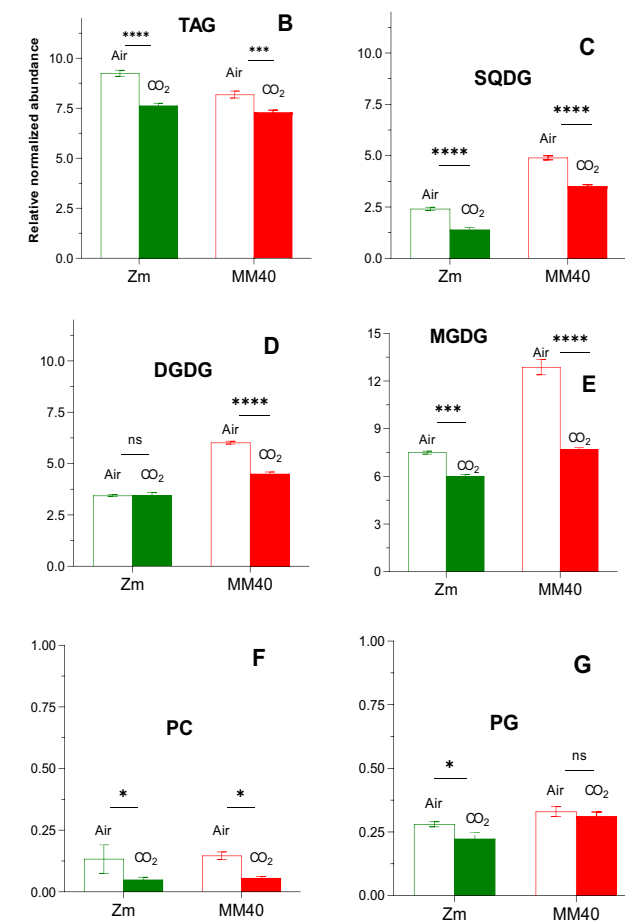
Fig. 7 Heatmap of each lipids compound found (**A**) and relative contents of TAG, SQDG, MGDG, DGDG, PC and PG (**B–G**) in the biomass of *Synechococcus nidulans* grown in different media and atmospheric conditions ($n=3$). MM: Martian medium, TAG: triglyceride,

SQDG: sulfoquinovosyldiacylglycerol, MGDG: monogalactosyldiacylglycerol, DGDG: digalactosyldiacylglycerol, PC: phosphatidylcholine, PG phosphatidylglycerol, ns: not significant. (*p value < 0.05; **p value < 0.01; ***p value < 0.001; ****p value < 0.0001)

increase in MGDG 32:1 (16:0/16:1) and 34:1 (16:0/18:1) was detected when using MM40. Other MGDGs remained substantially unchanged or slightly decreased (MGDG 32:2) under CO_2 (Fig. 8).

From Fig. 7D, DGDGs increased when using MM40 as growth medium. The effect of CO_2 was almost null when using ZM while it slightly reduced the overall content of DGDGs when using MM40. In particular we have measured an increase in DGDG 32:1 (16:0/16:1) when simultaneously using CO_2 and MM40 (Fig. 8).

The synthesis of PC was slightly inhibited by the CO_2 while it was substantially unaffected by the change of the growth medium (Fig. 7F). Specifically, under CO_2 the relative abundance of PC 32:2 increased at the expenses of PC 32:1 for both growth media adopted (Fig. 8).

PG is functionally linked to the core complexes of both photosystem I (PSI) and photosystem II (PSII) [52, 53]. The increase in these PGs and in particular those containing fatty

acids 16:1 (in our case PG 32:1, but the difference was not statistically significative) can be correlated to a stabilization of PSII [54]. The effect of the different growth media and atmosphere composition on total PGs relative abundance was roughly the same observed for PCs, i.e. CO_2 led to a slight decrease, while the effect of MM40 was almost undetectable (Fig. 7G). The specific lipid affected by the use of CO_2 was PG 34:1, whose relative abundance decreased under the Mars-like atmosphere.

By showing the cumulated relative abundance saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids, the results shown in Fig. 9 were obtained. No statistically meaningful effect was observed on SFA as both the growth medium and the atmosphere composition was changed. On the other hand, the use of MM40 significantly increased the total content of MUFAs, while the CO_2 acted in the opposite direction leading to a lower content of these lipids whatever the medium used. Same
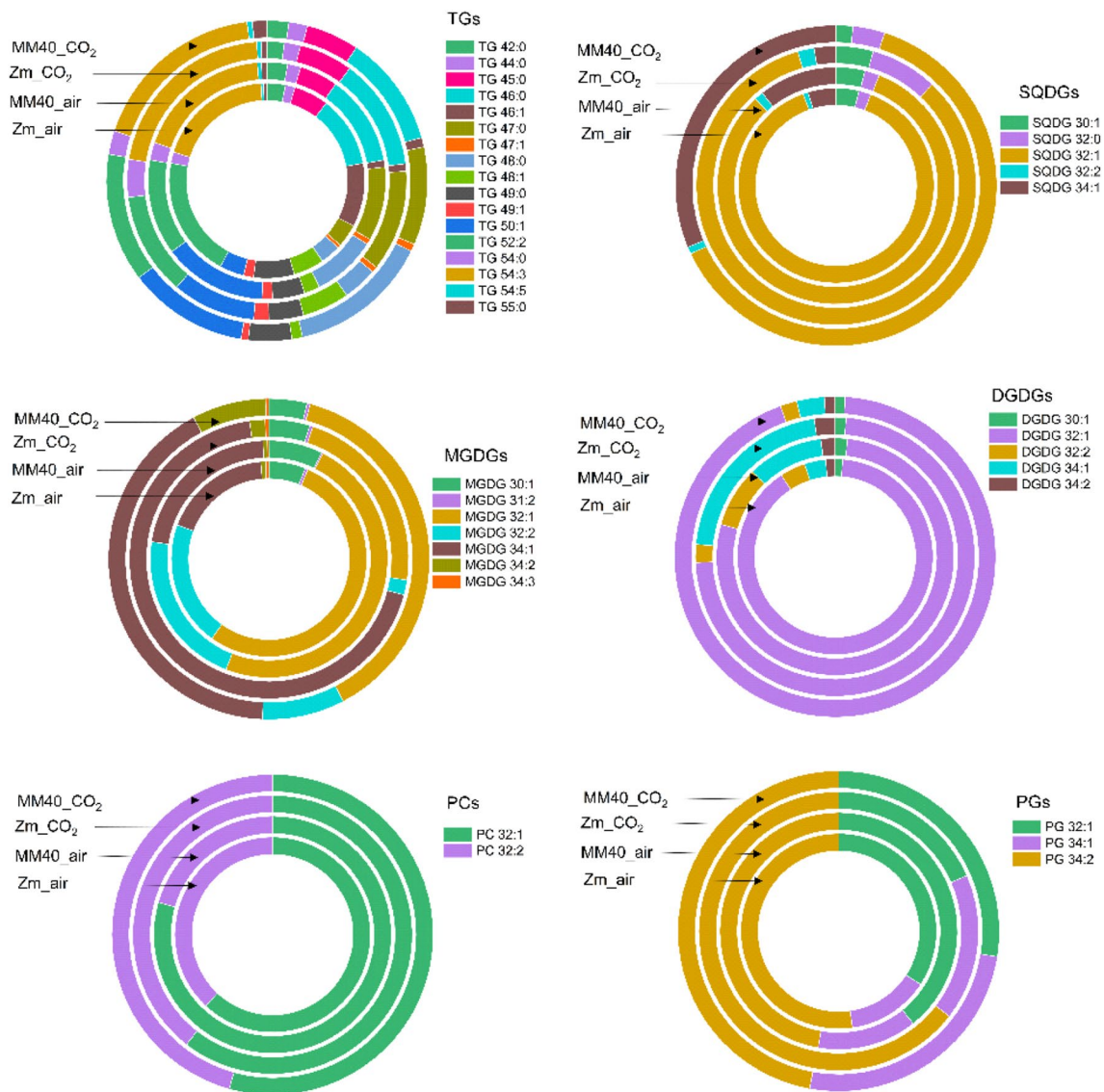
Fig. 8 Effect of experimental conditions on the relative abundance of each detected lipid in their corresponding class. MM: Martian medium, TG: triacylglycerol, SQDG: sulfoquinovosyldiacylglycerol, MGDG: monogalactosyldiacylglycerol, DGDG: digalactosyldiacylglycerol, PC: phosphatidylcholine, PG phosphatidylglycerol

qualitative effect of CO_2 was observed on total PUFAs, even if in this case no relevant effect could be observed because of the change of growth medium.

3.6 Discussion of main results

The possibility of growing and using *S. nidulans* during a crewed mission on Mars was investigated. The cyanobacterium was capable to grow in media containing MM per cent volumes equal to 40%vol and managed to survive in an atmosphere obtained by continuously flowing in pure CO_2 which led the pH to very low values. In view of reducing the nutrient requirements from Earth, the use of 40% v/v of MM

appears to be an optimal solution since the payload might be significantly reduced with respect to the case where the medium should be entirely brought from Earth.

The chemical composition of the resulting biomass mainly consists of proteins (approximately 40%), with carbohydrates and lipids in the ranges 15–20% and 7–8% respectively. This composition, which could be favourable for producing high-value products for astronauts, remained largely unchanged when MM was incorporated into the growth medium. Under CO_2, a decrease in proteins and carbohydrates and a corresponding increase in lipids were registered. It is known from the literature that lipid content, in microalgae and cyanobacteria, can increase when the cells
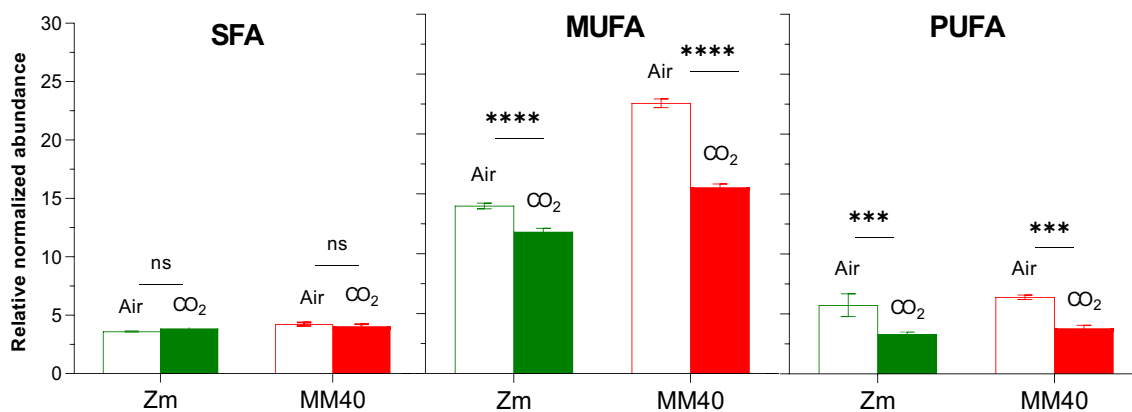
Fig. 9 Fatty acids levels found in complex lipids of *Synechococcus nidulans* biomass grown in different percentage of MM and under CO_2 ($n = 3$). MM: Martian medium, SFA: saturated fatty acid, MUFA: monounsaturated fatty acid, PUFA: polyunsaturated fatty acid, ns: not significant. (***p value < 0.001; ****p value < 0.0001)

are stressed by hostile conditions like nutrient starvation, salinity, pH, temperature, and light [55]. A possible explanation for enhanced lipid yield is that, under stressful conditions all CO_2 fixed by photosynthesis is channelled to lipid biosynthesis, for energy storage functions, rather than being used for the physiological metabolism of algae [55–57]. Some scientific reports have demonstrated that the increase in CO_2 concentration can lead to N_2 constraint and reroute carbohydrates to lipids biosynthesis. This is because lipid biosynthesis requires less N_2 compared to protein synthesis. N_2 starvation might cause an increase in the intracellular content of fatty acid acyl-CoA that activates diacylglycerol acyl transferase (DGAT) which in turn can convert fatty acid acyl-CoA to TAGs [57, 58].

The increase in antioxidant power using MM40 can be caused by the stress due to the relatively high salinity and heavy metal concentration of the medium which may have up regulated the biosynthesis of antioxidant compounds. Indeed, it is well known that microalgal cells synthetize antioxidant molecules when subjected to stress conditions [59]. While it is not possible to explain the exact biochemical mechanism underlying this phenomenon, our results clearly indicate that the use of a medium (MM) simulating that one obtainable from resources on Mars leads to the production of biomass with a higher antioxidant power. In cyanobacteria, respiration and photosynthesis are sources of ROS. When the equilibrium between the amount of oxidants and the production of antioxidants is disrupted, the organism experiences oxidative stress, which can result in cellular damage and potential cell death [54, 60]. The decrease in antioxidant activity under a continuous flux of CO_2 can determine a possible reduction in ROS production. This can be probably caused by the partial inhibition of photosynthetic activity, determined by the low pHs, which in turn leads to a reduction in dissolved oxygen available in solution for

the production of ROS. The decrease in antioxidant activity also suggests a possible decrease in lipid peroxidation, which is a process that involves the oxidative degradation of lipids in cell membranes. Nonetheless, despite the reduction in antioxidant power and, consequently, in the content of compounds with antioxidant properties, this result suggests the production of biomass with higher nutritional quality and increased safety. In fact, lipid peroxidation is not only responsible for cellular damage, but also reduces the quality of the oil produced from the biomass itself, as it is responsible for the so-called rancidity.

The application of CO_2 resulted in a decline in pigment production compared to the air content. It is documented in the literature that high levels of CO_2 can alter the production of pigments because in such condition, these pigments are not crucial for light absorption. Indeed, Gordillo et al. [61] proposed that in *S. platensis*, CO_2 could induce the degradation of pigments synthesized in excess, as they are not essential for light harvesting. As a result, the organism can downregulate the synthesis of such pigments and allocate resources to other metabolic functions, for example lipids biosynthesis. This reduction in pigment content might coincide with a decrease in photosynthetic efficiency, associated with a decline in Rubisco content and carbonic anhydrase activity, as demonstrated by García-Sánchez et al. [62] in their experiments with *Gracilaria tenuistipitata*. In such context, if the cultures have reduced levels of pigments, their ability to capture light is further compromised. Consequently, as observed in this work, CO_2-enriched cultures may reach the stationary phase earlier than cultures in air which have a higher pigment content and thus can better perform photosynthesis [47, 63].

TAGs do not have a structural function in cyanobacteria, microalgae, and higher plants. Fatty acids, which are synthetized in the chloroplast, act as the fundamental

components for TAGs and other lipids. In challenging environmental or stressful growth conditions, cyanobacteria can alter their lipogenesis with the formation and accumulation of neutral lipids, mainly in the form of TAG [64]. All glycolipids in cyanobacteria are derived from diacylglycerols (DAGs), which are the precursors of TAGs [65]. In our case, the DAGs are likely re-routed to the synthesis of glycolipids. Indeed, we detected a reduction in TAG synthesis and an increase in glycolipids synthesis, but this aspect needs to be further investigated. The glycosyl glycerides SQDG, MGDG and DGDG together with PG are contained in the thylakoids, wherein photosynthesis takes place. MGDGs are functionally more tightly associated with the core complexes of photosystems (PSI) and with the ATPase complex of chloroplasts [66, 67]. Their up-regulation occurred mainly when using MM40 and, in a lower extent due to CO_2, can indicate clearly a modified activity or structure around PSI to respond to high CO_2 levels. The accumulation of MGDGs can indicate a modification in the abundance of hexagonal structures within the lipid matrix of thylakoid membranes. These hexagonal structures can serve to stabilize membrane proteins under stressful conditions [53]. DGDGs were localized mainly in the reaction centre of PSII [66, 68]. Our results seem to demonstrate that the natural conversion of MGDG to DGDG improves under stress conditions. In fact, accumulation of DGDG, especially DGDG 32:1 (16:0/16:1), is mainly observed in samples grown in MM40.

A similar process involved phosphatidylethanolamines (PEs) and PC. Phosphatidylethanolamine, similar to MGDG, tends to form nonlamellar lipid structures. On the other hand, PC, similarly to DGDG, prefers the formation of lamellar phase structures [53]. However, we do not identify PE or significant changes in PC levels. PG is functionally linked to the core complexes of both PSI and PSII [69]. The increase in these PG and in particular those containing fatty acids 16:1 (in our case PG 32:1, but the difference was not statistically significative) can be correlated to a stabilization of PSII [69]. SQDG are lipid molecules carrying negative charges as the PG. They are found throughout the membranes of plant chloroplasts and cyanobacteria. SQDG primarily have a structural and functional role in PSI and PSII [70]. Their increment, observed when using especially MM40 and CO_2, could represent a compensatory mechanism in response to the increase in longer and unsaturated fatty acid contents. The synthesis of a stabilizing lipid species (SQDG) is a mechanism that could be implemented to maintain membrane integrity when cells experience stressful environmental conditions, such as high concentrations of CO_2 [71]. In particular, the increase in SQDG 32:1 (16:0/16:1) appears to be in line with this mechanism. However, further investigation is necessary to confirm this physiological aspect.

The increasing market trend and the growing demand from consumers for healthy and natural products have bolstered the prominence of microalgae and cyanobacteria as a rising source of nutrient-dense natural food supplements. Therefore, these physiological aspects are most important to understand the process of lipid biosynthesis. Moreover, it can be essential for the engineering of microalgae and cyanobacteria to improve lipid production in view of food production on Mars. Many studies have indicated that galactolipids obtained from plants, cyanobacteria, and green algae showed various biological properties in vitro and/or in vivo. These properties encompass inhibitory effects against tumour growth mitigation of inflammatory responses, and antiviral capabilities [72]. For example, MGDG 36:6 and MGDG 34:3 (that we have determined in *S. nidulans* as 16:0/18:3) isolated from fresh spinach demonstrated an inhibitory activity on Epstein-Bar virus [73]. Hence, these lipids have an important nutritional power and are carriers of important fatty acids. This aspect can become of great relevance when applied in harsh conditions, as Mars environment, wherein it is practically impossible to have access to sources of fresh food.

4 Conclusion

The possibility of growing *S. nidulans* CCALA 188 using local resources on Mars was investigated. The cyanobacterium was able to grow and to adapt to harsh environmental conditions modifying his cellular physiology and improving lipid biosynthesis. These findings, coupled with the significant presence of beneficial pigments like carotenoids, the nutritional benefits of galactolipids and phospholipids, and the strong antioxidant properties, make this strain a promising one for supplying astronauts with high-value products during long missions on Mars, where self-sufficiency relies on utilizing local resources.

Considering such outcomes, the proposed cultivation system for Mars could be relatively straightforward and require reduced payloads. Although further research is required to validate these observations, these initial findings suggest that the envisioned approach holds significant promise in meeting the nutritional requirements of astronauts on Mars. Optimizing food production is imperative for promoting missions in deep space or on Mars where the cost of Earth-supplied provisions is unsustainable. To achieve this goal in space, several key initiatives are essential. First and foremost, it is crucial to define the scale of the microorganism growth compartment. This involves improving our understanding of different species, elucidating the effects of cultivation and space-related factors on microorganism and plant productivity and quality, and refining ISRU techniques to utilize regolith as a viable plant substrate. Furthermore,

there is a pressing need to engineer microorganisms adapted to the Martian atmosphere, capable of utilizing CO_2 and N_2. Investigating the physiological effects and processes in microgravity is imperative, as is the selection of suitable species and the development of protocols for food processing. Cultivating microorganisms to obtain bioactive value products holds promise, not only for fostering higher plant cultivation but also for enhancing the overall quality of food for astronauts.

Declarations

Conflict of interest The authors declare no conflict of interest.

Ethical approval and informed consent Neither ethical approval nor informed consent was required for this study.

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