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In-situ resource utilization to produce *Haematococcus pluvialis* biomass in simulated Martian environment

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ABSTRACT

The production of food or nutraceutical compounds using in-situ available resources is one the most relevant challenges in view of the realization of crewed mission to Mars. The possibility to grow *Haematococcus pluvialis* CCALA 1081 in a medium obtained from Mars regolith and astronauts' urine simulants under an atmosphere of CO_2 mimicking the Martian one after pressurization, is investigated in this work. The experimental results showed that *H. pluvialis* could be grown using only such resources thus theoretically reducing to zero the payload related to fertilizers to bring from Earth. Total lipid content increased under Mars simulated conditions. Metabolomics showed that some steps of the tricarboxylic acid cycle were upregulated under Mars simulated atmosphere. Lipidomics revealed that triglycerides and diglycerides were overexpressed when cultivation was carried out under Mars simulated conditions. Accordingly, the growth of *H. pluvialis* on Mars seems not only feasible but also capable to improve the nutritional profile of the biomass.

1. Introduction

The colonization of Mars represents an epochal challenge, offering not only the prospect of a solution to crucial terrestrial issues such as resource depletion and overpopulation but also envisioning a future of human multiplanetary existence [1]. However, missions to Mars are today hardly achievable due to their high dependency on the volume and mass of embedded resources (payloads) that would require relevant economic investments. Furthermore, re-supply of resources from Earth is impractical because a single expedition to Mars can last from six to nine months and travel opportunities are limited by the launch window [2,3]. Accordingly, a future Mars colony should become self-sustaining in terms of air, water, and food production. This goal could be achieved using the resources available on Mars along with the biological waste generated by astronauts according to a paradigm well known under the acronym ISRU (In situ resource utilization). The ISRU approach emerges as a key strategy to ensure the sustainability of the Mars colony, maximizing the utilization of available resources on the red planet. Mars' atmosphere is rich in carbon dioxide (CO_2) which can be converted into useful organic products by photosynthetic microorganisms such as microalgae and cyanobacteria [4,5]. Moreover, the latter ones may grow by exploiting mixtures of in-situ available resources such as astronaut's urine and Mars regolith leachate to produce potentially edible biomass and nutraceuticals for crew members [6,7]. However, only few works investigated the possibility of using microalgae or cyanobacteria to convert in-situ available resources into useful products on Mars. On the contrary, research has been conducted on the feasibility of cultivating microorganisms in a simulated Martian regolith [6].

In particular, the ability to grow cyanobacteria species (*Anabaena cylindrica*, *Nostoc muscorum*, and *Arthrospira platensis*) and a green microalga (*Chlorella vulgaris*) using Mars global simulant (MGS-1) under an Earth-like atmosphere, was investigated by Macário et al. who reported that not all the tested species were able to grow by exploiting the Mars regolith extract [8]. They explained that *A. platensis* and *C. vulgaris* were unable to grow while *N. muscorum* and *A. cylindrica* prospered in a regolith leachate. Another recent work investigated the diazotrophic growth of cyanobacterium *Anabaena* sp. PCC 7938 under an artificial gas mixture consisting of 96 % N_2 and 4 % CO_2, resembling Martian

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conditions [9]. In this work *Anabaena* sp. was able to grow under controlled atmosphere and in a medium consisting of BG-11 and MGS-1 simulant of Mars regolith [9]. Billi et al. [10], showed also that lunar and Martian regolith simulants supplemented with a nitrogen source can support planktonic and biofilm growth of the cyanobacterium *Chroococcidiopsis* sp. 029. Recently, the possibility to cultivate *A. platensis* to produce food for astronauts in the context of a manned mission on Mars was investigated [6]. The experimental results clearly demonstrated that *A. platensis* could grow under simulated microgravity in a mixture containing up to 40 % (*v/v*) of a medium obtained by mixing Martian regolith leachate (JSC MARS-1) with synthetic human urine (MP-AU) in a CO_2 atmosphere resembling a compressed Mars atmosphere. While these results represent a relevant advancement in view of producing microalgae on Mars, at most 40%vol of the Zarrouk's medium could be replaced by a medium produced from in-situ resources, i.e. regolith and astronaut's urine. As a result, the remaining part of the growth medium (60 % *v/v*) consisted of Zarrouk medium whose production involves the use of synthetic compounds that should be brought from the Earth. A thorough examination of the long-term sustainability of Mars missions is of paramount importance, especially in the perspective of reducing payload requirements and influencing the techno-economic feasibility of long-duration space missions.

In the present study, we investigated, the growth of *Haematococcus pluvialis* CCALA 1081 in a medium produced by using simulant of Martian resources represented by Martian regolith, urine of astronauts and pressurized CO_2. Such cultivation conditions are investigated firstly to verify the feasibility of the IRSU process reported in Fig. 1 which represents a slight modification of the one envisioned by Cao et al. [11]. The process should take place within pressurized domes where photobioreactors (PBR) should produce microalgal biomass meant to supply food or nutraceuticals for astronauts. The medium would be produced from regolith and human urine while the gas phase would consist of pure pressurized CO_2 obtained from Mars atmosphere, pulsed at specific time intervals within the PBR.

The selection of *Haematococcus pluvialis* CCALA 1081 as a candidate strain is particularly significant due to its remarkable ability to produce high levels of high-value compounds like carotenoids, including astaxanthin (3,3-dihydroxy-β-carotene-4,4-dione), recognized as a "super antioxidant." These compounds are crucial for protecting the crew from oxidative stress and extreme environmental conditions on Mars [12–14]. Antioxidants like the latter one have been recommended to protect crew members and minimize the effects of oxidative stress caused by high levels of radiation and stress conditions on Mars [6,15–18]. Carotenoids are also essential for human vision. For instance, β-carotene is a precursor of retinal constituent of vitamin A while zeaxanthin and lutein protect eyes from high light intensity [19,20]. Consequently, the integration of these compounds into the diet of astronauts is essential because the human body is unable to produce most of these photoprotective compounds [19].

Other important bioactive compounds that could be obtained from *H. pluvialis* are lipids which can serve as a source of essential fatty acids, such as ω-3, that are essential precursors of anti-inflammatory

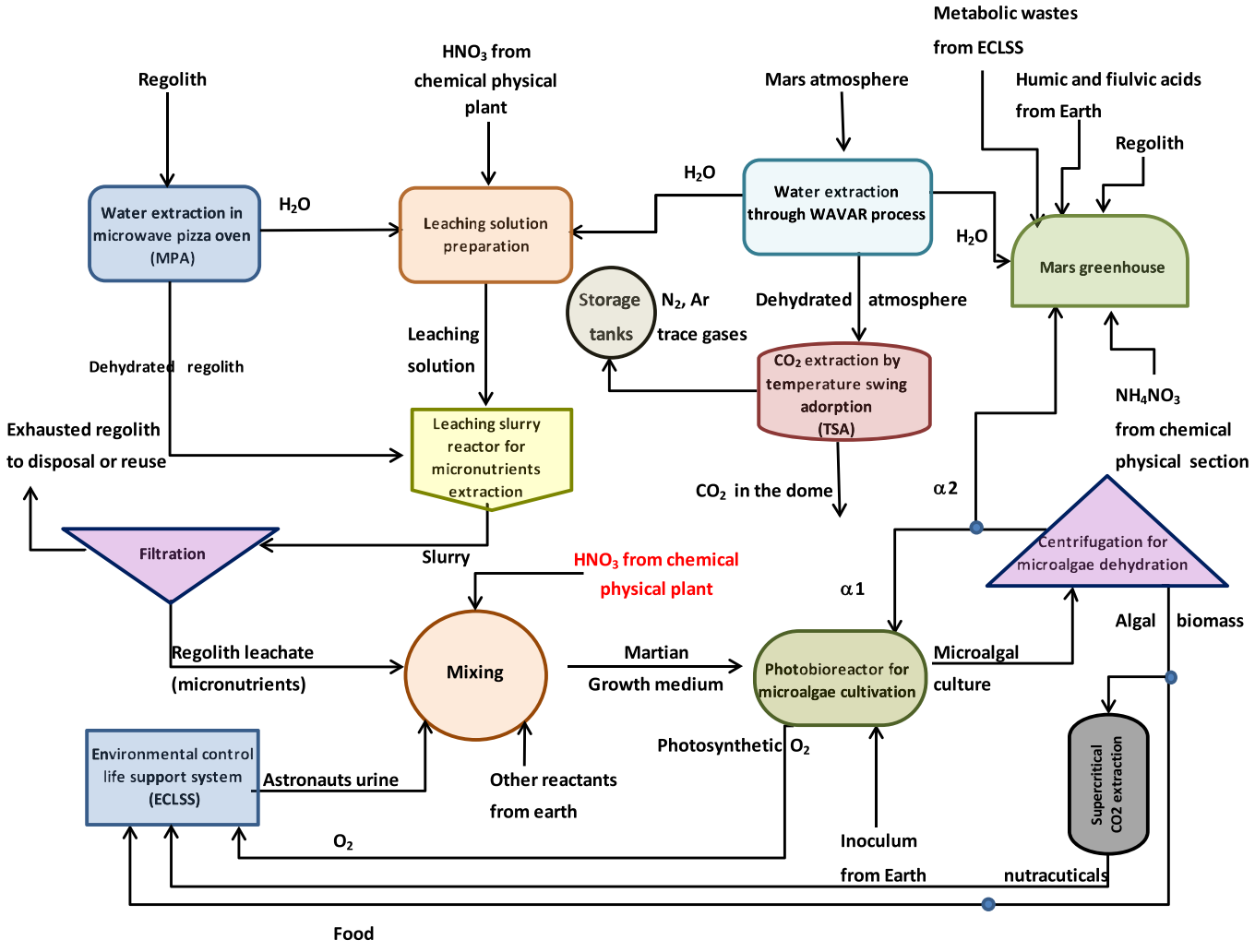

Fig. 1. Modified scheme of the biological section of the ISRU process patented by Cao et al., (2021). Adapted from Concas et al., (2023).

eicosanoids. Moreover, polar lipids in *H. pluvialis*, such as glycolipids have shown chemotherapeutic potential, anti-proliferative effects, as well as the capability to inhibit nitric oxide (NO) release, and anti-inflammatory activity [21,22]. Lipids and carotenoids might be extracted from the biomass using the available CO_2 on Mars, by employing supercritical fluid extraction technologies (SCFE) (cf. Fig. 1). The use of SCFE technologies, already widely employed for obtaining high-value molecules from microalgal biomass on Earth [23], reveals a promising approach for extracting lipids and carotenoids from microalgae biomass directly on Mars. Furthermore, the entire biomass of *H. pluvialis* could be theoretically used as food for crew members during missions on Mars. In fact, the European Food Safety Authority (EFSA) have assigned to *H. pluvialis* the qualified presumption of safety (QPS) with the qualification "for production purpose only" [24]. Additionally, it is worth mentioning that UK Food Standards Agency (FSA) has approved the status of "novel food" for supercritical CO_2 extracts from *H. pluvialis* [13,25,26]. Simultaneously, the US Food and Drug Administration (USFDA) has granted "GRAS" (Generally Recognized As Safe) status for astaxanthin obtained from *H. pluvialis* [13,25,26].

Considering the high value of *H. pluvialis* biomass, in this work we have used an untargeted metabolomic and lipidomic approach to study the effect of the different growth conditions taking place when implementing the process of Fig. 1 on Mars, on the microalgal physiology and biochemical composition. The final goal is to understand whether the *H. pluvialis* biomass produced on Mars using only in-situ available resources could be used as a source of food or supplements for the members of the crew.

2. Materials and methods

2.1. Microorganism growth

Haematococcus pluvialis CCALA 1081 was obtained from the Culture collection of autotrophic organisms (CCALA), Třeboň, Czech Republic. Microalgae were maintained under axenic conditions at the laboratory of the Interdepartmental Center of Environmental Sciences and Engineering (CINSA), University of Cagliari, Sardinia, Italy. The strain was cultured in 5 L laboratory bottles (DWK Life Sciences, Germany) containing 4 L of modified *Z*-medium (ZM), stoppered with a vented cotton plug and covered with aluminum foil. The modification of ZM consisted solely in the increase to 10 g/ L of Na_2CO_3 concentration with respect to the one available in the literature [7]. Prior to inoculation, the culture media and the bottles were sterilized at 121 °C and 1 atm for 20 min. The culture was maintained under photoautotrophic conditions in a chamber at a controlled temperature of 20 ± 1 °C. The photoperiod was fixed at 12:12 h light and dark periods with white light illumination of 50 μmol/m^2/s (Light meter Delta OHM, GHM group, Germany). Shaking was adjusted to 70 rpm.

2.2. Preparation of Martian medium (MM)

The Martian medium (MM) was prepared mixing a leachate of Martian regolith simulant (JSC MARS-1) and synthetic human urine (MP-AU) according to the procedure proposed by Fais et al., [6]. Briefly, the regolith leachate (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 slurry was stirred for 24 h at 25 °C within a 1 L Erlenmeyer flask with a cap and then filtered with bibulous paper. The main constituents of JSC MARS-1 are reported in Table S1. Synthetic human urine (MP-AU) was produced according to a methodology proposed in the literature [27] and then diluted with ultrapure water at a ratio of 1:10 *v*/v. Finally, the leachate of Martian regolith and diluted urine were mixed (1:1 *v/v*) to produce MM. The detailed chemical composition of MM is reported in Table 1. It should be noted that the high content of Ti in the MM was leached from mineral phases of regolith other than TiO_2 [6].

Table 1
Concentration of macro-nutrients and metals in the Martian Medium (MM).

Macronutrients	(g/L)	Micronutrients	(mg/L)
Na_2SO4	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		

2.3. Growth experiments

Growth experiments were performed using media consisting of mixtures of MM and ZM at different levels of MM (0, 20, 40, 60 and 100%vol) and a gas phase consisting of pure air or CO_2 at 1.2 atm for 12 h during the light illumination phase. Such operating mode was adopted to simulate the ISRU process shown in Fig. 1. The culture was grown in batch within 50 mL transparent vented cap flasks filled up to 35 mL. The optical density (OD) at the beginning of the experiments was 0.1 at 750 nm. The strain was phototrophically cultivated at 20 ± 1 °C under 12:12 h light-dark illumination of 80 μmol m^{-2} s^{-1} white light and continuous agitation at 70 rpm (Stuart SSM1, Italy). Experiments were performed at least in triplicate. It is important to note that the culture was not acclimatized to pressurized gas chamber conditions mimicking Mars conditions before testing it in MM culture medium. Microalgae growth was monitored for 20 days measuring spectrophotometric absorbance (Genesys 20, Thermo Scientific, Walthmanm Waltham, USA) of the optical density (OD) produced from the cultures at 750 nm. The biomass concentration was evaluated in g L^{-1} from OD measurements at 750 nm using a suitable calibration line (Fig. S2). MM and working solutions were sterilized at 121 °C for 21 min prior to use.

2.4. Biomass characterization

2.4.1. Chemicals

Analytical LC grade isopropanol, methanol, acetonitrile, acetic acid, formic acid, ammonium formate, ammonium acetate, were purchased from Sigma Aldrich (Milano, Italy). Sulfuric 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 (Val de Reuil Cedex, France). Sodium carbonate and Folin-Ciocalteau reagent were acquired by Sigma-Aldrich Inc. (St. Louis, MO, USA). Glucose, bovine serum albumin and vanillin standards were acquired by Sigma-Aldrich (Merck Kgaa, Darmstadt, Germany). Bi-distilled water was obtained by a MilliQ purification system (Millipore, Milan, Italy). A SPLASH® LIPIDOMIX® standard lipid component mixture was purchased from Sigma Aldrich (Milan, Italy). HNO_3 67-(69 %), and standards stock solution (~1000 mg/ L) of Al, As, Ba, Be, Ca, Cd, Co, Cr, Cu, Fe, K, Hg, Li, Mg, Mn, Mo, Na, Ni, Pb, Sn, Ti, and Zn were of ICP grade (Carlo Erba Reagents Milan, Italy).

2.4.2. Sample preparation

Thirty-five mL of *H. pluvialis* culture 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 three times. The cell pellet was frozen at −80 °C, lyophilized with LIO-5PDGT freeze-dryer (5 Pascal, Milano, Italy) and finely pulverized with a mortar and pestle. The dried powder was left in the dark before analysis.

2.4.3. Determination of macronutrients: total carbohydrates, lipids, and soluble proteins

Carbohydrates and soluble proteins were extracted using 5 mL of phosphate-buffered saline (PBS) (20 mM, pH 7.4) through ultrasonication with ExctractorOne (GM Solution, Cagliari, Italy). Carbohydrate levels were determined following a modified method by Dubois et al. [28], involving the addition of phenol and concentrated sulfuric acid, followed by spectrophotometric analysis at 490 nm with glucose as a reference standard. Results were reported as g/100 g of glucose. Total soluble protein content was measured using the Lowry et al. method [29], with bovine serum albumin (BSA) as a reference standard. Results were reported as g/100 g of BSA. Total lipids were determined using a method by Chen et al. [30], involving sonication, heating, and extraction with a methanol/chloroform solution. Lipids were extracted using the method proposed by Bligh and Dyer [31] and their quantification was performed by the colorimetric reaction proposed by Mishra et al. [32]. Results were expressed as g/100 g. All analyses were conducted in triplicate, and results are presented as mean ± standard deviation.

2.4.4. ICP-OES trace elements analysis

The samples were prepared as follows: 10 mg of lyophilized biomass were extracted using an aqueous solution of HNO_3 at 1 % and sonicated for three time using a sonicator (GM solutions). Then the samples were filtered with a 0.45 μm nylon filters. Agilent 5100 Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES, Agilent Technologies, Santa Clara, USA) was used to determine trace metals in lyophilized samples. The working conditions were set at 1200 W for radio frequency (RF), 0.7 L min^{-1} nebulizer flow, 1.00 L min^{-1} auxiliary flow, 12.0 L min^{-1} plasma flow, 12 rpm pump speed, axial plasma viewing, and read time 5 s. Control solvent samples were simultaneously prepared to avoid false positives and contamination during analysis. ICP multi-element intermediate standard solution was prepared at 2 mg L^{-1} in H_2O at 1 % HNO_3. The working solutions were prepared daily by diluting the multi-element intermediate standard solutions in H_2O at 1 % HNO_3. Calibration curve was built with five points and were considered acceptable when r2 ≥ 0.997. Data are expressed as mg/kg or g/kg of dried biomass (mean ± SD).

2.4.5. Sample preparation for metabolomics analysis

Lyophilised microalgae samples (n = 30) were extracted using a slight modification of the method by Folch et al. [33]. Briefly, 10 mg of each lyophilised biomass sample was transferred to a centrifuge tube and 5 mL of bi-distilled water was added. Solutions were then ultrasonicated for 2 min for three time using ExctractorOne. 1 mL of this solution was transferred into 15 mL centrifuge tube and 125 μL of chloroform and 250 μL of methanol were added. Then 10 μL of the internal standards, and 10 μL of a solution of Succinic acid-2,2,3,3-d_4 at 500 mg/L (to obtain a final concentration of 50 mg/L) were added. Solutions were vortexed every 15 min for 1 h. After 1 h, 380 μL of chloroform and 90 μL of aqueous 0.2 M potassium chloride were added. The suspension was centrifuged at 24104 rcf for 10 min. After centrifugation, 1 mL of the polar phase and 400 μL lipophilic layer were transferred into distinct glass vial and dried with a gentle nitrogen stream.

2.4.6. Untargeted GC/MS analysis

The aqueous phase obtained from the Folch extraction was derivatized with 50 μL of pyridine containing methoxamine hydrochloride at 10 mg/mL. After 17 h, 50 μL of *N*-methyl-N-(trimethylsilyl) trifluoroacetamide was added and the samples were heated at 70 °C for 1 h. One microliter of derivatized samples was injected splitless into a 6850-gas chromatograph coupled with a 5973 mass spectrometer (Agilent Technologies Inc., Santa Clara, CA). The injector temperature was 200 °C. The gas flow rate through the column was 1 mL min^{-1}. The fused silica capillary column was a DB5-MS column (30 m × 0.25 mm i.d.; J&W Scientific Inc., Folsom, CA). The initial temperature program was as follows: 3 min of isothermal heating at 50 °C, which was then increased to 250 at 3 °C/min and held at 250 °C for 25 min. The transfer line and the ion source temperatures were 280 and 180 °C, respectively. Ions have been generated at 70 eV with electron ionization and were recorded at 1.6 scans/s over the mass range m/z 50 to 550. The GC–MS data analysis was conducted by integrating each resolved chromatogram peak. Annotation of metabolites was performed using the standard NIST08 mass spectra library (http://www.nist.gov/srd/mslist.cfm, Max Planck Institute of Golm, Germany), Human metabolome database (HMDB) and, when available, by comparison with authentic standards.

2.4.7. Untargeted UHPLC-QTOF-MS/MS analysis

The organic phase obtained from the Folch extraction was dissolved in 20 μL of a mixture of methanol/chloroform (1:1 *v/v*) and diluted with 380 μL of a mixture of 2-propanol/acetonitrile/water (2:1:1 *v/v/v*) containing the internal standard PE 15:0–18:1 (d7). Then, the samples were analysed with a UHPLC-QTOF/MS coupled with an Agilent 1290 Infinity II LC system, injecting 5 and 8 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, Palo Alto, CA). The column was maintained at 50 °C at a flow rate of 0.2 mL min^{-1}. The mobile phase for positive ionization mode consisted of (A) 10 mM ammonium formate solution in 60 % milli-Q water and 40 % acetonitrile and (B) 10 mM ammonium formate solution containing isopropanol/acetonitrile (9:1 *v/v*). In the 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 source was operated with the following parameters: gas temperature, 200 °C; gas flow (nitrogen) 10 L min^{-1}; nebulizer gas (nitrogen), 50 psig; sheath gas temperature, 300 °C; sheath gas flow, 12 L min^{-1}; 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. Chromatographic separation of lipids was obtained with a Kinetex 5 μm EVO C18 100 A, 150 mm × 2.1 μm column (Agilent Technologies, Palo Alto, CA). The column was maintained at 50 °C at a flow rate of 0.2 mL min^{-1}. 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 LC-MS iterative method consists in injecting the same sample multiple times, while precursors previously selected for MS/MS fragmentation are excluded on a rolling basis. Five different iterative analyses were performed for maximizing the maximum number of lipid species detected and then all samples were acquired in ESI full scan mode. For all lipid classes, MS/MS spectra were analysed to further confirm the annotation using the pattern of fragmentation [34–37].

2.4.8. GC–MS fatty acids methyl ester analysis

Fatty acid methyl ester (FAME) analysis was conducted following the method proposed by G. Breuer et al., [38], with minor adjustments. In brief, 10 mg of lyophilized biomass were weighed into glass tubes. Approximately 150 mg of 1–1.3 mm glass balls were added, and the biomass was suspended in a 1 mL mixture of methanol/chloroform (4:5 *v*/v), including the internal standard tritridecanoin (TAG 39:0, 13:0/13:0/13:0) at a concentration of 50 mg/L. The samples underwent vortexing eight times for 60 s and subsequent sonication in an ultrasonic bath for 15 min at 5 °C. The solutions were transferred to glass centrifuge tubes, and the previous tubes were washed three times, each time with 1 mL of a chloroform: methanol mixture containing the internal standard. The solutions were vortexed for 10 s and then sonicated for 15 min at 5 °C. Next, 2.5 mL of MilliQ water containing 50 mM of 2-Amino-

2-hydroxymethyl-propane-1,3-diol (Tris) and 1 M NaCl, were added. The solutions were sonicated for 10 min at 5 °C. After centrifugation for 10 min at 177 rcf at 5 °C, the chloroform phase was transferred to glass tubes. The old samples were re-extracted using 1 mL of chloroform and sonicated for 10 min at 5 °C. After centrifugation, the chloroform phase was collected and combined with the previous one, then dried with a gentle nitrogen stream. Fatty acids were trans-esterified to obtain FAMEs by adding 3 mL of methanol containing 5 % (*v*/v) sulfuric acid to the tube containing dried lipids and incubating the samples for 3 h at 70 °C. After cooling, 3 mL of MilliQ and 3 mL of n-hexane were added. The samples were vortexed for 1 min and then centrifuged for 10 min at 177 rcf at 5 °C. 2 mL of the hexane phase was collected from each sample and washed with 2 mL of MilliQ twice. The hexane phase containing FAMEs was transferred to glass vials for GC–MS analysis. A gas chromatography trace 1300 equipped with a triple quadrupole mass spectrometry (TSQ 9000), a fused capillary column Agilent HP-5 (30 m × 0.25 i.d, 0.25 μm f.t.), an automatic sampler (AI 1310) with a split-splitless injector was used. The injector was set at 250 °C, and the carrier gas (helium) flow was 1.5 mL min^{-1}. The oven temperature program involved several steps, and the sample was injected in split mode with a split ratio of 1:20. Mass spectrometry parameters included a transfer line temperature and ion source set at 250 and 300 °C, respectively. Ions were generated at 70 eV with electron ionization and recorded over the mass range *m/z* 50 to 550 at 1.5 scans/s. Peak identification was achieved by comparing peak retention time with Supelco 37 component FAME Mix (Sigma Aldrich). Data are expressed as mg/g of dry weight (mean ± standard deviation) and calculated using the equation proposed by Breuer et al. [38].

$$FA\left(\frac{mg}{g}\right) = IS\ added * \frac{\frac{(Area\ of\ individual\ FAME)}{(Area\ of\ C13{:}0\ FAME * Rel.Resp.Factor\ individual\ FAME)}}{g\ of\ biomass\ added}$$

2.4.9. Statistical data analysis

Uni-variate analysis was performed with GraphPad Prism software (version 8.3.0, Dotmatics, Boston, Massachusetts). Mean differences between groups were tested for statistical significance using the Student's *t*-tests. The multivariate statistical analysis (MVA) was performed with the SIMCA-P+ program (Version 14.1, Umetrics, Sartorius, Germany). MVA refers to those statistical techniques that simultaneously analyse multiple measurements on samples under investigation. In this work, different tools were used: a) the unsupervised Principal Component Analysis (PCA) for data set overview, where results are shown in two dimensions as score (related to observations) and loading (related to variables) scatter plots; b) the supervised Partial Least Squares-Discriminant Analysis (PLS-DA) to assess class belonging of samples and its orthogonal variant (OPLS-DA) to find discriminant metabolites. Variables were mean centered and unit variance scaled column wise. The quality of the models and the optimum number of principal components were evaluated based on the cumulative parameters R^2X and R^2Y (goodness of fit) and their analogues in cross validation Q^2X and Q^2Y (goodness of prediction) and tested for overfitting using a Y-table permutation test ($n = 400$). The variable influence on projection (VIP) scores in the OPLS-DA predictive component describe the metabolite influence on sample classification; only variables having VIP score > 1 was taken into consideration and annotated [39]. To perform these statistical investigations the relative intensity of each metabolite peak is expressed as the ratio of the peak area to that of the internal standard and normalized peak areas were imported into the software.

3. Results and discussion

The experiments were conducted to verify whether the microalga *H. pluvialis* CCALA 1081 could thrive utilizing synthetic Martian regolith and astronaut's simulated urine under an atmosphere consisting of 100 % pressurized CO_2 which mimics the one obtainable from Mars environment through the process shown in Fig. 1. An additional objective of this study was to explore whether these challenging conditions might induce alterations in the production of macronutrients, such as carbohydrates, lipids, and proteins, by the microalgae, thereby potentially influencing the feasibility of utilizing microalgal biomass as a source of nutraceuticals for astronauts during their space missions. In this framework, two groups of experiments were performed. The first one involved a gas phase consisting of air at 1 atm while the second group was performed using a gas phase obtained by pulsing pure CO_2 every 24 h in the chamber to simulate the operating conditions within the pressurized dome on Mars proposed by Cao et al., [7]. Each group of experiments was carried out by using different growth media consisting of different levels of MM, i.e. 0, 20, 40, 60, and 100 % *v*/v. Levels of total carbohydrates, lipids, proteins, and pigments were also determined while the effects on microalgae metabolism and lipid composition were explored using metabolomics and lipidomics approaches, respectively. Metals, and heavy metals content, were also studied to investigate the safety of biomass. Hereby, the samples will be denoted by the acronym MM (Martian medium) followed by the volume percentage of ZM replaced with MM, and further followed by the letter A or C to signify the use of air or CO_2, atmosphere, respectively.

3.1. Growth kinetics and pH monitoring

The time evolution of the growth of *H. pluvialis* is shown in Fig. 2, where the average values of biomass levels were 0.042 ± 0.005 g/L and 0.034 ± 0.003 g/L under air and CO_2, respectively. After 20 days, the average concentration was 0.61 ± 0.07 g/L and 1.03 ± 0.04 g/L under air and CO_2, respectively. Compared to air, *H. pluvialis* cultures grew better under 100 % CO_2 providing a higher biomass concentration for all the tested percentages of MM ($p < 0.0001$). No statistical differences were observed when ZM was replaced by different percentages of MM. Therefore, it can be assumed that the limiting nutrient that controls the growth in ZM was the same of MM. For this reason, the replacement of

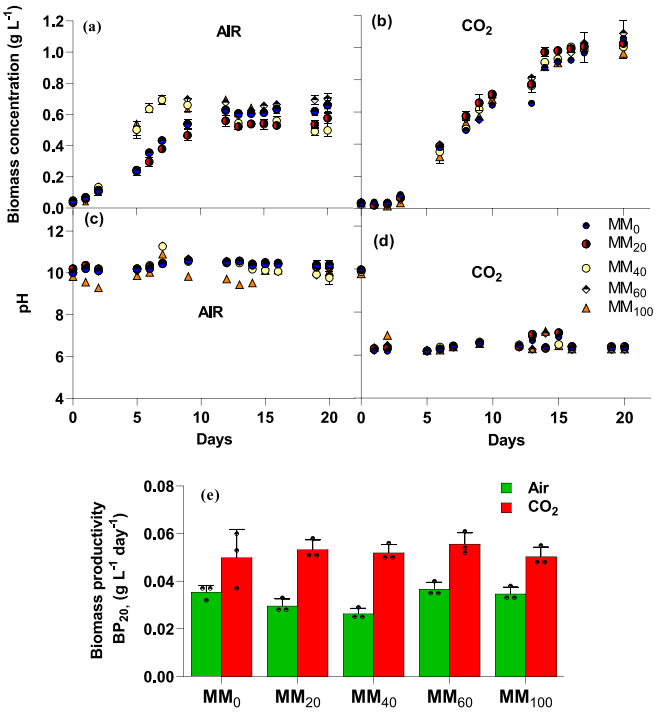

Fig. 2. Time evolution of biomass concentration and pH variations of *H. pluvialis* air (a, c) and CO_2 (b, d) for different MM volume percentages in the growht medium. Comparison of the biomass productivities after 20 days of cultivation (e). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Mean differences between groups were tested for statistical significance using the Student's *t*-tests ($N = 3$).

ZM with any percentage of MM did not produce a significant change in the growth kinetics. Based on the composition of ZM and MM, it can be assumed that such element might be iron. Though such inference should be properly corroborated by further experiments, the phenomenological evidence confirms that a growth medium (MM_{100}-C) produced by using only resources available in-situ, i.e. regolith, atmospheric CO_2 and metabolic waste of the crew, could be used to grow *H. pluvialis* on Mars. Assuming to adopt a batch cultivation strategy, the biomass productivity was evaluated after 20 days of cultivation (BP_{20}) since, as shown in Fig. 2e, the steady state of all cultures was achieved after this period. Interestingly, with the supply of a carbon source (CO_2), the productivity of biomass was increased 1.6-fold with respect to the case where air was used when considering MM_{100}. Such an increase might be due to that, after 20 days, the cultures under air probably experienced carbon starvation reaching the steady state at a lower biomass concentration than the ones cultivated under CO_2 where carbon was continuously supplied. These results are consistent with the ones by Lakshmikandan et al. [40] who reported that *Chlorella vulgaris* MSU AGM 14 at 8 % of CO_2 was able to produce 0.79 ± 0.04 g/ L of biomass with a productivity of 0.064 ± 0.003 g/ L day $^{-1}$ which was 23 % higher when compared to air. It is important to emphasize the significance of these results lies in the fact that *H. pluvialis* was able to grow in a CO_2 atmosphere using a growth medium produced using solely simulants of locally available resources, i.e. Martian regolith leachate and astronauts' urine. This would lead to a dramatic reduction of the payload associated to the nutrients in the framework of Mars missions. As a result, the techno-economic feasibility of the mission would be greatly improved.

The pH evolution during microalgae growth was also monitored (Figs. 2c and d). The results show that, under air, pH slightly increased because photosynthesis consumed dissolved CO_2 and bicarbonates then returned to its initial values when microalgae quit growing at the stationary phase. On the contrary, when cultivated under CO_2, the pH dropped significantly from 10.0 to 6.0 because of the excess of HCO_3^- formed in the medium which is not taken up by the microalgae. Subsequently the pH slightly re-increased because of the photosynthetic activity.

3.2. Total carbohydrate, lipids, and soluble protein

Several species of microalgae are known to be rich in lipids, some other in proteins and/or carbohydrates, as well as in specific bioactive compounds [41]. It is also widely recognized that microalgae are excellent sources of vitamins, including vitamin A, B1, B2, B6, B12, C and E, as well as microelements such as potassium, iron, magnesium, calcium, and iodin [42]. Considering these aspects, the *Haematococcus* spp. exploitation as a food supplement can be further developed to sustain life on Mars. In this perspective, to ascertain whether the composition of the produced biomass could improve the astronauts' nutritional requirements in terms of protein, carbohydrates, and lipids, their contents were analysed (Fig. 3).

In *H. pluvialis* cultivated under air, carbohydrates, lipids and proteins amounted on average to 21 ± 2 % wt, 18 ± 1 % wt and 42 ± 2 % wt on dry a basis, respectively. Under pure CO_2 the same components accounted for 18 ± 2%wt, 24 ± 2%wt and 37 ± 3 % wt, respectively. Therefore, a general increase of lipids' content was observed at the expense of carbohydrates and proteins when the strain was exposed to CO_2. In microalgae, the carbon substrate is crucial to promote lipid accumulation [5,43]. In fact, microalgae can use gaseous CO_2 through the CO_2-concentrating mechanism (CCM) thus stimulating the carboxylase activity of the ribulose 1,5-bisphosphate carboxylase/oxygenase Rubisco [44].

Fig. 3 also shows that the replacement of ZM with MM did not produce a significant change in terms of total lipids and proteins of *H. pluvialis,* whatever the volume replaced. However, a statistically significant change was registered for carbohydrate at $p < 0.05$.

A statistically significant increase of lipids (Fig. 3) was observed

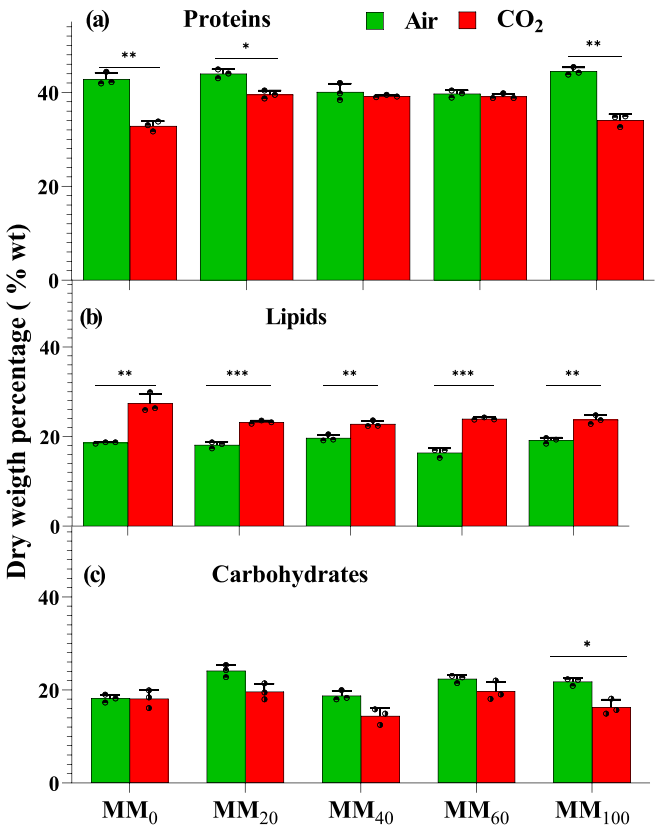

Fig. 3. Carbohydrates, lipids, and proteins content of *H. pluvialis* biomass after growth in MM_x on air and CO_2 atmosphere. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Mean differences between groups were tested for statistical significance using the Student's t-tests ($N = 3$).

when comparing the experiments under CO_2 (for all the MM %) with those carried out in the air. The increase of lipid production was 1.5-fold greater for MM_0 and 1.2-fold for MM_{40}. Varshney et al., reported an increase in neutral lipids when the green alga *Acutodesmus* sp. was grown under a CO_2 concentration of 5 and 10 % [45]. However, at higher CO_2 levels, i.e. 15 % and 20 %, a decrease in lipids content was observed. Recently, cultivated *Scenedesmus dimorphus, Scenedesmus* sp., *Ankistrodesmus convolutus, Coelastrum asteroidum* and *Chlorococcum* sp. at 0.5 %, 1.0 % and 1.5 % of CO_2 and the highest content of lipid was observed for the experiment conducted at 0.5 % of CO_2 [46]. Consistent with these results, *H. pluvialis* grew well under high CO_2 concentration without being affected the by the MM content in the growth medium. Accordingly, CO_2 from Mars atmosphere might be used to produce biomass with a high total lipids content which might represent a highly energetic food for the astronaut crew on Mars.

3.3. Minerals elements of H. pluvialis

It is known that microalgae are capable of assimilating micronutrients such as metals, both toxic and non-toxic. To further investigated the safety and the content of minerals of the biomass obtained from *H. pluvialis* grown using MM_{100} under CO_2, trace elements analysis was performed using ICP-OES. The corresponding results are shown in Table 2. There is no specific regulation that establishes the levels of toxic metals in microalgae biomass. Therefore, we have referred to the mineral content of *H. pluvialis* in compliance with the limits set by the EFSA (European Food Safety Authority) for toxic metals, such as Aluminum (Al), Arsenic (As), Barium (Ba), Cadmium (Cd), Mercury (Hg), Lead (Pb), and Titanium (Ti).The maximum levels for lead, cadmium, mercury, and titanium in various foods were established by the Commission Regulation (EC) No 1881/2006 of 19 December 2006 of the European

Table 2
Mineral element analysis for biomass cultivated in MM0 and 100 under air or pure CO_2.

Metals (mg/kg)	MM0_A	MM0_C	MM100_A	MM100_C
Al	56.8 ± 1.0	80.1 ± 1.1	67.0 ± 0.5	47.1 ± 0.3
Ba	1.5 ± 0.1	1.84 ± 0.03	5.12 ± 0.07	2.0 ± 0.1
Cu	2.2 ± 0.1	4.2 ± 0.1	1.83 ± 0.01	1.6 ± 0.2
Fe	225 ± 3	230 ± 5	205 ± 6	285 ± 5
Mn	10.22 ± 0.03	7.61 ± 0.07	6.40 ± 0.04	3.6 ± 0.1
Ti	27.7 ± 2.5	24.8 ± 2.7	128 ± 14	58.5 ± 7.7
Zn	7.2 ± 0.3	5.1 ± 0.4	11.2 ± 0.1	16.1 ± 0.3
Metals (g/kg)	MM0_A	MM0_C	MM100_A	MM100_C
Ca	1.73 ± 0.03	0.75 ± 0.03	0.78 ± 0.02	0.40 ± 0.02
K	0.93 ± 0.1	2.00 ± 0.04	2.27 ± 0.02	1.20 ± 0.04
Mg	0.61 ± 0.01	0.46 ± 0.01	0.45 ± 0.01	0.16 ± 0.01
Na	0.095 ± 0.001	0.073 ± 0.002	0.84 ± 0.01	0.37 ± 0.01

Concentrations for As, Be, Cd, Co, Cr, Hg, Li, Mo, Ni, Pb, Sn were lower to limit of detection (LOD), 0.15, 0.43, 0.15, 0.34, 0.42, 0.54, 1, 0.45, 1.71, 0.35, 0.37 mg/kg, respectively.

Union and range from 20 to 500 μg/kg, 50–500 μg/kg, 500–1000 μg/kg, and 500–2000 μg/kg of fresh weight, respectively. EFSA conducted a comprehensive evaluation of aluminum safety in food and set a Tolerable Weekly Intake (TWI) of 1 mg of aluminum per kilogram of body weight/week [47]. For barium, established a tolerable daily intake (TDI) of 200 μg/kg of body weight [48].

Considering these aspects and the specific metal content of *H. pluvialis* grown in MM100 shown in Table 2, the use of *H. pluvialis* biomass as a food supplement or its extract as a nutraceutical for humans is unlikely to pose a risk to human health. However, further investigations are needed to clarify all potential effects resulting from the use of *Haematococcus pluvialis* biomass.

3.4. Polar metabolites of H. pluvialis

GC–MS metabolomics analysis was carried out to simultaneously investigate polar metabolites and discover the changes of cellular metabolites caused by the replacement of ZM with MM or by the substitution of air with CO_2. Thirty samples of *H. pluvialis* biomass grown on different percentages of MM and Air/CO_2 atmosphere were analysed. After GC–MS analysis of trimethylsilylated microalgae, we were able to annotate 56 metabolites, including 16 aminoacids, 2 amines, 5 alcohols, 4 nucleobases, 15 organic acids, 9 sugars and 5 other compounds (Table S2 and Fig. S2). To study the changes of intracellular metabolites for each possible combination of MM percentage and atmosphere composition (air/CO_2), multivariate statistical analysis was performed. Unsupervised PCA was used to determine the major source of variance within the dataset. In the PCA score plot (Fig. 4-a), along the first principal component, that accounted for the 39.4 % of the variance, samples grown under a different composition of the gas phase were clearly separated. This trend suggests that the intracellular metabolic profiles of *H. pluvialis* differ significantly depending on the gas phase composition. The corresponding PCA loading plot (Fig. 4-b) shows the

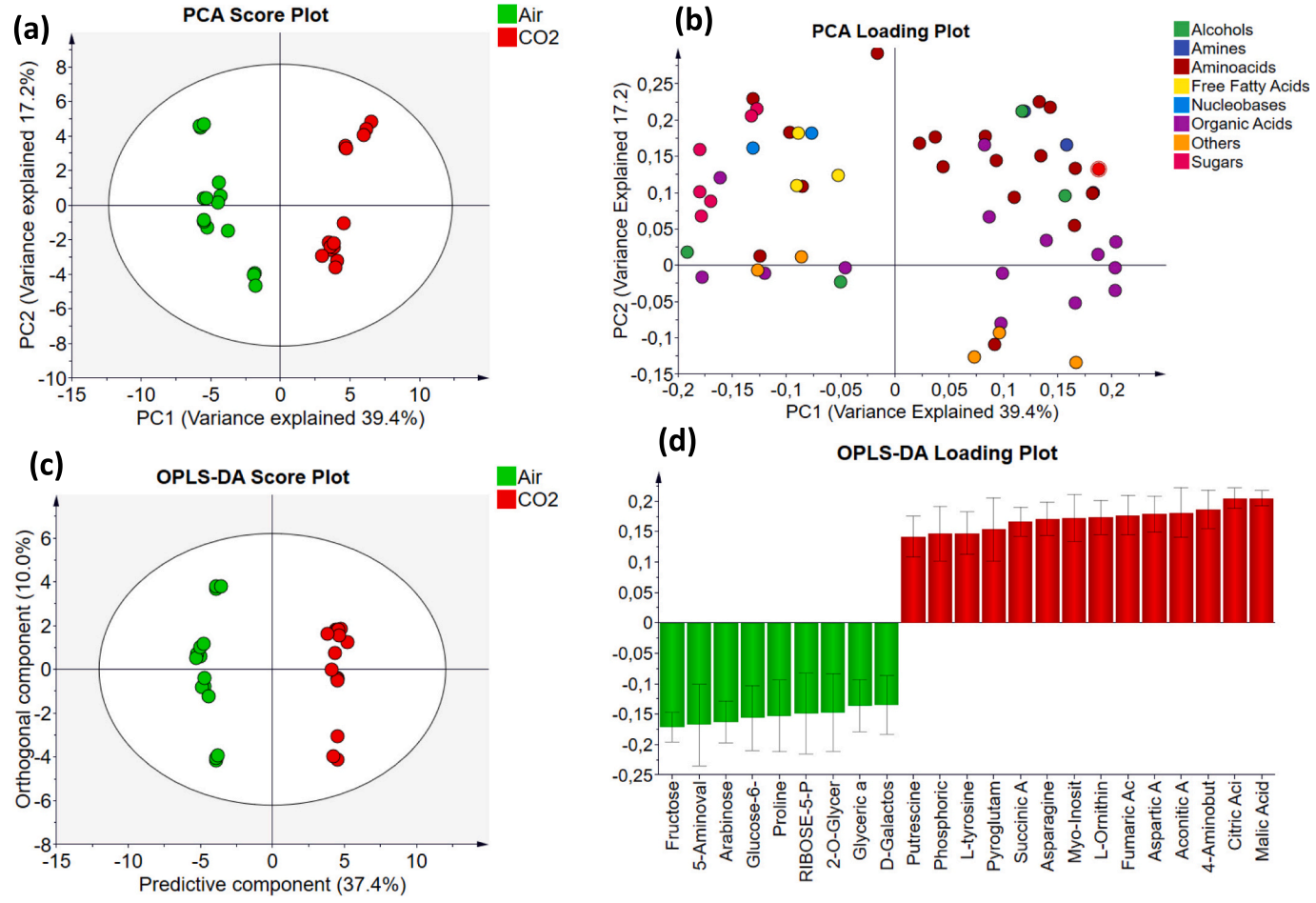

Fig. 4. (a) PCA score plot, (b) PCA loading plot, (c) OPLS-DA score plot, and (d) column loading plot of variables with VIP values >1 in the predictive component of OPLS-DA of GC–MS data obtained from *H. pluvialis* samples growing in air or CO_2 (n = 30). In the score plot the confidence interval is defined by Hotelling's T2 ellipse (95 % confidence interval); R2X (cum) = 0.77, Q2 (cum) = 0.60, R2Y cum = 0.99 and Q2Y cum = 0.98 for PCA and OPLS-DA, respectively. Variance explained was 39.4 % and 17.2 % for PC1 and PC2 respectively, with a total of 56.6 %.

variables that mostly contributed to samples clustering. The loading plot shows that the microalgal cells cultivated under CO_2 were characterized by higher levels of organic acids and amino acids while the cells grown under air showed higher levels of carbohydrates.

Supervised PLS-DA was also carried out to further validate the difference between air and CO_2 clusters identified through the PCA analysis. Data reported in Fig. S3 showed relevant differences between the two groups of *H. pluvialis* cells grown in air and CO_2. With the aim of finding the discriminant metabolites that differentiate air and CO_2 classes, pairwise OPLS-DA of GC–MS data was performed (Fig. 4-c). Criteria for screening differential metabolites were the variable influence of projection scores (VIP > 1) in the predictive component (Fig. 4-d) and the multiple *t*-test comparison to verify the significance of discriminant metabolites. Metabolites showing a higher classifying power underwent mean comparison tests. Malic acid, citric acid, aconitic acid, fumaric acid and succinic acid levels increased significantly ($p < 0.05$ for all samples) in the CO_2 experiment. Fig. 5 illustrates the alterations in these metabolites' levels at the conclusion of the experiments. These metabolites are involved in the citric acid cycle and then it can be speculated that high CO_2 concentrations can affect specific steps of the TCA cycle.

The obtained results are somehow corroborated by Guo et al., [49] who reported that, due to high concentration of CO_2, the contents of fumaric acid, malic acid and oxamic acid increased in microalgal cells as a result of their capability to store the extra carbon through their carbonyl groups. Hence, the increase of inorganic carbon (HCO_3^-) flow into the mitochondrion can promote, through the TCA cycle, carbon fixation as well as lipid and aminoacids biosynthesis [50]. Recently, using a GC–MS metabolomics approach to investigate the effects of ocean acidification on the microalga *Chlorella* sp., Tan et al., [51] observed that under high CO_2 the reactions of the TCA cycle were pushed to produce succinate.

From 16 annotated amino acids, only 5 showed a VIP value >1 and represented discriminant variable for the two treatments (Fig. 6). In particular, the levels of aspartic acid, ornithine, asparagine, pyroglutamic acid, and tyrosine increased in the microalgae cultivated under pure CO_2. Changes of tyrosine in MM_{40} and MM_{60} were not significative at $p < 0.05$. Interestingly proline levels decreased in CO_2 atmosphere. Under environmental stress conditions, *de-novo* protein synthesis is usually inhibited, and protein renewal along with proteolytic activity are increased leading to an increase in the levels of free amino acids [52–54]. In plants, the metabolism of nitrogen and carbon are highly linked because carbon skeletons of amino acids can be converted into

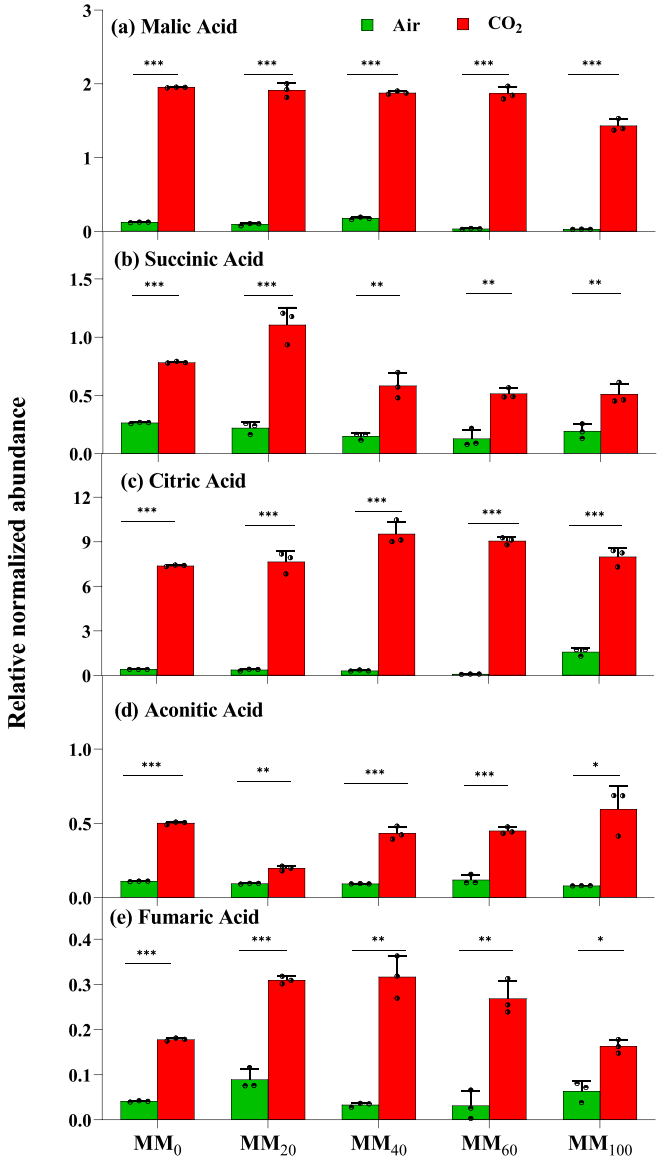

Fig. 5. Column plots of the relative content of TCA cycle intermediates (malic acid, citric acid, succinic acid, fumaric and aconitic acid) found in *H. pluvialis* biomass after growth in MM_x on air and CO_2 atmosphere. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Mean differences between groups were tested for statistical significance using the Student's t-tests ($N = 3$).

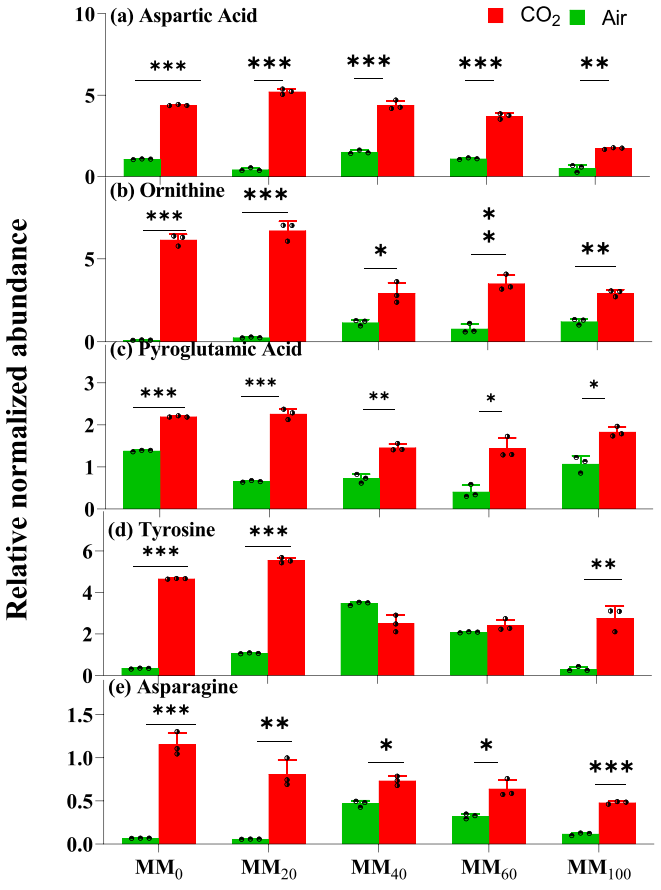

Fig. 6. Column plot of the relative content of discriminant aminoacids (Aspartic acid, Ornithine, Asparagine, Pyroglutamic acid and Tyrosine) of *H. pluvialis* biomass after growth in MMx on air and CO_2 atmosphere. Data are expressed as mean ± SD of normalized area of each peak (n = 3 for each dilution of MM and atmosphere). Results of Multiple t-test mean comparison with Bonferroni-Dunn correction are reported at: *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ levels.

precursor or intermediates of the TCA cycle to produce ATP [53]. For example, decarboxylation of glutamate can lead to the production of γ-aminobutyric acid (GABA) that in our analyses resulted to be over-expressed ($p < 0.05$) in the experiments under pure CO_2 (Fig. S4). GABA can in turn be oxidized to mitochondrial succinate [55–57] that we also found in higher levels in the algae cultivated under CO_2. Ornithine, together with arginine is implicated in the nitrogen cycle and serves as a precursor for polyamine (PA) synthesis [53,58]. The latter ones play a key role in the development of cell tolerance against high salinity and osmotic stress, and, as shown in the Fig. 7 we found that putrescine was up regulated under CO_2 condition ($p < 0.05$) [59]. In plants, these pathways can be induced also by carbohydrate starvation [53]. Other authors [50], studied the behaviour of various *Chlorella* strains under N starvation describing a metabolic pathway in which N recovered from amino acids catabolism is redistributed through glutamate-glutamine pathways and stored in other amino acids and succinate via the GABA pathway and TCA cycle. As for the C flow pathways, the authors reported that the excess of C was redistributed into lipid biosynthesis via the GABA pathways, glycolysis, and the TCA cycle [50].

Considering our experimental results, we can assume that the carbon excess in the experiment under CO_2 have determined a change in the C/N ratio which resulted in the re-distribution of N among specific amino acids such as ornithine, aspartate and asparagine that contributed to the observed increase of GABA. Then, it may be reasonably inferred that excess C is rechannelled towards the lipid biosynthesis via the GABA pathways and the TCA cycle. In particular, the excess of C may be diverted to lipid biosynthesis through the synthesis of citrate and acetyl-CoA. Finally, it can be reasonably stated that the excess C is not used during glycolysis since the levels of sugars in general, and glucose-6-phosphate, are down-regulated in microalgae grown in CO_2 (Fig. 7).

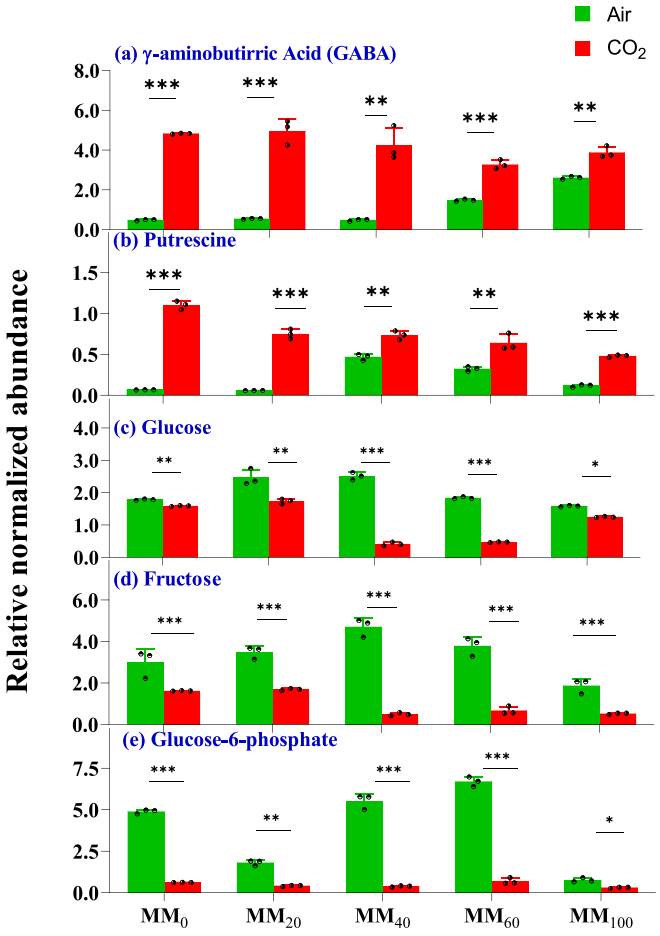

Fig. 7. Column plot of the relative content of GABA, Putrescine, glucose, fructose and glucose-6-phosphate of *H. pluvialis* biomass after growth in MMx on air and CO_2 atmosphere. Data are expressed as mean ± SD of normalized area of each peak (n = 3 for each dilution of MM and atmosphere). Results of Multiple t-test mean comparison with Bonferroni-Dunn correction are reported at: *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ levels.

3.5. Lipidome of *H. pluvialis*

The rise of total lipid content in *H. pluvialis* in response to exposure to high CO_2 levels is a significant factor in the strain's potential use as a source of bioactive compounds for astronauts. Indeed, the quality of lipids, as well as their quantity, is crucial since certain lipids such as TGs and DGs are essential for astronauts' nutrition, and their specific composition is important compared to other lipid classes.

For this reason, an untargeted lipidomic approach was adopted to characterize the lipid composition of *H. pluvialis* under different percentages of MM and gas phase composition. Three main classes of glycolipids (SQDG, MGDG and DGDG), two classes of betaine lipids (MGTS) and (DGTS), one of phospholipids (PC), two classes of neutral lipids (DG and TG) and free fatty acids were identified. For all the lipid species, the high-resolution mass spectrometry characteristics, are reported in Fig. S4, Figs. S5a-h and Table S3, respectively.

The lipid species found in *H. pluvialis* consisted of long chain of fatty acids (C15-C18), saturated and unsaturated with three double bonds. Polyunsaturated fatty acids (PUFA, ω-3 and ω-6) are important for human health to reduce the risk of cardiovascular disease and in particular medium and long chain triglycerides are implicated in the control of obesity [60,61]. During the space missions the crew members are forced to long-term confinement and isolation, exposure to radiation and different gravity forces, the risk of atherosclerosis is very high [62]. In the pathogenesis of atherosclerosis important factors are also the prothrombogenic ones that could be enhanced during space travels [63]. This risk could be reduced with moderate intensity exercise which induces a lowering lipid profile and by adopting a diet rich in ω-3 PUFA that could be integrated with edible biomass produced from *H. pluvialis* [63].

The PCA analysis of lipid species identified using QTOF/MS approach showed an evident clustering of the experimental groups air and CO_2 (Fig. 8-a). However, at MM_{100} the samples grown in air are clustered with those in CO_2. This behaviour is probably caused by the stress induced by the high salinity and heavy metals levels of the medium M_{100} on lipid biosynthesis which has the same effect of the stress determined by the low pH associated to the use of CO_2. The PCA loadings plot (Fig. 8-b) reveals that the lipid species identified as TG, DG, DGTS, and MGTS were more abundant in CO_2-exposed samples, while samples grown under air were richer in PC, DGDG and MGDG. To find the metabolites that mainly differentiate the two classes, pairwise OPLS-DA of LC-MS data was carried out and results reported in Fig. 8-c.

Univariate analysis showed that MGDG, DGDG and PC were more abundant in samples grown under air with percentages of MM ranging from MM_0 to MM_{60} (Fig. 9). MGDG are non-bilayer forming lipids, and DGDG are bilayer forming lipids both necessary for the integrity and functioning of photosynthetic apparatus (PSA) in the chloroplast. Their ratio can be modulated under stress conditions [64]. Also, PC levels under CO_2 conditions decreased dramatically at all dilutions of MM, except at MM_{100}.

The observed increase in the long-chain forms of TGs and DGs in *H. pluvialis* in CO_2 and different MM percentages (Fig. 8d and e) suggests that the microalgae underwent a process of membrane lipid remodelling and de novo fatty acid biosynthesis [64,65]. These changes in TG levels may have resulted from the microalgae's response to stress conditions, such as those experienced under simulated Martian conditions. Martian regolith contains various minerals and elements while it is generally poor of nitrogen and phosphorus. Therefore, the growth of microalgae in MM can lead to nutritional stress conditions, which determine the accumulation of TG in the cells. On the other hand, high CO_2 conditions

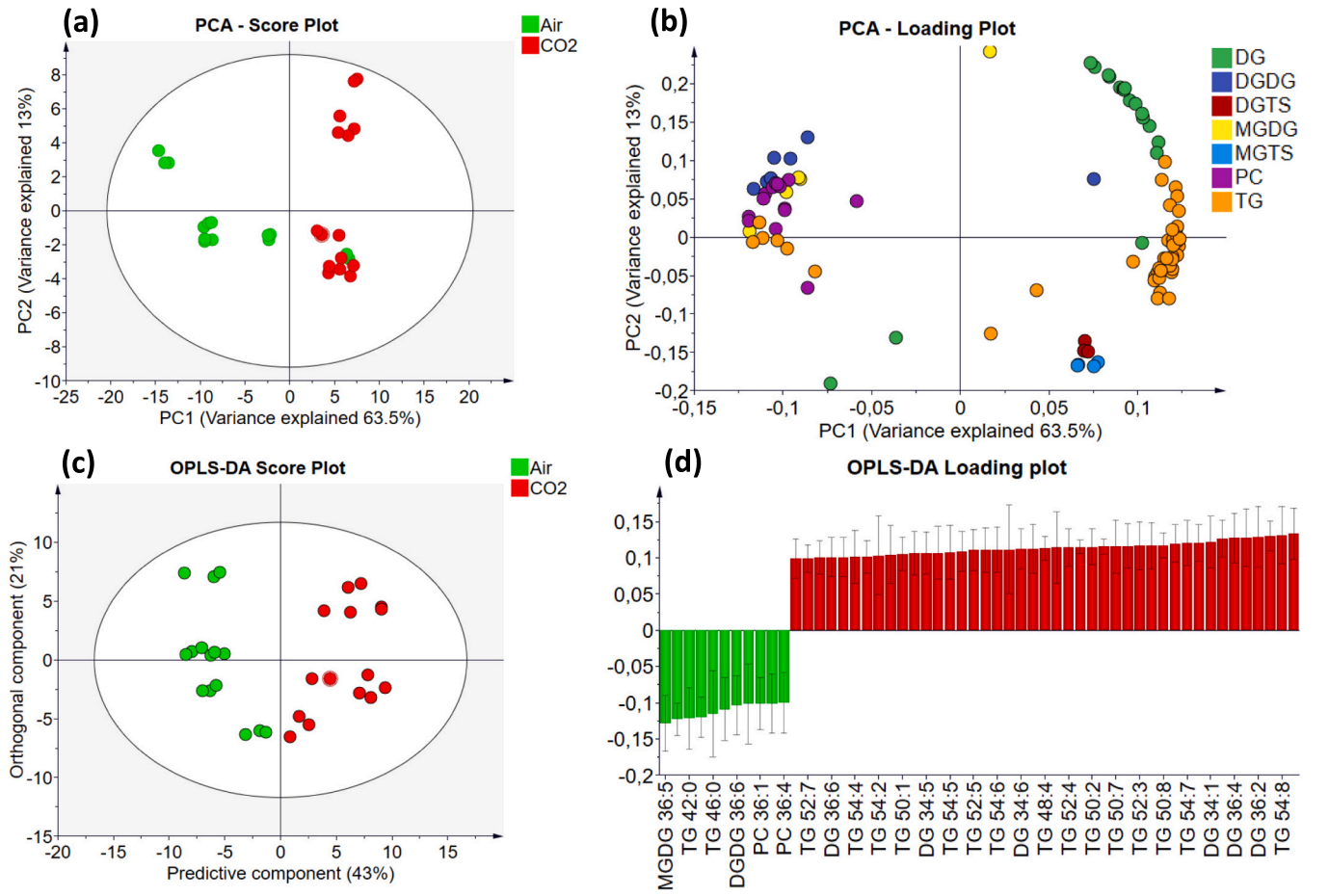

Fig. 8. (a) PCA score plot, (b) PCA loading plot, (c) OPLS-DA score plot, and (d) column loading plot of variables with VIP values >1 in the predictive component of OPLS-DA of LC-MS data obtained from *H. pluvialis* samples (n = 30). In the score plot the confidence interval is defined by Hotelling's T2 ellipse (95 % confidence interval); R^2X (cum) = 0.91, Q^2 (cum) = 0.87 and R^2Y cum = 0.85, Q^2Y cum = 0.76 for PCA and OPLS-DA, respectively. The explained variance was 63.5 % and 13 % for PC1 and PC2 respectively, with a total of 76.5 %.

contribute to a general increase of lipids due to the rechannelling of excess carbon inlet.

The increase in the long-chain forms of TGs and DGs is nutritionally significant, as these forms provide most of the energy value of *H. pluvialis* biomass. This is particularly important for space missions, where the availability of high-energy food sources is critical.

Therefore, the cultivation of *H. pluvialis* under simulated Martian conditions using in situ available resources appears to be not only feasible but also potentially capable to improve the nutritional profile of the obtained biomass.

3.6. Quantitative analysis of fatty acid methyl esters of H. pluvialis

To quantify the levels of fatty acids determined by the investigated operating conditions we have analysed only the samples obtained from MM0 and MM100 in air and CO_2 conditions. The levels of FAs found in *H. pluvialis* biomass were reported in Table 3. In all conditions, the most abundant FAs found were PUFAs (Polyunsaturated fatty acids, 19.1 ± 0.2) followed by SFAs (Saturated fatty acids, 9.2 ± 0.1) and MUFAs (Monounsaturated fatty acids 1.9 ± 0.1). Under stress conditions, an increase in the content of these fatty acids was registered, in agreement with Liang et al. [66]. We have registered a strong increase in important fatty acids such as Oleic acid (C18:1), Linoleic acid (18:2, ω-6), and Linolenic acid (C18:3, ω-6) that have many properties for human health. For example, oleic acid may help lower LDL (low-density lipoprotein) cholesterol [67]. Linoleic and linolenic acids are classified as essential fatty acids (EFAs) and play a vital role in metabolism, inflammation, and energy homeostasis [68]. Linoleic acid serves as a precursor to make arachidonic acid (AA ω-6), which is involved in inflammatory processes while Linolenic acid serves to make other omega-3 fatty acids, such as EPA (eicosapentaenoic acid, 20:5 ω-3) and DHA (docosahexaenoic acid, 22:6 ω-3), which are crucial for brain health and cardiovascular function [67]. These aspects are very important for human health and in particular for astronauts that are forced to follow a nutrient-poor diet due to the limited resources that can be loaded onto a spacecraft. However, we also registered an increase in levels of Myristic (C14:0), Palmitic (C16:0) and Stearic acid (C18:0). The EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS) offers a scientific evaluation that reassesses the safety of fatty acids (E 570, that include also Myristic acid, Palmitic, Stearic and Oleic acid) when employed as a food additive [69]. The available research on sub-chronic toxicity was restricted; however, no indications of harmful effects were observed at doses up to 10 % in the diet (equivalent to 9000 mg of lauric acid per kilogram of body weight per day) [69]. Based on these data, could be safe to use the biomass of *H. pluvialis* as a food additive for astronauts during space missions.

4. Conclusions

The microalga *H. pluvialis* can grow in a medium produced by using only resources that mimics the ones available on Mars, i.e. regolith and CO_2, plus astronauts' urine. This is a very promising result in view of future manned missions to Mars since food-supplements might be produced in-situ with a very low impact on the payload of the mission and

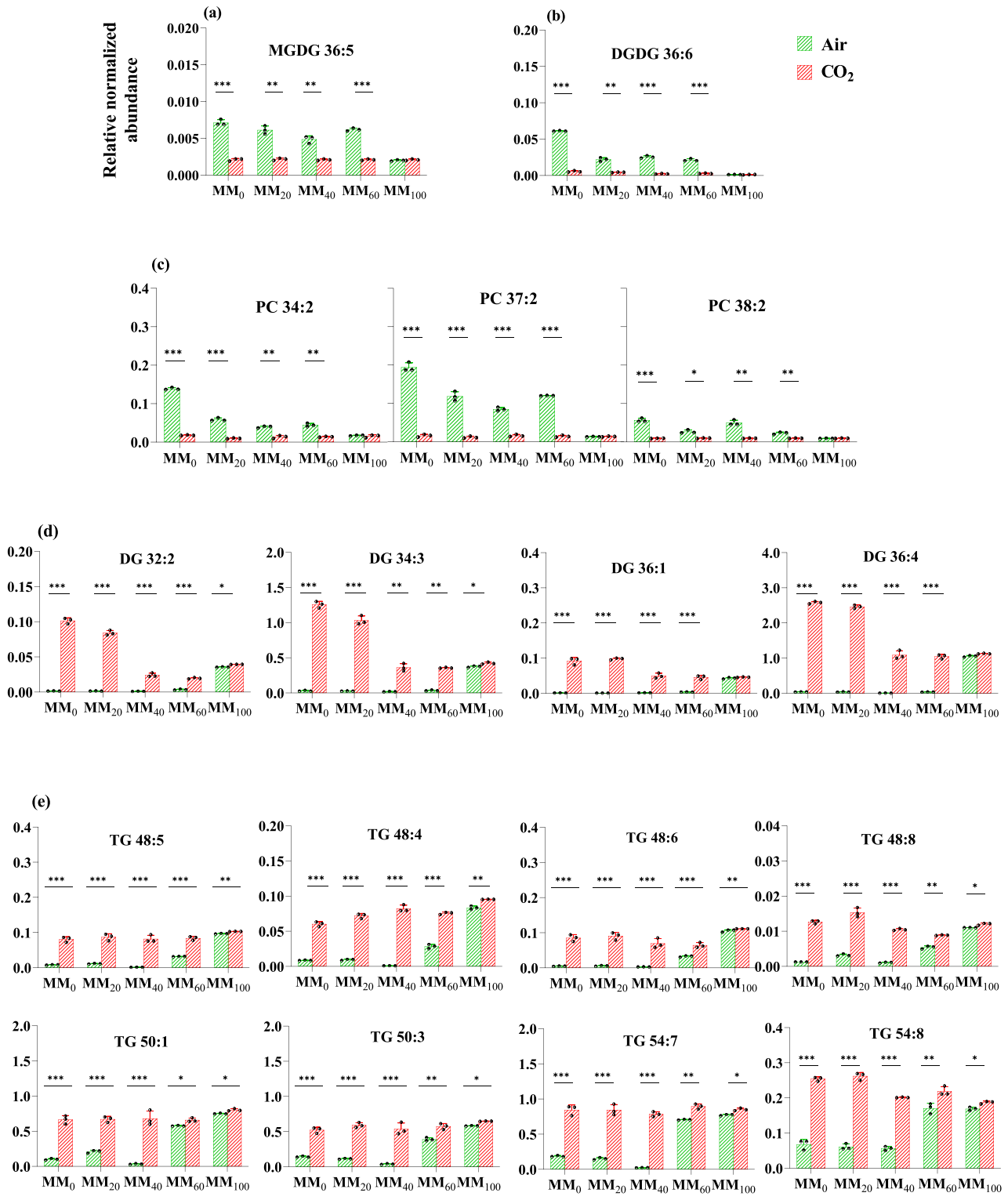

Fig. 9. (a) Column plot of the relative content of MGDG 36:5, (b) DGDG 36:6, (c) PC, (d) DG and (e) TG of *H. pluvialis* biomass after growing in air and CO_2. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Mean differences between groups were tested for statistical significance using the Student's t-tests ($N = 3$).

its corresponding technoeconomic feasibility.

From the quantitative point of view growth appeared to be substantially unaffected by the replacement of the control growth medium and gas phase with the corresponding ones obtainable on Mars in the framework of a manned mission.

The biochemical analysis of the obtained biomass demonstrated that, the use of simulants of Mars resources determines an increase of the total lipid content of the microalgal biomass.

This increase can be ascribed mainly to triglycerides and diglycerides that are highly desirable categories of lipids for the astronauts' diet due to their high energy value and anti-inflammatory potential. Another positive impact of the Mars simulated conditions is that even the production of GABA is enhanced with potential good effects on the psychological wellness of astronauts. Ultimately, this preliminary study

Table 3
Fatty acids (as FAMEs) content in *H. pluvialis* biomass expressed as mg/g (mean ± SD).

Fatty acids	C:N	MM0_A	MM100_A	MM0_C	MM100_C
Myristic acid	C 14:0	0.076 ± 0.001	0.21 ± 0.04	0.105 ± 0.005	0.21 ± 0.03
Pentadecanoic acid	C 15:0	0.064 ± 0.03	0.15 ± 0.01	0.196 ± 0.042	0.14 ± 0.01
Palmitic acid	C 16:0	7.7 ± 0.1	24 ± 2	12 ± 0.5	23 ± 1.7
7-Hexadecenoic acid	C 16:1	0.32 ± 0.01	1.1 ± 0.1	0.90 ± 0.05	0.99 ± 0.088
7,10-Hexadecadienoic acid	C 16:2	1.27 ± 0.02	3.3 ± 0.3	4.4 ± 0.2	4.1 ± 0.3
7,10,13-Hexadecatrienoic acid	C 16:3	2.94 ± 0.06	5.6 ± 0.5	4.7 ± 0.2	5.9 ± 0.5
Margaric acid	C 17:0	0.16 ± 0.01	0.24 ± 0.02	0.19 ± 0.01	0.32 ± 0.03
Margaroleic acid	C 17:1	0.060 ± 0.001	0.11 ± 0.01	0.20 ± 0.02	0.83 ± 0.09
Stearic acid	C 18:0	1.2 ± 0.1	2.9 ± 0.2	1.4 ± 0.1	2.4 ± 0.2
Oleic acid	C 18:1	0.30 ± 0.02	1.1 ± 0.2	0.62 ± 0.04	1.1 ± 0.2
Linoleic acid	C 18:2	3.9 ± 0.1	8.8 ± 0.4	9.5 ± 0.5	11.0 ± 0.7
Linolenic acid	C 18:3	11.0 ± 0.5	37.0 ± 1.2	21.0 ± 0.6	37 ± 1.0
Arachidic acid	C 20:0	0.007 ± 0.002	0.092 ± 0.007	0.004 ± 0.002	0.071 ± 0.015
Gadoleic acid	C 20:1	0.006 ± 0.003	0.051 ± 0.005	0.045 ± 0.015	0.078 ± 0.006
Behenic acid	C 22:0	0.006 ± 0.002	0.043 ± 0.003	0.006 ± 0.001	0.024 ± 0.004
Erucic acid	C 22:1	1.20 ± 0.09	1.2 ± 0.1	0.6 ± 0.1	0.7 ± 0.1
Lignoceric acid	C 24:0	0.008 ± 0.003	0.039 ± 0.002	0.012 ± 0.003	0.047 ± 0.011
Σ SFAs		9.2 ± 0.1	28 ± 2	14.2 ± 0.7	26 ± 2
Σ MUFAs		1.9 ± 0.1	3.5 ± 0.2	2.3 ± 0.2	3.7 ± 0.2
Σ PUFAs		19.1 ± 0.2	55 ± 2	40 ± 2	58 ± 2

seems to corroborate the hypothesis that edible biomass *H. pluvialis* CCALA 1081 can be cultivated on Mars using only in-situ available resources. However, further research, aimed to assess the effect of high radiation levels and reduced gravity, is needed to confirm this conclusion.

CRediT authorship contribution statement

Mattia Casula: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Conceptualization. **Pierluigi Caboni:** Writing – review & editing, Supervision, Resources, Project administration, Conceptualization. **Debora Dessì:** Writing – review & editing, Investigation. **Paola Scano:** Software, Formal analysis. **Nicola Lai:** Writing – review & editing, Formal analysis. **Alberto Cincotti:** Writing – review & editing, Formal analysis. **Giacomo Cao:** Writing – review & editing, Validation, Investigation, Conceptualization. **Alessandro Concas:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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