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Cultivation and nutritional characteristics of *Chlorella vulgaris* cultivated using Martian regolith and synthetic urine

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ABSTRACT

Long-term spatial missions will require sustainable methods for biomass production using locally available resources. This study investigates the feasibility of cultivating *Chlorella vulgaris*, a high value microalgal specie, using a leachate of Martian regolith and synthetic human urine as nutrient sources. The microalga was grown in a standard medium (BBM) mixed with 0, 20, 40, 60, or 100 % Martian medium (MM). MM did not significantly affect final biomass concentrations. Total carbohydrate and protein contents decreased with increasing MM fractions between 0 % and 60 %, but biomass in the 100% MM showed the highest levels of carbohydrates and proteins (25.2 ± 0.9 % and 37.1 ± 1.4 % of the dry weight, respectively, against 19.0 ± 1.7 % and 32.0 ± 2.7 % in the absence of MM). In all MM-containing media, the fraction of the biomass represented by total lipids was lower (by 3.2 to 4.5%) when compared to BBM. Conversely, total carotenoids increased, with the highest value (97.3 ± 1.5 mg/100 g) measured with 20% MM. In a three-dimensional principal component analysis of triacylglycerols, samples clustered according to growth media; a strong impact of growth media on triacylglycerol profiles was observed. Overall, our findings suggest that microalgal biomass produced using regolith and urine can be used as a valuable component of astronauts' diet during missions to Mars.

1. Introduction

Mars, by its proximity and relatively mild surface conditions, stands out as a prime candidate for human settlement. However, providing consumables such as oxygen, water, and food to keep humans alive is a challenge. Granted, the International Space Station has been inhabited for over twenty years, but the life-support systems the crews have relied on for consumables are not sufficient to sustain human life on Mars. The success of settlement efforts hinges not only on life-support systems but also on capabilities to produce essential resources locally, including food (Detrell, 2021; Mapstone et al., 2022).

Arguably, nutrition will be the key to maintaining physical strength, mental well-being, and endurance during space missions. Furthermore, astronauts face unique challenges such as bone loss and muscle mass loss (Arora et al., 2022), A balanced and high-quality diet is a critical element of spaceflight mission design (Bychkov et al., 2021).

Food production beyond Earth is highly challenging; traditional agriculture is impractical. Production of edible microalgae may be part of the solution due to their remarkable adaptability, their ability to thrive in a wide range of environmental conditions, their high photosynthetic efficiency which can lead to a high rate of biomass and oxygen production, as well as the richness in nutrients such as proteins, vitamins, lipids and bioactive compounds (Dolganyuk et al., 2020, Detrell, 2021; Mapstone et al., 2022). Fais et al. (2022) proposed that using Martian regolith and astronaut urine when growing microalgae could help minimize the number of resources imported from Earth. Indeed, Martian regolith and astronaut urine could provide minerals, nitrogen and water essential for microalgal growth.

The eukaryotic microalga *Chlorella vulgaris* and different cyanobacterium species such as "spirulina" (e.g., *Limnospira indica*), are being used on Earth as food supplements (Detrell, 2021). However, their physiology and nutritional benefits under spaceflight conditions must be better understood before concluding for their relevance as food supplements for astronauts. The green alga *Chlorella* is being studied for the

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applications in the field of human nutrition, biofuel production and environmental uses (Mallick et al., 2012; Sakarika and Kornaros, 2019; Abreu et al., 2023). Oils obtained from *Chlorella vulgaris,* are considered nutraceuticals (Maurício et al., 2023). *C. vulgaris* is widely adaptable to different cultivation conditions but with indigestible cell walls that must be broken before human consumption (Mason, 2001; Detrell, 2021).

The present study aimed at understanding whether biomass generated from Martian resources and astronaut urine could cover, at least in part, the nutritional requirements of astronauts during their stay on Mars. *C. vulgaris* was cultivated in a standard medium mixed with a leachate of a Martian regolith and synthetic human urine. The percent production of proteins, carbohydrates, carotenoids, minerals, and lipids, with the study of their triacylglycerol profile, was then quantified.

2. Materials and methods

2.1. Microalgae strain

The green microalga *C. vulgaris* CCALA 269 was obtained from the Culture collection of autotrophic organisms (CCALA), Třeboň, Czech Republic. It was routinely grown in Bold Basal Medium (BBM; Table S1) at 20 °C in a 5-L laboratory bottle under a light intensity of 40 μmol_{ph} $m^{-2}s^{-1}$ (as measured with a Delta Ohm HD2102.1 portable luxmeter, GHM GROUP, Germany) with a 12 h/12 h day/night cycle. Cultures were agitated by using a rotary shaker (Stuart SSM1 Orbital Shaker, Biosigma, Italy) set at 70 rpm.

2.2. Culture medium based on a simulant of Martian regolith and human urine: Martian medium (MM)

Martian medium (MM), a mixture of regolith leachate and (RL) and synthetic human urine (MP-AU), was prepared as described by Fais et al. (2022). Briefly, the regolith leachate (RL) was obtained by adding 50 g of regolith simulant (JSC Mars-1; grain size <1 mm) to 500 mL of ultrapure water at a pH of 6.80, stirring the resulting slurry for 24 h at 25 °C in a 1-L Erlenmeyer flask with a cap, and filtering with filter paper. The MP-AU was produced according to Sarigul et al. (2019) and 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. Dilutions of MM were prepared to produce experimental growth media: MM_0 (BBM only), MM_20 (80 % BBM and 20% MM), MM_40 (60 % BBM and 40% MM), MM_60 (40 % BBM and 60% MM) and MM_100 (MM only). The detailed chemical composition of MM, as determined by Fais et al. (2022), is given in Table S2.

2.3. Growth experiments

A culture of *C. vulgaris* CCALA 269 in exponential phase was used to inoculate 15 transparent, vented cap flasks filled with 40 mL each of one among the five experimental growth media described above, in triplicate. The strain was cultivated at 20 ± 1 °C under a white light intensity of 40 μmol_{ph} $m^{-2}s^{-1}$ (measured at the surface of the culture) administered from above and continuous agitation at 70 rpm (Figure S1). Microalgal growth was monitored through daily OD measurements. Biomass concentrations were inferred from OD based on an OD-biomass calibration line.

2.4. Chemicals

JSC Mars-1 regolith simulant was purchased from Orbital Technologies Corporation (Madison, WI, USA). Analytical LC-grade isopropanol, methanol, acetonitrile, acetic acid, formic acid, ammonium formate, and ammonium acetate were purchased from Sigma Aldrich (Milano, Italy). Analytical-grade sulphuric acid 96 %, orthophosphoric acid 85 %, sodium nitrate, potassium chloride, phenol, copper sulphate, sodium hydroxide, and sodium potassium tartrate were purchased from Carlo Erba (Val-de-Reuil, France). Sodium carbonate and Folin-Ciocalteu reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA). Glucose, bovine serum albumin and vanillin standards were purchased from Sigma-Aldrich (Merck Kgaa, Darmstadt, Germany). Ultrapure water was generated with a Milli-Q® 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^{-1}) of Al, As, 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).

2.5. Biomass characterization

After 17 days of cultivation, 35-mL culture samples were taken and centrifuged at 1968 g for 10 min at 20 °C. Supernatants were discarded and pellets were resuspended in ultrapure water. The washing procedure was repeated three times. Pellets were frozen at −80 °C, lyophilized with an LIO-5PDGT freeze-dryer (Cinquepascal, Milano, Italy), and finely pulverized with a mortar and pestle.

Quantification of total carbohydrates and soluble proteins. Briefly, 2 mg of lyophilized sample were weighted using a 5-decimal place analytical balance ABT 120–5DNM (division, 0.01–0.1 and reproducibility, 0.02–0.1; KERN & SOHN GmbH, Germany) within a glass tube, and cell walls were ruptured in 5 mL of phosphate-buffered saline (PBS; 20 mM, pH 7.4) by sonication with an ExctractorOne (GM Solution, Cagliari, Italy).

Carbohydrate concentrations were determined using a slightly modified version of the method suggested by Dubois et al. (1956). Briefly, 1 mL of phenol 5 % (*w/v*), and 5 mL of concentrated sulfuric acid were added to 1 mL of the extract in a glass tube. After 10 min at room temperature, the reaction was stopped by immersing the samples in cold water for 20 min. Samples were filtered with 0.45-μm PTFE membrane filters and their absorbance at 490 nm was determined by using a spectrophotometer. A quantitative analysis was performed using the external standard method with glucose as a standard. A 5-point calibration curve was built by correlating absorbance with glucose concentration. Total soluble protein contents were determined according to Lowry et al. (1951). Briefly, 500 μL of the sonicated samples were set to react with 500 μL of 1 N sodium hydroxide for 5 min at 100 °C. After cooling the solution for 10 min, 2.5 mL were added of a solution of 5% (*w/v*) sodium carbonate, 0.5% (*w/v*) cupric sulphate and 1 % (*w/v*) sodium potassium tartrate. After 10 min, 0.5 mL of 1 N Folin-Ciocalteu reagent were added, and absorbance was measured at 750 nm. A quantitative analysis was performed using the external standard method by correlating absorbance with concentration of bovine serum albumin. The results are expressed in g/100 g (% dwt, mean ± SD).

Quantification of total lipids. Cell walls were ruptured as described by Chen and Vaidyanathan, (2013), with slight modifications. Briefly, 2 mg of lyophilized sample were weighted within a glass tube, and 100 μl of PBS and 1.5 mL of 1 N sodium hydroxide in 25 % methanol were added. The suspension was sonicated for 10 min and incubated at 100 °C for 30 min. After boiling, samples were cooled and centrifuged at 1968 g. Total lipids were extracted according to Folch et al., (1957), with slight modifications. Briefly, 1.5 mL of methanol, 3 mL of chloroform and 0.5 mL of a 0.2 M potassium chloride solution were added to each sample, after which samples were agitated and centrifuged for 5 min at 1968 g. The colorimetric reaction was carried out according to Mishra et al. (2014). Briefly, 100 μL of concentrated sulfuric acid were added to 1 mL of the chloroform phase after drying under a gentle nitrogen stream. Samples were heated for 10 min at 90 °C and, after cooling, 2.4 mL of phospho-vanillin 68 % (*w/v*) were added. Absorbance was measured at 530 nm. Lipid quantification was performed by means of the external standard method and results are expressed in g/100 g (% dwt, mean ± SD).

Quantification of total carotenoids. Total carotenoids and chlorophyll-a (data not shown) content were determined according to Zavrel et al.

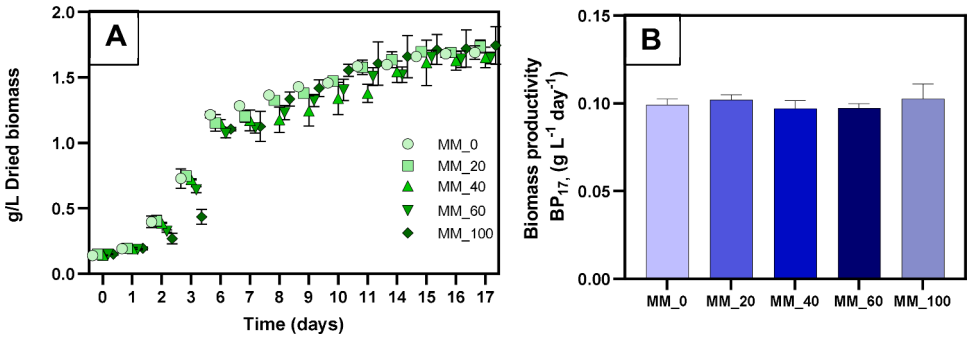

Fig. 1. A) Biomass concentration of *Chlorella vulgaris* CCALA 269 over the 17-day cultivation period. B) Comparison of the average biomass productivities over the cultivation period (the differences are not statistically significant).

(2015). Briefly, 10 mg of lyophilized biomass were weighted inside Eppendorf tubes. Glass beads and one mL of neutralized methanol were added. Each sample was sonicated in an ultrasonic bath for 30 min at 10 °C and stored at 4 °C. After 24 h, the samples were vortexed and sonicated again for 30 min at 10 °C. The solutions were centrifuged for 10 min at 12,298 g. Chlorophyll a and total carotenoid contents were estimated based on absorbance at 470, 665 and 720 nm, measured after a blank measurement of methanol at 470 nm. The following correlations, proposed by Ritchie (2006) and Wellburn (1994), were used to estimate the total carotenoid concentrations:

$$Carotenoids\,[\mu g/mL] = (1000(A_{470} - A_{720}) - \\ - 2.86\,Chlorophyll\,a\,[\mu g/mL])/221$$

Results are expressed in mg/100 g (mean ± SD).

ICP-OES analysis of metals and metalloids. Sample preparation for trace metal analysis was carried out by suspending 10 mg of lyophilized biomass in 10 mL of 1 % HNO_3 in water, before sonicating three times for 2 min with the sonicator ExtractorOne (GM solutions). Samples were then filtered with 0.45-µm nylon filters.

An Agilent 5100 Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES: Agilent Technologies, Santa Clara, USA) was used to quantify trace elements. The settings were as follows: 1200 W radio frequency; 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 5 s read time. Solvent samples were run to control for false positives and contamination during analysis. A 5 mg L^{-1} ICP multi-element standard solution was prepared in 1 % HNO_3 in water. The working solutions were prepared on the day of use by diluting the multi-element standard solution in 1 % HNO_3 in water. Calibration curves were built with five points and were considered acceptable when $r^2 \geq 0.997$.

Triacylglycerol profiling. TAGs from *C. vulgaris* biomass were extracted using a method similar to that described above for total lipids. Briefly, for each sample, 10 mg of lyophilized biomass were weighed within a glass tube before adding 5 mL of a chloroform:methanol solution (2:1 *v/v*). The suspension was sonicated three times using ExctractorOne (GM solution, Cagliari). After 1 hour, 0.5 mL of aqueous 0.2 M potassium chloride were added. The suspension was centrifuged at 24,104 g for 10 min at 4 °C and the lipophilic layer was picked up and dried under a gentle nitrogen stream. The dried extracts were resuspended in 20 µL of a methanol/chloroform (1:1 *v/v*) mixture and diluted with 980 µL of a 2-propanol/acetonitrile/water (2:1:1 *v/v/v*) mixture containing standards (Splash, Lipidomics, Sigma Aldrich, Milan, Italy). Samples were analysed with a UHPLC-QTOF/MS coupled with an Agilent 1290 Infinity II LC system, injecting 1 µL in the positive ionization mode. Chromatographic separation of lipids was performed with a Kinetex 5 µm EVO C18 100 A and a 150 mm x 2.1 µm column (Agilent Technologies, Palo Alto, CA). The column was maintained at 50 °C at a flow rate of 0.4 mL/min. The mobile phase consisted of a 10 mM ammonium formate solution in 60 % of ultrapure water and 40 % of acetonitrile (A) and a 10 mM ammonium formate solution containing 90 % of isopropanol and 10 % of acetonitrile (B). 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 to 1 % in 5 min, a stay at this percentage for 1.9 min, and an increase back to the initial conditions in 1 min. The Agilent jet stream source was operated with the following parameters: gas temperature, 200 °C; gas flow (nitrogen), 10 L/min; nebulizer gas (nitrogen), 50 psig; sheath gas temperature, 300 °C; sheath gas flow, 12 L/min; capillary voltage, 3500 V; 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; mass precursor per cycle, 3; threshold for MS/MS, 5000 counts. Before analysis, the instrument was calibrated using an Agilent tuning solution at the mass range of *m/z* 50–1700. Samples were acquired first with an auto MS/MS iterative mode with a mass error tolerance of 20 ppm a retention exclusion tolerance of 0.2 min, and then in full-scan mode to annotate chromatographic area. The relative concentration of triacylglycerols (TAGs) in mg/L were calculated as follows:

$$C_s = (A_s / A_i) \times C_i$$

where C_s is the analyte concentration in the sample, and C_i the concentration of the internal standard (TG 48:1 d7). A_s and A_i are the chromatographic peak area of molecular species in the samples and the internal standard, respectively. The nomenclature used here for lipids (TAGs) is based on Lipids Maps (Fahy et al., 2009).

2.6. Statistical analysis

The significance of mean differences for total proteins, carbohydrates, lipids, and carotenoids were investigated using a one-way ANOVA followed by Dunnett's correction for multiple comparisons, with a confidence interval of 95 %. This was performed using the GraphPad Prism software (version 8.3.0, Dotmatics, Boston, Massachusetts).

The effects of different cultivation media on the triacylglycerols produced by *C. vulgaris* was investigated using a principal component analysis (PCA) as well as a 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. The variable influence on projection (VIP) scores of the OPLS-DA predictive component describe the metabolite influence on sample classification; only variables with VIP score >1 were taken into consideration and annotated (Eriksson et al., 2013). Theses analyses were performed using the SIMCA-*P*+ program (Version 14.1, Umetrics, Sartorius, Germany). Subsequently, a univariate analysis of VIP obtained comparing MM_0 and MM_100 was performed in the GraphPad Prism software. The significance of mean difference was obtained using one-way and two-way ANOVA.

Table 1
Effect of different dilutions of Martian medium (MM) on macronutrient fraction (% dwt) after 17 days of cultivation. Values are reported as mean ± standard deviation. n= 3.

Macronutrients		MM_0	MM_20	MM_40	MM_60	MM_100
Total Proteins (TP)		32.0 ± 2.7	23.8 ± 2.2	24.2 ± 0.7	21.2 ± 2.0	37.1 ± 1.4
Total Carbohydrates (TC)		19.0 ± 1.7	15.3 ± 1.3	15.2 ± 1.4	14.0 ± 0.8	25.2 ± 0.9
Total Lipids (TL)		18.5 ± 0.2	15.3 ± 0.7	14.0 ± 0.5	15.0 ± 0.4	15.2 ± 0.5
p-value[1]	TP		< 0.005	< 0.005	< 0.0005	< 0.05
	TC		< 0.05	< 0.05	< 0.005	< 0.0005
	TL		< 0.0001	< 0.0001	< 0.0001	< 0.0001

[1] p-value were obtained by comparing the mean of each MM dilution with the mean of MM_0 using a one-way ANOVA and Dunnett's correction for multiple comparisons.

Table 2
Effect of different dilutions of Martian medium (MM) on total carotenoids (mg/100 g) after 17 days of cultivation.

Macronutrients	MM_0	MM_20	MM_40	MM_60	MM_100
Total Carotenoids	77.0 ± 2.1	97.3 ± 1.5	90.6 ± 3.9	80.3 ± 3.4	85.5 ± 3.7
p-value[1]		< 0.0001	< 0.001	ns	< 0.05

[1] p-values were obtained by comparing the mean of each MM dilution with the mean of MM_0 using a one-way ANOVA and Dunnett's correction for multiple comparisons.

Table 3
Metals and metalloid concentrations (mean ± SD) in biomass cultivated in MM_0 and MM_100.

Metals	MM_0	MM_100
Al (mg/kg)	78.7 ± 0.8	262 ± 7
Ca (g/kg)	1.75 ± 0.03	1.61 ± 0.01
Cu (mg/kg)	56.5 ± 0.7	8.4 ± 0.5
K (g/kg)	8.08 ± 0.02	2.98 ± 0.02
Fe (mg/kg)	96.0 ± 1.7	97.7 ± 6
Mg (g/kg)	0.941 ± 0.002	0.422 ± 0.002
Mn (mg/kg)	51.2 ± 3	6.6 ± 0.5
Na (g/kg)	11.67 ± 0.03	3.57 ± 0.01
Ti (mg/kg)	5.6 ± 0.9	7.4 ± 0.2
Zn (mg/kg)	43.7 ± 0.3	28.0 ± 1.7

Concentrations for As, Be, Cd, Co, Cr, Hg, Li, Mo, Ni, Pb, Sn were below the limit of detection (LOD, 0.15, 0.43, 0.15, 0.34, 0.42, 0.96, 1, 0.45, 1.71, 0.35, 0.37 mg/kg, respectively).

3. Results and discussion

3.1. Growth of *Chlorella vulgaris* in Martian medium

The cyanobacteria *Spirulina platensis, Synechococcus nidulans*, and *Chroococcidiopsis thermalis*, were previously reported to be well adapted to MM, in which they can produce sources of nutrients, nutraceuticals, or bioactive compounds (Fais et al., 2022; Casula et al., 2024, 2024). The present study explores the impact of using MM on the growth and nutrients production of *Chlorella vulgaris* CCALA 269. The biomass production profile of cultures in MM_20, MM_40, and MM_60 over a 17-day cultivation period (shown in Fig. 1A) was similar to MM_0 cultures in. However, cultures in MM_100 initially exhibited lower growth rates, likely due to an adaptation phase. Nonetheless, the type of medium did not significantly impact final biomass concentrations. The biomass production was as follows: MM_0, 1.70 ± 0.05 g L^{-1}; MM_20, 1.74 ± 0.05 g L^{-1}; MM_40, 1.65 ± 0.08 g L^{-1}; MM_60, 1.65 ± 0.04 g L^{-1}; MM_100, 1.75 ± 0.14 g L^{-1}. Consistently, the biomass productivity over the 17-day cultivation period (BP_{17}; Fig. 1B) did not differ significantly across culture media, highlighting the microalga's flexibility and resource utilization capabilities. These results contrast with responses observed in cyanobacteria: while *S. platensis, S. nidulans*, and *C. thermalis* exhibited suboptimal growth in MM_100, *C. vulgaris* effectively acclimated to this medium. This has significant implications for the sustainability of Mars missions: cultivating these species using Martian regolith and astronaut urine could help reduce the payload mass associated with hauling nutrients from Earth. Part of the needed water, and of macro- and microelements, could indeed be sourced from urine, while the remaining water would be extracted from the hydration and adsorbed water of Martian regolith, along with trace elements, following the process patented by Cao et al. (2021).

3.2. Total proteins, lipids, and carbohydrates

A balanced diet is crucial to the health and performance of astronauts during long-term space missions, as emphasized by Neubek (2005). Proteins play a crucial role in the support of muscle tissue maintenance and immune system function (Smith and Zwart, 2008; Heer et al., 2015). Lipids serve as dense reservoirs of energy which is essential for sustaining optimal brain and nervous system function in space (Bruce et al., 2017). Carbohydrates, particularly sugars, serve as immediate energy sources critical for meeting the metabolic demands (Smith and Zwart, 2008; Heer et al., 2015). The results presented in Table 1 shows macronutrient composition changes across different cultivation media (MM_0 to MM_100). Firstly, as the MM concentration increases from MM_0 to MM_60, there is a decrease in both total carbohydrate and protein contents. For instance, the total protein levels decreased from 32.0 ± 2.7 % in MM_0 to 21.2 ± 2.0 % in MM_60, while total carbohydrate contents decrease from 19.0 ± 1.7 % in MM_0 to 14.0 ± 0.8 % in MM_60. Conversely, the biomass obtained using MM_100 exhibits significantly higher levels of carbohydrates (25.2 ± 0.9 %) and proteins (to 37.1 ± 1.4 %) when compared to MM_0. Moreover, total lipid contents are consistently lower in biomass from all MM-containing media compared to MM_0. The total lipid content decreased from 18.5 ± 0.2 % in MM_0 to 15.2 ± 0.5 % in MM_100. These findings highlight the importance of MM concentration in modulating the nutritional contents of the biomass.

A possible explanation for the reduction in carbohydrate and protein content from MM0 to MM60 and for the increase in MM100 could be related to changes in the concentrations of certain microelements in the medium, such as urea nitrogen and iron. For example, Chen et al. (2015) demonstrated that variations in urea and iron concentrations can lead to changes in protein synthesis in *Chlorella vulgaris*. Similarly, variations in the content of N, P, and Fe can cause changes in carbohydrate production in *Chlorella vulgaris*, as demonstrated by Al-Rubiaee and Al-Safaar (2021). Additionally, Rizwan et al. (2017) showed that the microalga *Dunaliella tertiolecta* can alter carbohydrate production depending on the iron complex used in the culture medium. Hence, as the fraction of MM increases, the normal nutrient concentrations are diluted, potentially causing stress or a decrease in the metabolic efficiency of the microalga. Instead, the full MM may represent a good source of N, P and Fe to improve carbohydrates and proteins synthesis. However, with the available information, we are not yet able to explain this non-linear trend.

Table 4
Levels of triacylglycerols (TAGs) identified in *Chlorella vulgaris* biomass grown in different dilutions of Martian medium (MM).

TAG (C#:sat#)	Fatty Acids	% dwt				
		MM_0	MM_20	MM_40	MM_60	MM_100
42:0	12:0/14:0/16:0	0.013 ± 0.001	0.017 ± 0.001	0.016 ± 0.001	0.016 ± 0.001	0.016 ± 0.001
44:0	14:0/14:0/16:0	0.035 ± 0.002	0.040 ± 0.001	0.040 ± 0.002	0.037 ± 0.001	0.036 ± 0.001
45:1	14:0/15:0/16:1	0.015 ± 0.004	0.017 ± 0.001	0.018 ± 0.001	0.016 ± 0.001	0.015 ± 0.002
45:0	14:0/15:0/16:0	0.038 ± 0.002	0.044 ± 0.001	0.043 ± 0.002	0.041 ± 0.002	0.041 ± 0.001
46:1	14:0/16:0/16:1	0.038 ± 0.003	0.047 ± 0.002	0.043 ± 0.001	0.044 ± 0.001	0.041 ± 0.002
46:0	14:0/16:0/16:0	0.107 ± 0.003	0.120 ± 0.02	0.119 ± 0.004	0.116 ± 0.002	0.11 ± 0.01
47:2	15:1/16:0/16:1	0.015 ± 0.001	0.012 ± 0.001	0.012 ± 0.001	0.006 ± 0.001	0.006 ± 0.001
47:1	15:0/16:0/16:1	0.041 ± 0.002	0.048 ± 0.002	0.046 ± 0.002	0.048 ± 0.002	0.044 ± 0.001
47:0	15:0/16:0/16:0	0.094 ± 0.004	0.106 ± 0.001	0.105 ± 0.002	0.103 ± 0.003	0.10 ± 0.01
48:2	16:0/16:1/16:1	0.039 ± 0.001	0.048 ± 0.001	0.044 ± 0.001	0.042 ± 0.001	0.043 ± 0.001
48:1	16:0/16:0/16:1	0.092 ± 0.003	0.106 ± 0.001	0.099 ± 0.002	0.102 ± 0.001	0.10 ± 0.01
48:0	16:0/16:0/16:0	0.175 ± 0.006	0.203 ± 0.002	0.199 ± 0.005	0.204 ± 0.004	0.195 ± 0.002
49:1	15:0/16:0/18:1	0.064 ± 0.002	0.080 ± 0.002	0.071 ± 0.001	0.076 ± 0.002	0.076 ± 0.001
49:0	16:0/16:0/17:0	0.061 ± 0.002	0.071 ± 0.001	0.071 ± 0.003	0.072 ± 0.002	0.069 ± 0.001
50:6	16:0/16:3/18:3	0.042 ± 0.002	0.052 ± 0.002	0.029 ± 0.001	0.030 ± 0.001	0.058 ± 0.002
50:5	16:0/16:3/18:2	0.032 ± 0.001	0.040 ± 0.001	0.024 ± 0.002	0.025 ± 0.001	0.041 ± 0.001
50:4	16:0/16:3/18:1	0.071 ± 0.002	0.087 ± 0.002	0.049 ± 0.001	0.051 ± 0.002	0.095 ± 0.002
50:3	16:0/16:2/18:1	0.075 ± 0.003	0.090 ± 0.001	0.061 ± 0.001	0.060 ± 0.001	0.093 ± 0.002
50:2	16:0/16:1/18:1	0.24 ± 0.001	0.273 ± 0.004	0.23 ± 0.01	0.25 ± 0.01	0.28 ± 0.01
50:1	16:0/16:0/18:1	0.84 ± 0.03	1.02 ± 0.01	0.87 ± 0.03	0.93 ± 0.02	0.98 ± 0.01
50:0	16:0/16:0/18:0	0.191 ± 0.005	0.226 ± 0.003	0.212 ± 0.005	0.22 ± 0.01	0.215 ± 0.003
52:9	16:3/18:3/18:3	0.010 ± 0.004	0.014 ± 0.001	0.008 ± 0.001	0.006 ± 0.001	0.013 ± 0.001
51:2	16:0/17:1/18:1	0.058 ± 0.001	0.070 ± 0.001	0.060 ± 0.001	0.064 ± 0.002	0.074 ± 0.002
52:8		0.013 ± 0.001	0.017 ± 0.001	0.010 ± 0.001	0.010 ± 0.001	0.019 ± 0.001
51:1	16:0/17:0/18:1	0.048 ± 0.001	0.056 ± 0.002	0.053 ± 0.002	0.054 ± 0.002	0.061 ± 0.002
52:7	16:3/18:1/18:3	0.076 ± 0.003	0.097 ± 0.001	0.051 ± 0.001	0.051 ± 0.001	0.093 ± 0.002
51:0	16:0/17:0/18:0	0.034 ± 0.001	0.039 ± 0.001	0.038 ± 0.004	0.040 ± 0.003	0.036 ± 0.001
52:6	16:2/18:1/18:3	0.065 ± 0.002	0.083 ± 0.004	0.047 ± 0.001	0.044 ± 0.002	0.073 ± 0.001
52:5		0.104 ± 0.004	0.129 ± 0.003	0.077 ± 0.002	0.074 ± 0.002	0.13 ± 0.01
52:4	16:0/18:1/18:3	0.35 ± 0.01	0.419 ± 0.005	0.258 ± 0.006	0.27 ± 0.01	0.440 ± 0.005
52:3	16:0/18:1/18:2	0.65 ± 0.02	0.763 ± 0.015	0.636 ± 0.016	0.64 ± 0.01	0.758 ± 0.008
52:2	16:0/18:1/18:1	2.5 ± 0.1	2.96 ± 0.03	2.619 ± 0.063	2.8 ± 0.1	2.74 ± 0.04
52:1	16:0/18:0/18:1	0.39 ± 0.01	0.485 ± 0.05	0.406 ± 0.010	0.45 ± 0.02	0.502 ± 0.006
52:0	16:0/18:0/18:0	0.29 ± 0.01	0.344 ± 0.07	0.328 ± 0.008	0.34 ± 0.01	0.324 ± 0.004
53:0		0.024 ± 0.001	0.047 ± 0.002	0.043 ± 0.002	0.042 ± 0.001	0.047 ± 0.001
53:3	17:1/18:1/18:1	0.039 ± 0.002	0.052 ± 0.001	0.046 ± 0.001	0.051 ± 0.001	0.054 ± 0.001
53:2	17:0/18:1/18:1	0.045 ± 0.001	0.028 ± 0.001	0.027 ± 0.002	0.028 ± 0.001	0.028 ± 0.001
54:6	18:1/18:2/18:3	0.075 ± 0.003	0.091 ± 0.002	0.054 ± 0.003	0.054 ± 0.001	0.083 ± 0.002
54:5	18:1/18:1/18:3	0.30 ± 0.01	0.364 ± 0.004	0.240 ± 0.006	0.223 ± 0.004	0.317 ± 0.003
54:4	18:1/18:1/18:2	0.54 ± 0.017	0.64 ± 0.08	0.55 ± 0.02	0.53 ± 0.01	0.57 ± 0.01
54:3	18:1/18:1/18:1	2.24 ± 0.05	2.68 ± 0.03	2.51 ± 0.07	2.60 ± 0.05	2.44 ± 0.02
54:2	18:0/18:1/18:1	0.59 ± 0.01	0.704 ± 0.008	0.65 ± 0.01	0.65 ± 0.01	0.660 ± 0.008
54:1		0.098 ± 0.001	0.116 ± 0.001	0.107 ± 0.003	0.11 ± 0.02	0.118 ± 0.002
54:0	18:0/18:0/18:0	0.077 ± 0.005	0.094 ± 0.001	0.089 ± 0.003	0.090 ± 0.003	0.086 ± 0.001
55:0	16:0/17:0/22:0	0.034 ± 0.001	0.040 ± 0.002	0.037 ± 0.002	0.038 ± 0.001	0.035 ± 0.001
56:3	18:1/18:1/20:1	0.049 ± 0.007	0.059 ± 0.001	0.055 ± 0.002	0.057 ± 0.001	0.052 ± 0.002
56:2	18:1/18:1/20:0	0.106 ± 0.004	0.126 ± 0.002	0.12 ± 0.04	0.121 ± 0.003	0.118 ± 0.002
56:1	16:0/18:1/22:0	0.035 ± 0.003	0.044 ± 0.001	0.043 ± 0.002	0.043 ± 0.001	0.045 ± 0.001
56:0	16:0/18:0/22:0	0.041 ± 0.004	0.050 ± 0.001	0.047 ± 0.002	0.048 ± 0.001	0.045 ± 0.002
58:2	18:1/18:1/22:0	0.037 ± 0.002	0.044 ± 0.001	0.042 ± 0.002	0.043 ± 0.001	0.042 ± 0.001
58:0	16:0/20:0/22:0	0.022 ± 0.001	0.026 ± 0.002	0.026 ± 0.001	0.026 ± 0.001	0.025 ± 0.002
60:2		0.019 ± 0.001	0.024 ± 0.003	0.024 ± 0.002	0.025 ± 0.002	0.022 ± 0.001
Σ TAGs		11.3 ± 0.3	13.5 ± 0.1	11.7 ± 0.3	12.1 ± 0.2	12.7 ± 0.1
Σ Saturated TAGs		1.24 ± 0.04	1.47 ± 0.02	1.41 ± 0.04	1.43 ± 0.03	1.38 ± 0.02
Σ Monosaturated TAGs		1.68 ± 0.05	2.02 ± 0.02	1.76 ± 0.05	1.88 ± 0.05	1.98 ± 0.03
Σ Polyunsaturated TAGs		8.7 ± 0.3	10.4 ± 0.1	8.9 ± 0.2	9.2 ± 0.2	9.8 ± 0.1

3.3. Total carotenoids

Carotenoids, are integral to a spectrum of physiological processes, including vision, gene regulation, and immune function (Ahmed et al., 2013). Due to their antioxidative properties, their ingestion by astronauts may help mitigate the deleterious effects of oxidative stress, which is exacerbated during space travel and exploration (Goodwin and Christofidou-Solomidou, 2018), notably due to reduced gravity and radiation exposure (Montesinos et al., 2021; Bychkov et al., 2021). The inclusion of carotenoids in astronauts' diets could thus contribute to maintaining astronaut health and performance (Bychkov et al., 2021). The quantification of carotenoid contents in the microalgal biomass was therefore conducted, to assess their suitability as a dietary supplement.

The results (Table 2) revealed intriguing variations in carotenoid levels across MM concentrations. Comparing MM_0 to MM_100, one can observe a substantial increase in total carotenoids, from 77.0 ± 2.1 % in MM_0 to 85.5 ± 3.7 % in MM_100, with an increase of approximately 11 %. Furthermore, carotenoid levels were consistently higher in the biomass from all MM-containing media compared to MM_0, with the highest fractions observed in MM_20 and MM_40 (although the difference was not statistically significant for MM_60). This suggests that the inclusion of MM in the culture medium fosters the production of carotenoids, possibly due to the presence of specific nutrients or stressors that stimulate their synthesis (Encarnação et al., 2012). In fact, the observed phenomenon may be ascribed to the presence of metals at high concentrations and the elevated urea content (urea serving as a nitrogen

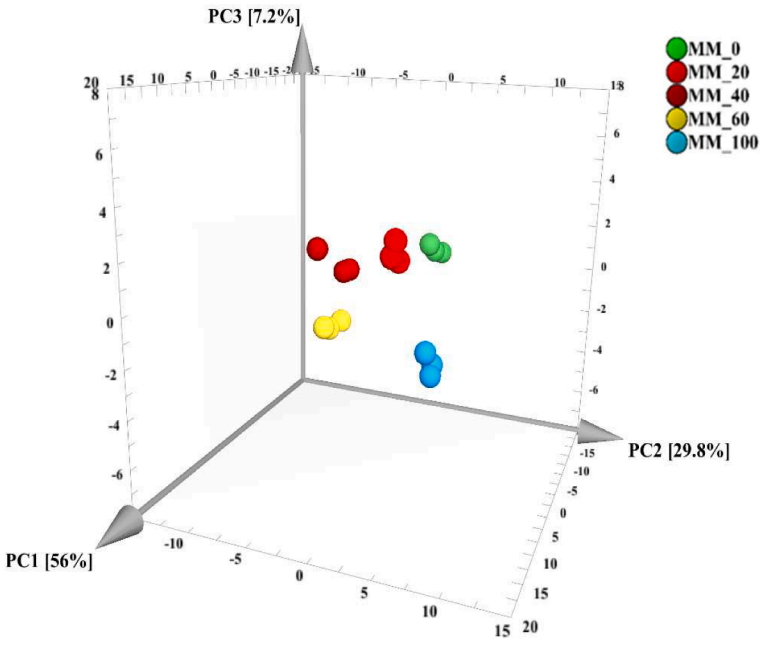

Fig. 2. 3D-PCA score plot of triacylglycerol profiles from *Chlorella vulgaris* CCALA 269 grown in Martian medium (MM). The total variance explained with the 3 principal component is 93 %, ($R^2X = 0.929$, $Q^2 = 0.868$).

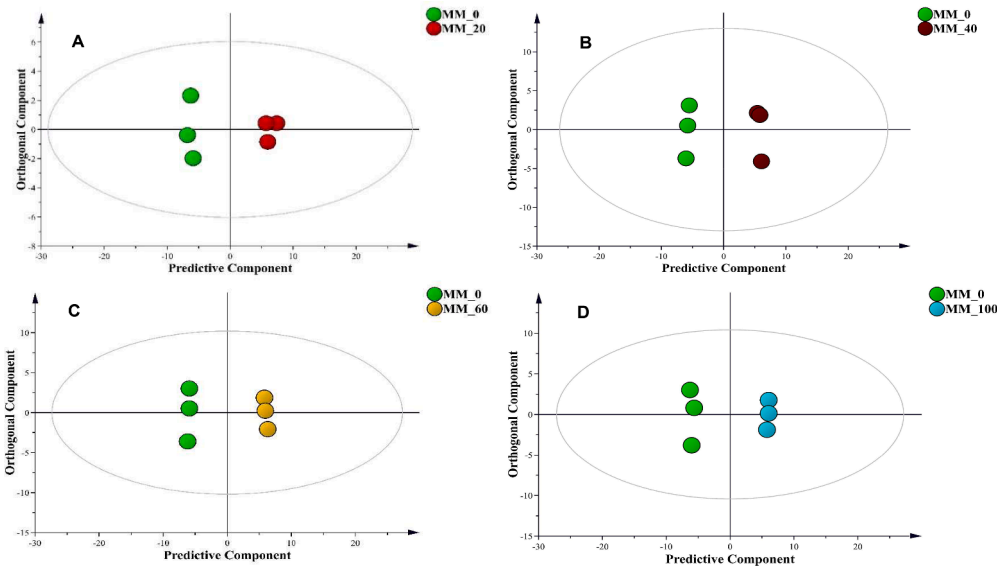

Fig. 3. OPLS-DA score plots comparing triacylglycerol profiles of *Chlorella vulgaris* CCALA 269 biomass produced in each Martian medium dilution (MM_20–100) with that of biomass produced in standard medium (MM_0). A) 1 + 1 + 0; $R^2Y = 0.992$, $Q^2 = 0.952$; B) 1 + 1 + 0; $R^2Y = 0.998$, $Q^2 = 0.995$; C) 1 + 1 + 0; $R^2Y = 0.999$, $Q^2 = 0.996$; D) 1 + 1 + 0; $R^2Y = 0.999$, $Q^2 = 0.985$.

source in the synthetic urine). In the case of metals, for instance, their accumulation from MM_100 within microalgae cells may foster the generation of reactive oxygen species (ROS). These ROS species can engage in detrimental interactions with lipids, proteins, and nucleic acids, thereby inducing oxidative stress. To counteract this stress, microalgae employ various defence mechanisms, such as the synthesis of antioxidant compounds. These may include enzymes (e.g., superoxide dismutase, catalase), which function to neutralize ROS and convert metal ions into less reactive forms (Cassier-Chauvat and Chauvat, 2014; Monteiro et al., 2012; Concas et al., 2015), but also pigments – such as carotenoids. Our findings suggest that optimizing MM composition could be a viable strategy for enhancing the antioxidant content of microalgal biomass, thereby improving its potential as a dietary supplement.

3.4. Minerals

The assimilation of excessive amounts of micronutrients, such as metals, may make microalgal consumption unsafe for humans. Trace element in the microalgal biomass were quantified; results are shown in Table 3. There are no upper limits for toxic metals in microalgal biomass, while for foodstuff the maximum levels for contaminants is set by the EFSA (European Food Safety Authority). These limits are presented in the Commission Regulation (EC) No 1881/2006 of 19 December 2006 of the European Union and specify limits for lead (20–500 μg/kg of fresh weight), cadmium (50–500 μg/kg), mercury

Table 5
Discriminant metabolites in pairwise OPLS-DA comparing triacylglycerol profiles of standard medium (MM_0) and Martian medium (MM_100).

TAG	VIP-value	Expression*
50:4	1.10	*up*
53:2	1.09	*down*
50:6	1.08	*up*
47:2	1.08	*down*
52:7	1.07	*up*
51:1	1.07	*up*
52:8	1.06	*up*
50:0	1.06	*up*
56:3	1.05	*up*
56:1	1.05	*up*

* Expression levels are relative to MM_100.

(500–1000 μg/kg) and titanium (500–2000 μg/kg). Our results showed that titanium levels exceed the limits established by the European Union for food, suggesting a very moderate use of *C. vulgaris* biomass for astronauts' diet. Furthermore, it is use as a direct source of food is unlikely due to its poor digestibility. However, this does not exclude the use of biomass as a source of bioactive compounds necessary for astronauts' diet.

The EFSA also conducted a comprehensive evaluation of aluminium safety in food and set a Tolerable Weekly Intake (TWI) of 1 mg of aluminium per kilogram of body weight per week (Aguilar et al., 2008), which, based on our results, would correspond to ca. 1.9 kg of biomass a week for a 70-kg crewmember.

In terms of metal contents, the use of moderate amounts of *C. vulgaris* biomass produced in MM_100 – directly as a dietary supplement is unlikely to pose a risk to human health.

Although the composition of the Martian Medium (MM) does not include copper and zinc, the microalgae were grown with Bold's Basal Medium (BBM) before being fed with MM. Thus, they may have incorporated amounts of zinc and copper that remain detectable analytically. In addition, microalgae can absorb heavy metals from the environment and concentrate them within the cells.

3.5. Triacylglycerol profiles

Lipids extracted from *C. vulgaris* grown in MM were subjected to UHPLC separation followed by MS detection. A total of 52 triacylglycerol (TAG) species were identified and quantified (Table 4). High resolution mass spectrometry characteristics of TAGs can be found in the supplementary materials (Table S3). Interestingly, TAG fatty acids (FAs) were annotated as $\omega-3$, such as hexadecatrienoic acid (HTA, C16:3) and α-linolenic acid (ALA, C18:3); $\omega-6$, such as linoleic acid (C18:2); and $\omega-9$, such as oleic acid (C18:1). These results are consistent with fatty acid methyl ester (FAME) compositions reported in literature (Teh et al., 2021). TAGs represent fractions of the biomass of 11.3 ± 0.3 (MM_0), 13.5 ± 0.1 (MM_20), 11.7 ± 0.3 (MM_40), 12.1 ± 0.2 (MM_40) and 12.7 ± 0.1 % dwt (MM_100), and 60–80 % of the total lipids.

A principal component analysis was performed to further investigate the changes in TAGs profiles across the different growth media (Fig. 2). The PCA score plot displays distinct clusters: MM_40 and MM_60 are distributed along PC1 and separated along PC3, while MM_0, MM_20, and MM_100 are spread across PC2 and separated along PC3.

To further investigate the differences underscored by the PCA, four pairwise OPLS-DA (MM_0 *vs.* each of the MM dilutions) were performed (Fig. 3).

Pairwise OPLS-DA revealed that the triacylglycerol profile is profoundly influenced by the type of culture medium (Fig. 3). Metabolites whose level changed significantly between MM_0 and MM_100 were screened based on the variable influence of projection scores (VIP > 1) in the predictive component, and the significance of differences was tested using multiple *t*-test comparisons. Table 5 reports metabolites showing a high classifying power.

The discriminant metabolites obtained by pairwise OPLS-DA were subjected to mean comparison tests to further assess the significance of their mean differences (Fig. 4). All tested mean differences, except the levels of TAG 47:2, were statistically significant ($p < 0.05$).

C. vulgaris TAGs exhibited different levels across MM dilution (Table 3). The levels of TAG species such as 50:4, 50:6, 52:7, and 51:1 demonstrate an upregulation in MM_100 compared to MM_0 (Table 4). Conversely, TAG species like 53:2 and 47:2 show a downregulation in MM_100. These findings suggest an alteration in the response of TAG metabolism in *C. vulgaris* to different growth media conditions.

These changes may reflect an adaptive responses of *C. vulgaris* to varying environmental conditions, such as nutrient availability and stressors associated with different MM. Previous studies have underscored the plasticity of lipid metabolism in microalgae in response to environmental cues. Understanding these metabolic adaptations is crucial for optimizing microalgae-based biotechnological processes, including lipid production for various applications. Accordingly, numerous studies have investigated the influence of various factors on the lipid composition of microalgae, including silicon deficiency, phosphate limitation, high salinity, cadmium exposure-induced stress, co-immobilization with the bacterium *Azospirillum brasilense* in alginate beads, light intensity, and iron content in the growth medium (Lynn et al., 2000; Reitan et al., 1994; Rao et al., 2007; Guschina and Harwood, 2006; Lebsky et al., 2002; de-Bashan et al., 2002; Kojima and Zhang, 1999). Specifically, nitrogen deficiency has been shown to increase lipid contents in microalgae (Illman et al., 2000). As an example, research on *Chloroidium ellipsoideum* and *Chlorococcum infusionum* has revealed that total lipid contents peaks in the absence of nitrogen in the culture

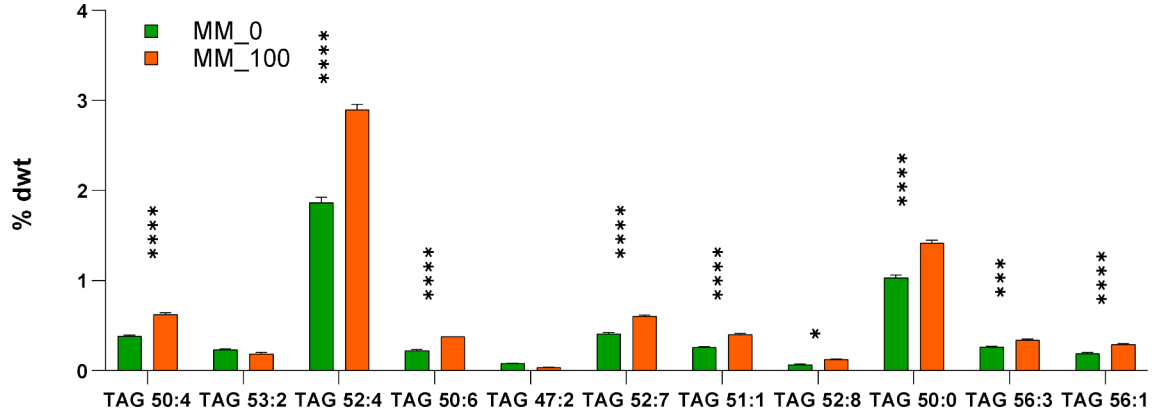

Fig. 4. Univariate analysis of pairwise OPLS-DA discriminant metabolites (standard medium, MM_0 *vs.* Martian medium, MM_100). Mean differences were tested using two-way ANOVA.

medium, and demonstrated an inverse correlation between nitrate and lipid contents (Satpati and Pal, 2015). Stress conditions can re-route microalgal metabolism to TAGs production, which serve as storage energy and are rapidly degraded when physiology is compromised (Cakmak et al., 2014). For example, nitrogen starvation has been demonstrated to increase total lipid contents but reduce the production of polyunsaturated fatty acids (Chisti, 2007). The significantly higher nitrogen contents of MM, compared to those of BBM, underscore the potential influence of nitrogen availability on lipid synthesis in microalgae and could explain the rise of polyunsaturated TAGs. On the other hand, high nitrogen contents could have been expected to cause an increase in protein content.

In addition to nitrogen starvation, other various mineral element deprivation generally can stimulate changes in neutral lipids (Cakmak et al., 2014). It has been previously reported that phosphorus deprivation can increase lipid contents in different algal species (Reitan et al., 1994). Changes in magnesium and sulphur levels can be followed by an increase or decrease in neutral lipids contents (Cakmak et al., 2014). Increasing the iron levels in the culture medium notably enhances the synthesis of neutral lipids in *C. vulgaris* (Liu et al., 2013). This effect on lipid production has been observed in other microalgal species. For instance, an increase in fatty acids and consequently in triacylglycerol contents has been documented in *Botryococcus* spp. exposed to growing concentrations of Fe^{3+} (Yeesang and Cheirsilp, 2011). Similarly, supplementation with ferrous ammonium sulphate has resulted in elevated carbohydrate contents in *Dunaliella tertiolecta*, while iron provided in the form of EDTA-chelated compound is able to stimulate lipid biosynthesis (Rizwan et al., 2017). The higher levels of iron ions in MM, compared to BBM, may thus contribute to the observed increase in total triacylglycerol production by *C. vulgaris*. Further investigations into the biochemical pathways associated with these TAG species could uncover potential targets for metabolic engineering to enhance lipid productivity in microalgae.

In the context of space exploration, the ability to modulate lipid metabolism in microalgae like *C. vulgaris* could have significant implications for supporting space missions. By understanding how TAG metabolism responds to different growth conditions, scientists can develop strategies to optimize microalgae cultivation systems onboard spacecraft, ensuring a sustainable and reliable source of dietary lipids. However, the biological significance of these lipids remains multifactorial and not fully elucidated, and the identification of specific TAG species with altered expression in response to MM_100 could have implications for biomedical research and biotechnology applications. Some TAGs have been linked to bioactive properties, including antioxidant and anti-inflammatory effects (Zhang et al., 2019). Overall, the results highlight the importance of studying TAG metabolism in microalgae like *C. vulgaris* and its relevance for space exploration, biotechnology, and human health. Further research in this area holds promise for advancing our understanding of lipid metabolism in microorganisms and leveraging their metabolic potential for various practical applications in space and terrestrial contexts.

Noteworthy, regolith may contain toxic compounds such as perchlorate ions at levels potentially toxic to plants (Oze et al., 2021). Furthermore, Martian surface concentrations of perchlorate exhibit spatial and depth-dependent variability, with levels reaching 0.4–2 % wt (Billi et al., 2021; Oze et al., 2021). In this work, *Chlorella vulgaris* was cultivated using a Martian regolith not containing perchlorates. However, further experiments are underway to investigate the effects of perchlorates on microalgae growth and preliminary data (not shown) indicated that doses exceeding 0.6 % may reduce the growth of microalgae.

4. Conclusion

The challenges related to providing resources for long-term, crewed missions to Mars has led to extensive research efforts for the production of fuels and of a range of life support materials, especially food. Capabilities to produce edible microalgal biomass could help address this challenge: in addition to the macronutrients this biomass would provide, bioactive compounds may reduce damage from psychological stress, reduced gravity and radiation exposure. In the present work, the possibility of producing high-value biomass was investigated by cultivating the microalga *C. vulgaris* CCALA 269 using Martian medium (MM), obtained by mixing a leachate of Martian regolith simulant (JSC Mars-1) and synthetic human urine. Despite an initially slower growth rate, *C. vulgaris* achieved a similar productivity overall in all tested media, including pure Martian medium (MM_100). When the MM concentration was increased from 0 to 60 % (MM_0 to MM_60), the total carbohydrate and protein contents decreased. This decline suggests a potential impact of MM concentration on the synthesis of these macronutrients. Conversely, the biomass obtained using MM_100 exhibited higher levels of carbohydrates and proteins compared to MM_0. Lipid contents, however, decreased (compared to MM_0) when culture media contained MM. Overall, the biomass produced in MM_100 was found to have a macronutrient profile ideal for the astronaut's diet. Adding 20 % and 40 % of MM (leading to MM_20 and MM_40) to BBM increased total carotenoids contents. *C.vulgaris* biomass also showed high levels of calcium, potassium, manganese, and sodium. Triacylglycerols analyses suggest that MM affects the levels of saturated, monounsaturated, and polyunsaturated TAG with a source of ω−3 fatty acids. Overall, these results suggest that the use of urine and local regolith to cultivate *Chlorella vulgaris* could be a versatile and sustainable solution to help meet the dietary and nutritional needs of astronauts during missions to Mars.

CRediT authorship contribution statement

Mattia Casula: Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Giacomo Fais:** Conceptualization. **Cristina Manis:** Writing – original draft, Formal analysis, Data curation. **Paola Scano:** Validation, Software, Methodology. **Cyprien Verseux:** Writing – original draft, Supervision, Methodology, Investigation, Conceptualization. **Alessandro Concas:** Writing – original draft, Data curation, Conceptualization. **Giacomo Cao:** Investigation, Conceptualization. **Pierluigi Caboni:** Writing – original draft, Visualization, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of interests

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.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.lssr.2024.06.003.

References

Abreu, A.P., Martins, R., Nunes, J., 2023. Emerging applications of chlorella sp. and spirulina (Arthrospira) sp. Bioeng. 10 (8), 955.

Aguilar, F., Autrup, H., Barlow, S., Castle, L., Crebelli, R., Dekant, W., Engel, K.H., Gontard, N., Gott, D., Grilli, S., Gurtler, R., Larsen, J.C., Leclercq, C., Leblanc, J.C., Malcata, F.X., Mennes, W., Milana, M.R., Pratt, I., Rietjens, I., Tobback, P., Toldrá, F., 2008. Safety of aluminium from dietary intake scientific opinion of the panel on food additives, flavourings, processing aids and food contact materials (AFC). EFSA J 754, 1–34.

Ahmed, F., Fanning, K., Schuhmann, H., Netzel, M., Schenk, P., 2013. Microalgae: a valuable source of natural carotenoids with potential health benefits. Carotenoids: Food Sources, Production and Health Benefits. Nova Biomedical, New York, NY, pp. 143–163.

Al-Rubiaee, G.H., Al-Safaar, A.T, 2021. Effect of nutrients condition on the growth and lipid content of microalgae Chlorella vulgaris and Chroococcus minor. J. Genetic and Env. Res. Conservation 9 (1), 110–121.

Arora, S., Puri, S., Bhambri, N., 2022. A designer diet layout for astronauts using a microbiome mediated approach. FEMS Microbiol. Lett. 369 (1) fnac049.

Billi, D., Fernandez, B.G., Fagliarone, C., Chiavarini, S., Rothschild, L.J., 2021. Exploiting a perchlorate-tolerant desert cyanobacterium to support bacterial growth for in situ resource utilization on Mars. International Journal of Astrobiology 20 (1), 29–35.

Bruce, K.D., Zsombok, A., Eckel, R.H., 2017. Lipid processing in the brain: a key regulator of systemic metabolism. Front Endocrinol (Lausanne) 8, 60.

Bychkov, A., Reshetnikova, P., Bychkova, E., Podgorbunskikh, E., Koptev, V., 2021. The current state and future trends of space nutrition from a perspective of astronauts' physiology. Int. J. Gast. Food Sc. 24, 100324.

Çakmak, Z.E., Ölmez, T.T., Çakmak, T., Menemen, Y., Tekinay, T., 2014. Induction of triacylglycerol production in Chlamydomonas reinhardtii: comparative analysis of different element regimes. Bioresour. Technol. 155, 379–387.

Cao, G., Concas, A., Fais, G., Gabrielli, G., Manca, A., Pantaleo, A., Process and Kit to Investigate Microgravity Effect on Animal/vegetable Cells under Extraterrestrial Cultivation Conditions and Cultivation Process Thereof to Sustain Manned Space Missions, PCT/IB2012/053754, 2021.

Cassier-Chauvat, C., Chauvat, F., 2014. Responses to oxidative and heavy metal stresses in cyanobacteria: recent advances. Int. J. Mol. Sci. 16, 871–886.

Casula, M., Fais, G., Sidorowicz, A., Caboni, P., Cao, G., Concas, A., 2024. Effects of a novel bioprocess for the cultivation Synechococcus nidulans on Mars on its biochemical composition: focus on the lipidome. Biotechnol. Bioprocess Eng. 1–16.

Chen, C.Y., Lee, P.J., Tan, C.H., Lo, Y.C., Huang, C.C., Show, P.L., Lin, C.H., Chang, J.S., 2015. Improving protein production of indigenous microalga Chlorella vulgaris FSP-E by photobioreactor design and cultivation strategies. Biotechnol. J. 10 (6), 905–914.

Chen, Y., Vaidyanathan, S., 2013. Simultaneous assay of pigments, carbohydrates, proteins and lipids in microalgae. Anal. Chim. Acta 776, 31–40.

Chisti, Y., 2007. Biodiesel from microalgae. Biotechnol. Adv. 25 (3), 294–306.

Liu, B., Vieler, A., Li, C., Jones, A.D., Benning, C., 2013. Triacylglycerol profiling of microalgae chlamydomonas reinhardtii and nannochloropsis oceanica. Bioresour. Technol. 146, 310–316.

Concas, A., Pisu, M., Cao, G., 2015. Microalgal cell disruption through fenton reaction: experiments, modeling and remarks on its effect on the extracted lipids composition. Chem. Eng. Trans. 43, 367–372. https://doi.org/10.3303/CET1543062.

De-Bashan, L.E., Bashan, Y., Moreno, M., Lebsky, V.K., Bustillos, J.J., 2002. Increased pigment and lipid content, lipid variety, and cell and population size of the microalgae Chlorella spp. When co-immobilized in alginate beads with the microalgae-growth-promoting bacterium Azospirillum brasilense. Can. J. Microbiol. 48 (6), 514–521.

Detrell, G., 2021. Chlorella vulgaris photobioreactor for oxygen and food production on a Moon base—Potential and challenges. Frontiers in Astronomy and Space Sciences 8, 700579.

Dolganyuk, V., Belova, D., Babich, O., Prosekov, A., Ivanova, S., Katserov, D., Patyukov, N., Sukhikh, S., 2020. Microalgae: a promising source of valuable bioproducts. Biomolecules 10 (8), 1153.

DuBois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.T., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28 (3), 350–356.

Encarnação, T., Burrows, H.D., Pais, A.C., Campos, M.G., Kremer, A., 2012. Effect of N and P on the Uptake of Magnesium and Iron and on the Production of Carotenoids and Chlorophyll *by* the Microalgae Nannochloropsis sp. J. Agricul. Sci. Technol. A 2 (6A), 824.

Eriksson, L., Byrne, T., Johansson, E., Trygg, J., Vikström, C., 2013. Multi-and Megavariate Data Analysis Basic Principles and Applications. Umetrics Academy (Vol. 1).

Fahy, E., Subramaniam, S., Murphy, R.C., Nishijima, M., Raetz, C.R., Shimizu, T., Spener, F., van Meer, G., Wakelam, M.J.O., Dennis, E.A, 2009. Update of the LIPID MAPS comprehensive classification system for lipids1. J. Lipid Res. 50, S9–S14.

Fais, G., Casula, M., Sidorowicz, A., Manca, A., Margarita, V., Fiori, P.L., Pantaleo, A., Caboni, P., Cao, G., Concas, A., 2024. Cultivation of Chroococcidiopsis thermalis Using Available In Situ Resources to Sustain Life on Mars. Life 14 (2), 251.

Fais, G., Manca, A., Concas, A., Pantaleo, A., Cao, G., 2022. A novel process to grow edible microalgae on Mars by exploiting in situ-available resources: experimental investigation. Acta Astronaut. 201, 454–463.

Folch, J., Lees, M., Stanley, G.S., 1957. A simple method for the isolation and purification of total lipides from animal tissues. Journal of biological chemistry 226 (1), 497–509.

Goodwin, T.J., Christofidou-Solomidou, M., 2018. Oxidative stress and space biology: an organ-based approach. Int. J. Mol. Sci. 19 (4), 959.

Guschina, I.A., Harwood, J.L., 2006. Lipids and lipid metabolism in eukaryotic algae. Prog. Lipid Res. 45 (2), 160–186.

Heer, M., Titze, J., Smith, S.M., Baecker, N., 2015. Nutrition Physiology and Metabolism in Spaceflight and Analog Studies. Springer International Publishing (No. 11420).

Illman, A.M., Scragg, A.H., Shales, S.W., 2000. Increase in Chlorella strains calorific values when grown in low nitrogen medium. Enzyme Microb. Technol. 27 (8), 631–635.

Kojima, E., Zhang, K., 1999. Growth and hydrocarbon production of microalga Botryococcus braunii in bubble column photobioreactors. J. Biosci. Bioeng. 87 (6), 811–815.

Lebsky, V.K., González, L.E., Carrillo, A., Bashan, Y., 2002. Ultrastructure of coimmobilization of the microalga Chlorella vulgaris with the plant growth-promoting bacterium Azospirillum brasilense and with its natural associative bacterium Phyllobacterium myrsinacearum in alginate beads. Nitrogen Fixation: From Molecules to Crop Productivity. Springer Netherlands, Dordrecht (pp. 583-583).

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. biol. Chem 193 (1), 265–275.

Lynn, S.G., Kilham, S.S., Kreeger, D.A., Interlandi, S.J., 2000. Effect of nutrient availability on the biochemical and elemental stoichiometry in the freshwater diatom Stephanodiscus minutulus (Bacillariophyceae). J. Phycol. 36 (3), 510–522.

Mallick, N., Mandal, S., Singh, A.K., Bishai, M., Dash, A., 2012. Green microalga Chlorella vulgaris as a potential feedstock for biodiesel. J. Chem. Technol. Biotechnol. 87 (1), 137–145.

Mapstone, L.J., Leite, M.N., Purton, S., Crawford, I.A., Dartnell, L., 2022. Cyanobacteria and microalgae in supporting human habitation on Mars. Biotechnol. Adv. 59, 107946.

Mason, R., 2001. Chlorella and Spirulina: green supplements for balancing the body. Alternative & Complementary Therapies 7 (3), 161–165.

Maurício, T., Couto, D., Lopes, D., Conde, T., Pais, R., Batista, J., Melo, T., Pinho, M., Moreira, A.S., Trovão, M., Barros, A., 2023. Differences and similarities in lipid composition, nutritional value, and bioactive potential of four edible Chlorella vulgaris strains. Foods 12 (8), 1625.

Mishra, S.K., Suh, W.I., Farooq, W., Moon, M., Shrivastav, A., Park, M.S., Yang, J.W., 2014. Rapid quantification of microalgal lipids in aqueous medium by a simple colorimetric method. Bioresour. Technol. 155, 330–333.

Monteiro, C.M., Castro, P.M.L., Malcata, F.X., 2012. Metal uptake by microalgae: underlying mechanisms and practical applications. Biotechnol. Prog. 28, 299–311.

Neubek, D.J. (2005). *Nutrition Requirements, Standards, and Operating Bands for Exploration Missions* (No. JSC-E-DAA-TN71711).

Montesinos, C.A., Khalid, R., Cristea, O., Greenberger, J.S., Epperly, M.W., Lemon, J.A., Boreham, D.R., Popov, D., Gorthi, G., Ramkumar, N., Jones, J.A., 2021. Space radiation protection countermeasures in microgravity and planetary exploration. Life 11 (8), 829.

Oze, C., Beisel, J., Dabsys, E., Dall, J., North, G., Scott, A., Lopez, A.M., Holmes, R., Fendorf, S., 2021. Perchlorate and agriculture on Mars. Soil Syst. 5 (3), 37.

Rao, A.R., Dayananda, C., Sarada, R., Shamala, T.R., Ravishankar, G.A., 2007. Effect of salinity on growth of green alga Botryococcus braunii and its constituents. Bioresour. Technol. 98 (3), 560–564.

Reitan, K.I., Rainuzzo, J.R., Olsen, Y., 1994. Effect of nutrient limitation on fatty acid and lipid content of marine microalgae 1. J. Phycol. 30 (6), 972–979.

Ritchie, R.J., 2006. Consistent sets of spectrophotometric chlorophyll equations for acetone, methanol and ethanol solvents. Photosyn. Res. 89, 27–41.

Rizwan, M., Mujtaba, G., Lee, K., 2017. Effects of iron sources on the growth and lipid/carbohydrate production of marine microalga Dunaliella tertiolecta. Biotechnol. Bioprocess Eng. 22, 68–75.

Sakarika, M., Kornaros, M., 2019. Chlorella vulgaris as a green biofuel factory: comparison between biodiesel, biogas and combustible biomass production. Bioresour. Technol. 273, 237–243.

Sarigul, N., Korkmaz, F., Kurultak, İ., 2019. A new artificial urine protocol to better imitate human urine. Sci. Rep. 9 (1), 20159.

Satpati, G.G., Pal, R., 2015. Rapid detection of neutral lipid in green microalgae by flow cytometry in combination with Nile red staining—An improved technique. Ann. Microbiol. 65, 937–949.

Smith, S.M., Zwart, S.R., 2008. Nutritional biochemistry of spaceflight. Adv. Clin. Chem. 46, 87–130.

Teh, K.Y., Loh, S.H., Aziz, A., Takahashi, K., Effendy, A.W.M., Cha, T.S., 2021. Lipid accumulation patterns and role of different fatty acid types towards mitigating salinity fluctuations in Chlorella vulgaris. Sci. Rep. 11 (1), 438.

Wellburn, A.R., 1994. The spectral determination of chlorophylls a and b, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. J. Plant Physiol. 144 (3), 307–313.

Yeesang, C., Cheirsilp, B., 2011. Effect of nitrogen, salt, and iron content in the growth medium and light intensity on lipid production by microalgae isolated from freshwater sources in Thailand. Bioresour. Technol. 102 (3), 3034–3040.

Zavřel, T., Sinetova, M.A., Červený, J., 2015. Measurement of chlorophyll a and carotenoids concentration in cyanobacteria. Bio Protoc 5 (9) e1467-e1467.

Zhang, Y., Li, M., Gao, H., Wang, B., Tongcheng, X., Gao, B., Yu, L., 2019. Triacylglycerol, fatty acid, and phytochemical profiles in a new red sorghum variety (Ji Liang No. 1) and its antioxidant and anti-inflammatory properties. Food Sci Nutr 7 (3), 949–958.