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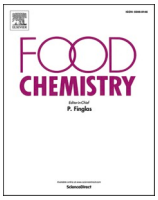

The production of FAHFA is enhanced when *Haematococcus pluvialis* is grown in CO_2

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ABSTRACT

Microalgae are considered as a potential source of bioactive compounds to be used in different fields including food and pharmaceutical industry. In this context, fatty acid esters of hydroxy-fatty acids (FAHFA) are emerging as a new class of compounds with anti-inflammatory and anti-diabetic properties. An existing gap in the field of algal research is the limited knowledge regarding the production of these compounds. Our research questions aimed to determine whether the microalga *H. pluvialis* can synthesize FAHFA and whether the production levels of these compounds are increased when cultivated in a CO_2-rich environment. To answer these questions, we used a LC-QTOF/MS method for the characterization of FAHFA produced by *H. pluvialis* while an LC-MS/MS method was used for their quantitation. The cultivation conditions of *H. pluvialis*, which include the utilization of CO_2, can result in a 10–50-fold increase in FAHFA production.

1. Introduction

Fatty acid esters of hydroxy-fatty acids (FAHFA) are a class of novel complex lipids, conserved from yeast to mammals (Celis Ramírez et al., 2020; Yore et al., 2014), with growing interest due to their distinctive characteristics and potential health advantages, demonstrating promising anti-inflammatory, antioxidant, and anti-diabetic properties (Benlebna et al., 2021; Olajide & Cao, 2022; Syed et al., 2019). Consequently, they are considered appealing prospects for the development of innovative food additives and functional foods. Yore and colleagues were the pioneers in identifying these compounds in mice adipose tissue and establishing their association with insulin sensitization (Yore et al., 2014). Their structure is composed of a fatty acid (FA) in which the carboxylic group is linked with the hydroxyl group of a hydroxy-fatty acid (HFA) through an ester bond (Riecan, Paluchova, Lopes, Brejchova, & Kuda, 2022). FAHFAs can be classified based on the position of the ester bond into two main categories. The first category consists of in-chain-branched FAHFAs, which play a role in biochemical regulation and immune reactions. The second category includes "linear" (ω-hydroxylated) FAHFAs, associated with skin protection characteristics.

FAHFAs are naturally synthesized in various organisms, including mammals, insects, and yeast (Olajide & Cao, 2022; Patel et al., 2022; Pham et al., 2019). In mammals, FAHFA are synthetised by Adipose Triglyceride Lipase (ATGL) by a transacylation reaction that esterifies HFA with FA originating from triglycerides or diglycerides (Patel et al., 2022). Furthermore, their synthesis and thus their levels depend on substrate (i.e. HFA) availability (Patel et al., 2022).

FAHFA can be found naturally in breast milk and various food sources, including cereals, fruits, vegetables, oils, eggs, and meat (Olajide & Cao, 2022). However, the levels of these compounds are relatively low, ranging from 45 to 320 ng/g of fresh food. Moreover, there is a scarcity of information concerning their absorption and bioavailability (Dyall et al., 2022). Recently, we reported the existence of branched FAHFA in the microalga *Coccomyxa melkonianii* (Fais et al., 2021). Microalgae, along with cyanobacteria, constitute a category of photosynthetic microorganisms recognized for their potential as rich sources of diverse bioactive compounds. (Jacob-Lopes et al., 2019).

In this research, our primary investigation focused on determining the presence of branched FAHFAs in the green microalga *Haematococcus pluvialis*. Subsequently, we explored the potential impact of CO_2 utilization to enhance the production of these compounds. *H. pluvialis* is known to be rich in lipids, proteins, carbohydrates and abundant in

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astaxanthin, which possesses potent antioxidant properties. The lipid content of this microalga is influenced by a range of factors, including growth conditions, climate, substrate, and cultivation method (Damiani, Popovich, Constenla, & Leonardi, 2010). On average, the lipid content of this microalga typically falls within the range of 15 to 30% of dry weight. However, under specific conditions, this value has been observed to exceed 40%. (Damiani et al., 2010; Saha et al., 2013; Wu, Yang, Hu, & Yu, 2013). Furthermore, it is known that environmental stressors and nutrient starvation play a crucial role in promoting lipid accumulation in microalgae (Sajjadi, Chen, Raman, & Ibrahim, 2018; Ma et al., 2015). We then hypothesized that supplementing microalgae cultivation with CO_2 could be a promising strategy for modulating their lipid production. Indeed, microalgae can uptake CO_2 through the CO_2-concentrating mechanism, thereby stimulating the carboxylase activity of the Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) (Su, 2021). Saifuddin et al. demonstrated that culturing *Nannochloropsis* sp. aerated with 15% of CO_2 may increase lipid production (Saifuddin, Aisswarya, Juan, & Priatharsini, 2015). Moreover, the addition of 2–10% CO_2 during growth has been shown to enhance the levels of polyunsaturated fatty acids (PUFAs) (Tang, Han, Li, Miao, & Zhong, 2011). Li et al. reported that the culture of mutated *H. pluvialis*, when cultivated with 15% of CO_2 increased lipid production (Li et al., 2017).

Over the years, several liquid chromatography coupled to mass spectrometry methods have been developed for the analysis of FAHFAs in both food and biological samples. (Brejchova et al., 2022; Brezinova et al., 2018; Fais et al., 2021; Matsuzawa et al., 2021; Zhu et al., 2017). These methods enable fast, highly sensitive, and precise identification of FAHFA species, even when they are present in trace quantities in food and biological samples. Close attention must be given to the annotation challenges associated with overlapping isomeric and isobaric species. Recently, Nelson et al., revealed that several fatty acid dimers were misidentified as FAHFA compounds (Nelson, Chow, Hughey, Crawford, & Puchalska, 2022). Furthermore, Kofeler et al., suggested that higher concentration of lipids could be cause the aggregate of FAs causing misidentification and distortion of quantitative data (Köfeler et al., 2021).

In this study, we aimed to enhance our understanding of the potential biotechnological and nutritional applications of this class of lipids. To address these objectives, we sought answers to the following questions: Can the green algae *H. pluvialis* produce FAHFA lipids? What levels of FAHFA are measured under standard growing conditions? Additionally, what levels of FAHFA, and their substrates HFA are observed when *H. pluvialis* is cultivated in a CO_2-enriched environment? Another key question in our investigation is whether the levels of carotenoids are influenced under these conditions.

2. Materials and methods

2.1. H. pluvialis growth

The green microalga *H. pluvialis* CCALA 1081 was purchased from the Culture Collection of Autotrophic Organisms in Třeboň, Czech Republic and was cultivated under axenic conditions at the laboratory of the Interdepartmental Center of Environmental Sciences and Engineering (CINSA, University of Cagliari, Italy). Two sets of batch experiments, each conducted in triplicate, were performed. The first experiment utilized *Z*-medium with air as the cultivation condition (ZM_Air). In the second experiment, Z-medium was employed with a gas phase represented by CO_2 (ZM_CO_2), pulsed at 1 atm for 3 min into a chamber sealed for 12 h during the light illumination phase of photosynthesis. We proceeded first removing, using a vacuum pump, the air inside the chamber where microalgae were grown. Then we daily introduced CO_2 (at 1 atm) in the sealed chamber.

The chemical composition of Z-medium is detailed in Table S1. Each experimental sample was obtained by cultivating the microalgae in transparent vented cap flasks filled up to 35 mL. The cultivation was carried out at 25 ± 1 °C under a 12:12 h light-dark illumination cycle of 40 μmol photons m^{-2} s^{-1} white light, with continuous agitation at 40 rpm using a Stuart SSM1 (Italy) apparatus. The optical density (OD at 750 nm) at the beginning of the experiments was 0.1 and the growth was monitored daily measuring spectrophotometric absorbance (Genesys 20, Thermo Scientific, Walthmanm Waltham, USA) of the chlorophyll-a at the same wavelength. The biomass concentration was obtained in g L-1 from OD through a calibration curve.

2.2. Biomass characterization

2.2.1. Chemicals

Analytical LC grade ethyl acetate, n-hexane, isopropanol, methanol, chloroform, acetonitrile, acetic acid, formic acid, ammonium formate, ammonium acetate, and equisplash lipidoMix were purchased from Sigma Aldrich (Milano, Italy). Solid phase extraction (SPE) cartridge S-C18 were purchased from Agela Technologies (Ca, USA). Analytical standard 10-OAHSA and 10-POHSA were purchased from Cayman chemical (Michigan, USA).

2.2.2. FAHFA extraction for untargeted and targeted analysis

The cultures of *H. pluvialis* were centrifuged at 1968 rcf for 10 min at 20 °C. After eliminating the supernatant, the pellet was resuspended in Milli-Q water and the washing procedure was repeated three times. The cell pellets were 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. Modified Folch extraction method was applied to obtain the lipid fraction (Folch, Lees, & Sloane Stanley, 1957). Briefly, 10 mg of lyophilized biomass were weighted into a polypropylene centrifuge tube, dissolved in 2 mL of deionized water, and disrupted with a sonication cycle for three times of 3 min using ExstractorOne (GM solution, Cagliari, Italy). Then, 1 mL of solution was transferred to a falcon tube and 125 μL of chloroform and 250 μL of methanol were added. Samples were then vortexed at 15-min intervals over the next hour for 1 min. Then 380 μL of chloroform and 90 μL of a solution of KCl 0.2 M were added. The samples were vortexed for 30 s. Then samples were centrifuged at 1968 rcf for 15 min at 5 °C. Four hundreds μL of the lipophilic phase was collected and dried under a gentle nitrogen steam.

For the targeted FAHFA analysis with the aim to maximize their recovery, FAHFAs extraction was performed using the modified method proposed by Kolar et al., (Kolar et al., 2018). Briefly 10 mg of lyophilized biomass were weighted within centrifuge tube and 1.5 mL of PBS, 1.5 mL of methanol and 3.0 mL of chloroform were added. The solutions were sonicated 2 min for 3 times using a ExctractorOne (GM solution, Italy). After centrifugation at 1968 rcf for 15 min the lipophilic phase was collected in a clean centrifuge tube. The lipophilic phase was dried under a gentle nitrogen stream. The dried lipophilic phase was purified using the method proposed by Kolar et al. (Kolar et al., 2018). Briefly, SPE cartridge (S C18, 200 mg/3 mL) were pre-washed with 3 mL of ethyl acetate for 2 times and then conditioned with 3 mL of n-hexane for 2 times. The lipophilic phase of each sample was reconstituted with 200 μL of chloroform and then vortexed for 60 s and applied on the SPE cartridge. Neutral lipids were eluted using 6 mL of 5% ethyl acetate in hexane, followed by elution of FAHFAs using 4 mL of ethyl acetate. Then the FAHFAs fraction was dried under a gentle nitrogen stream.

The lipophilic phase was dissolved in 20 μL of methanol/chloroform (1:1 *v/v*) and diluted with 380 μL of a 2-propanol/acetonitrile/water (2:1:1 *v/v/v*) mixture containing the internal deuterated standard. Then, the samples were analyzed with a QTOF/MS coupled to an UHPLC Agilent 1290 Infinity II LC system, injecting 8 μL in the 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 0.3 mL/min. The mobile phase for negative

ionization mode consisted of (A) 10 mM ammonium acetate solution in 60% milli-Q water and 40% acetonitrile plus acetic acid at 0.1% and (B) 10 mM ammonium acetate solution containing isopropanol/acetonitrile (9:1 *v/v*) plus acetic acid at 0.1%. Chromatographic separation was obtained with the following gradient: initially 60% of A, then a linear decrease from 60% to 50% of A in 2.1 min, then in 12.1 min at 30%. For 6.9 min staying at this percent and then brought back to the initial conditions in 2 min. Mass spectrometry parameters are reported in Table S2. Samples were acquired using an iterative auto MS/MS method with a mass error tolerance of 20 ppm with a retention time 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 and this method allows to recover a major lipids component. Then, mass spectra were used to study FAHFA ion fragmentations. FAHFAs were identified using a $[M\text{-}H]^-$ adducts and characteristic fragments such as the fatty acid (FA), the hydroxy fatty acid (HFA), and the HFA dehydration product (HFA-H_2O). Putative annotation of FAHFAs was carried out by comparing the fragment of HFA and the FA with the mass reported in an *in-silico* MS/MS library for automatic annotation of novel FAHFA lipids proposed by Fiehn lab (Ma et al., 2015). This database covered the following fatty acyl groups: 14:(0–1), 16:(0–1), 18:(0–3), 20:(0–5), 22:(0–6), 24:(0–6). Based on this information we developed a targeted MS method. Accurate masses were also compared searching each FAHFA on the online database metabolomics workbench (Powell & Moseley, 2023). The extracted ion chromatograms (EICs) and the mass spectra of each FAHFA families detected in our study were shown in supplementary materials (Fig. S1-S7). High resolution mass spectrometry of the compounds detected in this experiment are shown in Table S3.

Subsequently samples were acquired in full scan mode and the chromatographic area of each lipid was compared to the internal standards. For the hydroxy fatty acids annotation, we monitored the loss of 46 amu (as C_2H_5OH) for 16-OH hexadecanoic acid, 16-OH hexadecenoic acid, 18-OH octadecanoic acid and 18-OH octadecenoic were assigned detecting the presence of *m/z* 225, 223, 253 and 251, respectively. 13-OH hydroxyoctadecadienoic acid and 13-OH hydroxyoctadecatrienoic acid were putatively annotated detecting the characteristic presence of *m/z* 195 and 193 (loss of 100 Da involving the alcohol group) and 223 and 221 (loss of 72 Da) in the ms/ms spectrum of the precursors (*m/z* 295 and 293). Unfortunately, the position of the hydroxy group in the hexadecadienoic acid and hexadecatrienoic acid were not discerned from the ms/ms spectrum. Experimental data was subjected to statistical univariate analysis. Overlay extracted chromatograms of FA detected in our study were shown in Fig. S8A-B.

Dried FAHFAs fraction for the targeted analysis was reconstituted using 50 μL of 2-propanol and then vortexed 1 min for 2 times. Quantitative analysis was performed using an Agilent 1260 Infinity system coupled with a triple-quadrupole mass spectrometer 6470B (Agilent Technologies, Palo Alto, CA) injecting 10 μL in the negative ionization mode. Chromatographic separation of lipids was obtained with the same method described previously for untargeted analysis. Mass spectrometry parameters are reported in Table S4.

For the quantitation of FAHFA, a multiple reaction monitoring (MRM) method was employed, which involved following the MS/MS transitions for one quantifier ion and three qualifier ions (refer to Table S5 for details). The collision energies (CEs) for the MRM transitions are as follows: FA 29 V, HFA 28 V and HFA-H_2O 27 V. Quantitative analysis was performed using the following analytical standard, 10-POHSA, 10-OAHSA referring FAHFAs with fatty acyl chain with 16 carbon atoms to 10-POHSA and fatty acyl chain with 18 carbon atoms to 10-OAHSA. Six working solutions in 2-propanol in the range 1 μg/L to 500 μg/L, were carried out to build a calibration line (Fig. S9). The calibration line was considered acceptable when $R^2 > 0.997$. The results were expressed as pmol/g (mean ± SD). In fig. S10 and S11 were shown examples of extracted ion chromatogram of FAHFA, and the analytical standards used with the fragment detected.

2.2.3. Total carotenoids and chlorophyll-a determination

Total carotenoids and chlorophyll-a were estimated from dried samples modifying the method proposed by Zavrel et al. (Zavřel, Sinetova, & Červený, 2015). Briefly, 10 mg of lyophilized biomass were weighted, and 1 mL of neutralized methanol was added. Glass balls were added to each sample to form approximately a layer of 150 mm. The samples were sonicated in an ultrasonic bath for 30 min at 10 °C. The solutions were left overnight in the fridge and after 24 h the samples were first vortexed and then and sonicated in ultrasonic bath for 30 min at 10 °C. The solutions were centrifuged for 10 min at 12298 rcf. The content of chlorophyll-a and total carotenoids was estimated with a spectrophotometric analysis at λ = 720 nm, λ = 665 nm, respectively while λ = 470 nm was used for methanol as a blank. The following correlations proposed by Ritchie and Wellburn (Ritchie, 2006; Wellburn, 1994), were used to estimate the total carotenoids and chlorophyll-a concentrations, respectively:

$$\text{Chla}[\text{g/mL}] = 12.9447\ (A_{665} - A_{720})$$

$$\text{Carotenoids}[\text{g/mL}] = (1000\ (A_{470} - A_{720}286\text{Chla}[\text{g/mL}]/221$$

2.3. Statistical data analysis

Uni-variate analysis was performed with GraphPad Prism software (version 8.3.0, Dotmatics, Boston, Massachusetts). Means of each group were compared for statistical significance using the Student *t*-test. The significance levels based on the *p* -values are indicated by asterisks (*). No asterisks denotes *p*-values >0.05, * corresponds to $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$ and **** to $p < 0.0005$.

3. Results and discussion

3.1. Growth curve

The growth of *H. pluvialis* is shown in Fig. 1. After 10 d of cultivation the average concentration was 0.55 ± 0.07 g/L and 0.80 ± 0.04 g/L for ZM_Air and ZM_ CO_2, respectively. As depicted in Fig. 1, microalgae grown in CO_2 showed a longer adaptation phase compared to those grown in air, this is probably due to the pH change caused by the dissolution of CO_2 in water. Once the adaptation phase is completed, microalgae can produce higher amounts of biomass by using CO_2 as a carbon source. Indeed, after 8 d of cultivation under CO_2, the biomass of *H. pluvialis* shows a tendency to be higher when compared to cultivation under air.

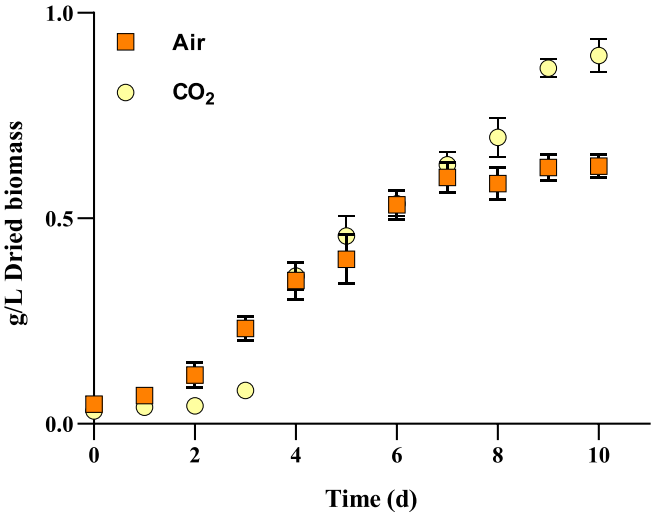

Fig. 1. Growth curves of *H. pluvialis* in ZM_Air and ZM_CO_2 grown for 10 days.

3.2. FAHFA analysis

In this study, a UHPLC-QTOF-MS platform was employed to investigate the presence of fatty acid esters of hydroxy fatty acids in *H. pluvialis* cultivated in ZM_Air and ZM_CO2 conditions. Seven families of branched FAHFA were quantified using an LC-MS/MS targeted analysis. Chemical structure of FAHFAs is reported in Fig. 2.

The detected levels of FAHFA ranged from 13 ± 4 pmol/g to 55 ± 6 pmol/g under ZM_Air conditions. Conversely, under CO2 (ZM_CO2), the levels reached values ranging from 20 to 2995 pmol/g, representing an increment of 54 times for the most abundant FAHFA. (Fig. 3 and Table 1). Higher CO_2 levels may provide more carbon substrate for microalgae metabolic processes, including FAHFAs lipid biosynthesis. Microalgae can synthesize FAHFA as part of stress-related metabolic pathways, serving as signalling molecules or upregulating specific genes that may contribute to FAHFA production. In particular, high levels of CO_2 may upregulate the activity of enzymes involved in FAHFA synthesis and thus facilitating the conversion of fatty acids into FAHFA. Further studies are needed to corroborate these assumptions.

FAHFA compounds were composed of palmitic acid (PA, C16:0), oleic acid (OA, C18:1), and the omega-3 essential fatty acid α-linolenic acid (ALA, C18:3), with the most abundant compounds being those containing palmitic acid. Indeed, α-linolenic acid must be obtained by humans exclusively through their diets. The absence of the necessary 12- and 15-desaturase enzymes makes de novo synthesis from stearic acid impossible. Dietary intake of alpha-linolenic acid (ALA) has been shown to positively impact lipid profiles by reducing levels of triglycerides, total cholesterol, high-density lipoprotein (HDL), and low-density lipoprotein (LDL) (Yue et al., 2021).

For comparison, *C. melkonianii* was found to be characterized by a unique profile of FAHFAs, incorporating acyl chains with 18 and 20 carbon atoms and varying degrees of unsaturation ranging from 1 to 4 (Fais et al., 2021).

Liberati-Čizmek et al., 2019 Olajide et al. recently reviewed the occurrence and analytical techniques used for the determination of FAHFA in foods (Olajide & Cao, 2022). For comparison levels of FAHFA produced by *H. pluvialis* are comparable with the levels found in edible mushrooms (Martínez-Ramírez, Riecan, Cajka, & Kuda, 2023).

3.3. Effect of CO_2 on fatty acids and hydroxy fatty acid biosynthesis

We also explored the changes in the levels of free fatty acids and hydroxy fatty acids, which serve as the building blocks of FAHFA, when *H. pluvialis* is cultivated in a CO_2-enriched environment. The analysis of free fatty acids by LC-QTOF/MS revealed that linolenic acid (C18:3), linoleic acid (C18:2), oleic acid (C18:1), hexadecatrienoic acid (C16:3), hexadecadienoic acid (C16:2), palmitic acid (C16:0), and stearic acid (C18:0) constituted major fatty acid components in *Haematococcus*

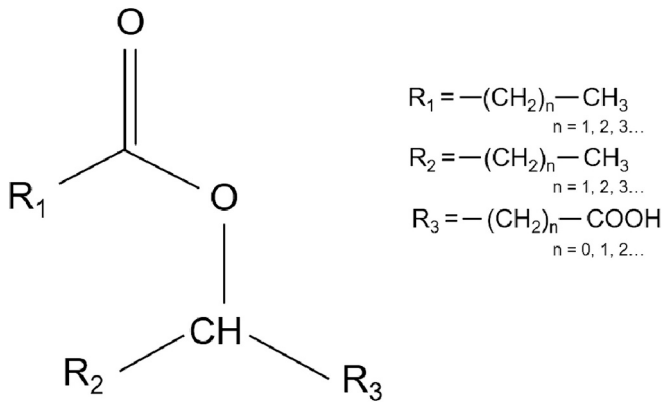

Fig. 2. General structure of FAHFAs produced by *H. pluvialis*.

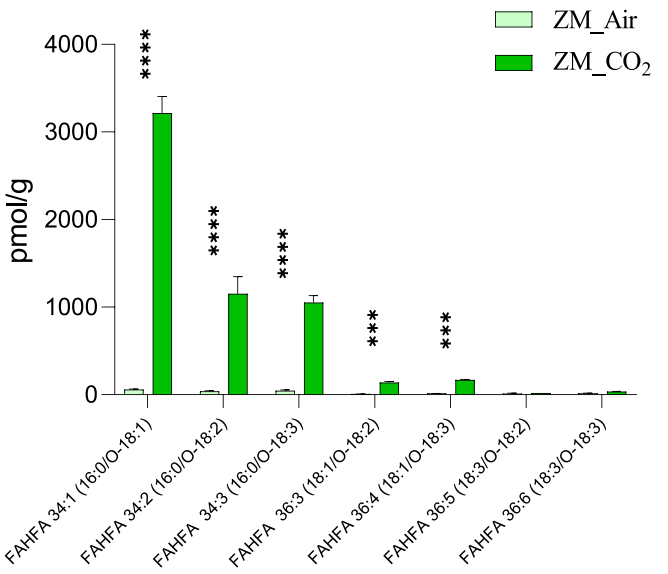

Fig. 3. Column plot of FAHFAs detected in *H. pluvialis*. The *p*-value was obtained performing Student *t*-test with Graphpad Prism software.

Table 1
Levels of FAHFAs in *H. pluvialis* grown under air and CO_2. Results are expressed as a pmol/g (mean ± SD).

FAHFA	ZM_Air	ZM_CO_2
34:3 (16:0/O-18:3)	49 ± 9	1184 ± 313
34:2 (16:0/O-18:2)	41 ± 4	1152 ± 197
34:1 (16:0/O-18:1)	55 ± 7	2995 ± 567
36:6 (18:3/O-18:3)	15 ± 3	35 ± 3
36:5 (18:3/O-18:2)	13 ± 4	18 ± 1
36:4 (18:1/O-18:3)	13 ± 2	170 ± 4
36:3 (18:1/O-18:2)	8 ± 2	144 ± 12

pluvialis grown in air. Palmitoleic acid (C16:1), methyloctadecenoic acid (C19:1), margaroleic acid (C17:1), margaric acid (C17:0), and arachidic acid (C20:0) were identified as minor components in the free fatty acid profile of *H. pluvialis* grown in air (Fig. 4). On the other hand, with CO_2 a significant increase ($p < 0.005$ and < 0.001) the levels of linolenic acid, oleic acid, linoleic acid, hexadecatrienoic acid, palmitic acid, and hexadecadienoic acid was observed (ranging from 1.8 to 3 times). However, levels of stearic acid and methyloctadecenoic acid showed a decrease (ranging from 0.8 to 1.2 times) (Fig. 3). These findings agree with the results reported by Tang et al. (Tang et al., 2011). Carbon dioxide is crucial for photosynthesis and could be an important factor to enhance the biosynthesis of fatty acids during microalgal growth (Ota et al., 2009). Our results showed that CO_2 may increase the levels of unsaturated and polyunsaturated fatty acids, and particularly the levels of oleic acid. A plausible explanation for the up regulation of unsaturated fatty acid levels could be the inhibition of enzymatic desaturation under low oxygen levels. (Tang et al., 2011; Vargas et al., 1998). Instead, low levels of CO_2 may be able to promote the accumulation of saturated fatty acids (Tang et al., 2011). Various studies, including those by Tsuzuki, Ohnuma, Sato, Takaku, and Kawaguchi (1990), Ota et al. (2009), Tang et al. (2011), Lam, Lee, and Mohamed (2012), Wang, Liang, Luo, Chen, and Gao (2014), King et al. (2015), and Liang et al. (2020), have investigated the impact of CO_2 on the biosynthesis of fatty acids using different microalgae and cyanobacteria. These studies have concluded that CO_2 has the potential to influence both the composition and content of intracellular fatty acids, and this effect may be specific to the species under consideration.

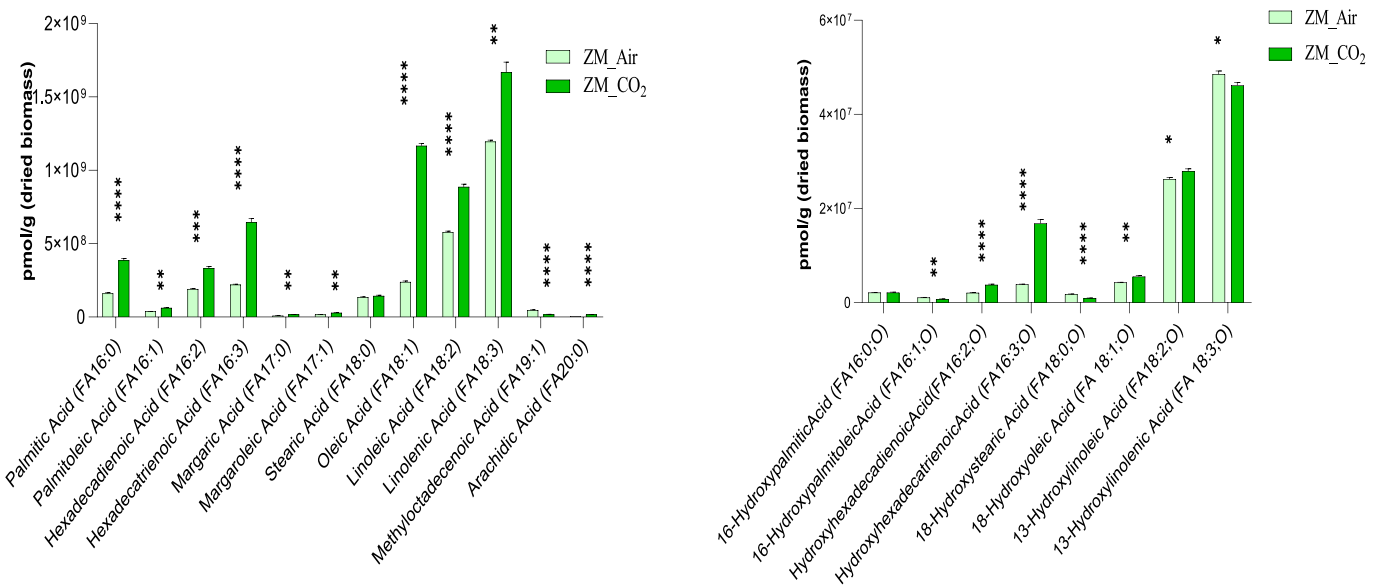

Fig. 4. Column plot of free fatty acids and hydroxy fatty acids (A and B) levels in *H. pluvialis* expressed as a ρmol/g (mean ± SD). The *p*-value was obtained performing Student *t*-test with Graphpad Prism software.

3.4. Total carotenoids and chlorophyll-a

The effect of high CO_2 on total carotenoids and chlorophyll-a produced by *H. pluvialis* vegetative cell was evaluated and compared with those obtained under normal growth conditions. The results are shown in Fig. 4. Under normal growth conditions (ZM_Air) total carotenoids levels reached 1.4 ± 0.1%. These results agree with previous work (García-Malea et al., 2005; Nobre et al., 2006). On the other hand, under CO_2 (ZM_CO_2) levels of total carotenoids decreased to the value of 1.18 ± 0.04 ($p < 0.05$). The same trends have been observed for the chlorophyll-a, but the differences were not statistically significant (see Fig. 5).

In general, it is known that high light irradiance and CO2 play a crucial role in the production of carotenoids in microalgae. In particular, Cheng et al. (2017) reported that *H. pluvialis* is able to synthesize more astaxanthin when grown under light at 250 μmol photons/m2/s2 and CO2. Furthermore, we recently observed that in the cyanobacterium Synechococcus nidulans grown under CO2 the levels of pigment are decreased (Casula et al., 2024). To explain this trend, Gordillo, Jiménez, Figueroa, and Niell (1998) hypothesized that in the cyanobacterium Spirulina platensis, CO2 may promote the degradation of pigment synthetized in excess because not essential for light harvesting. Other plausible explanation could be attributed to the effect caused by CO2 on the mevalonic pathway (MVA). In microalgae an increase in the levels of CO2 may lead to the upregulation of the mevalonic pathway (MVA) (Huang et al., 2021), as higher CO2 levels provide more carbon substrate for photosynthesis and subsequent metabolic processes. *S. nidulans* grown under CO2 conditions may allocate more carbon to other metabolic pathways or storage compounds rather than isoprenoid synthesis via the MVA pathway. Furthermore, we observed that the levels of glucose were lower when *S. nidulans* was grown in CO2. As a result, the organism can downregulate the MVA pathway due to the lack of available glucose and allocate resources to other metabolic functions, for example lipids biosynthesis. On the other hand, it is known that elevate CO2 can enhance acetyl-CoA biosynthesis and re-route more carbon flow into fatty acids biosynthesis (Liang, Wang, Wang, Zhu, & Jiang, 2019). Finally, we hypothesize that due to the combination of low light intensity and high CO2, part of acetyl-CoA synthesized through the Calvin cycle, was re-routed from MVA pathway to fatty acids biosynthesis, by reducing carotenoids biosynthesis.

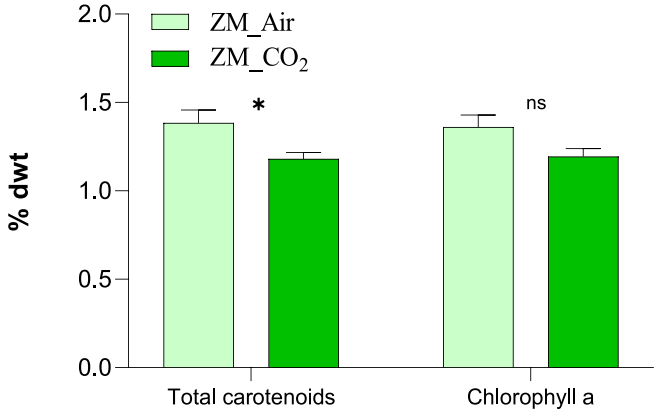

Fig. 5. Column plot of total carotenoids and chlorophyll *a* level in green vegetative palmella *H. pluvialis*, expressed as a g/100 g (mean ± SD). The p-value was obtained performing Student t-test with Graphpad Prism software.

4. Conclusions

In this work we investigated the potential use of CO_2 to improve FAHFA biosynthesis by *H. pluvialis*. Through an LC-QTOF-MS platform we were able to characterize different families of FAHFAs. On the other hand, levels of these compounds were measured with a LC-MS/MS system. We observed that the levels of these compounds were strongly upregulated when *H pluvialis* was grown in a CO_2 atmosphere with an increase of the levels up to 50 times when compared to standard growth conditions. Given that *H. pluvialis* is not directly consumed as a food source, its extract grown in CO_2, which is abundant in FAHFA and astaxanthin, can be utilized as a dietary supplement for its potential health and antioxidant advantages.

CRediT authorship contribution statement

Mattia Casula: Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Cristina Manis:** Writing – original draft, Formal analysis. **Paola Scano:** Visualization. **Alessandro Concas:** Writing – review & editing, Writing – original draft, Visualization,

Validation, Supervision. **Giacomo Cao:** Writing – review & editing, Data curation. **Pierluigi Caboni:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, 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

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2024.139165.

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