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Comparative analysis of isoprostanoid profiles in *Chlorella sorokiniana* grown under autotrophic and heterotrophic conditions

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ABSTRACT

Oxylipins are bioactive lipid mediators derived from polyunsaturated fatty acids (PUFAs), with roles in oxidative stress responses, immunomodulation, and inflammation. While microalgae are recognized as valuable sources of oxylipins, their profiles remain less studied across different species and cultivation conditions. In this study, we characterized the non-enzymatic oxylipin profile of *Chlorella sorokiniana* grown under autotrophic and heterotrophic conditions to assess the influence of cultivation strategies on their production. A total of 22 isoprostanoids, mainly Phytoprostanes (PhytoP), Phytofurans (PhytoF), Isoprostanes (IsoP), and Neuroprostanes (NeuroP). Autotrophic cultivation resulted in a higher accumulation of isoprostanoids, particularly the α-linolenic acid (ALA) derivatives, PhytoP and PhytoF species, likely due to oxidative stress induced by fluctuating light and temperature conditions. In contrast, heterotrophic growth, performed under controlled conditions, yielded lower overall oxylipin levels highlighting the presence of 10(*R*)-10-F_{4t}-NeuroP which was only present in heterotrophic *Chlorella*. We observed a correlation between the PUFA composition of *Chlorella* and its non-enzymatic oxylipin profile. Notably, several oxylipins identified in *Chlorella* have been associated with anti-inflammatory, immunomodulatory, and neuroprotective properties, emphasizing the potential of this microalga as a source of high-value bioactive oxylipins. This study paves the way to the utilization of *Chlorella* as a source of bioactive oxylipins, as well as to develop cultivation strategies to enhance the production of these lipid mediators.

1. Introduction

Microalgae are notorious reservoirs of naturally occurring bioactive lipids with diverse applications spanning as food, functional foods, supplements, cosmetics, nutraceuticals [1]. *Chlorella* sp. is a microalga approved for human consumption as food and nutraceuticals with one of the highest market-values among microalgae, namely USD 412.3 million by 2028, and an annual production of 5000 tons of dry matter [2]. This microalga is particularly rich in polyunsaturated fatty acids (PUFAs), including essential omega-3 (*n*-3) and omega-6 (*n*-6) species such as linoleic acid (LA, C18:2 *n*-6) and α-linolenic acid (ALA, C18:3 *n*-3) [3]. These PUFAs include the essential FA and are also critical dietary components due to their health-promoting properties [4], including antioxidant activity, roles as precursors of bioactive lipid mediators, and potential benefits in preventing neurodegenerative diseases and cognitive decline [5–7]. PUFAs are integral to complex polar lipids such as phospholipids (PLs) and glycolipids (GLs), which play important roles in the maintenance of cellular function and membrane integrity and properties [8], but also with intrinsic bioactivities, including anti-inflammatory, antiviral, and anti-tumor effects [9–13]. Other classes

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of bioactive lipids have been less reported, particularly a promising class of oxidized FA metabolites known as oxylipins, that are well known derivatives from PUFA [14].

Polyunsaturated FA are highly susceptible to oxidation due to the presence of carbon-carbon double bonds in their bis-allylic systems, leading to the formation of oxylipins [15]. They are bioactive lipids recognized as key lipid mediators playing critical roles in animals and plants, namely they regulate the immune response and participate in defense mechanisms against external aggressors [16,17]. Oxylipins are often considered pro-inflammatory mediators with some species reported with adverse effects [18], while other possess health-promoting properties such as anti-inflammatory, cardiovascular, and neuroprotective benefits, as well as tissue regeneration and analgesic properties [17,19–21], positioning oxylipins as promising candidates for bioprospection as novel nutraceuticals and pharmaceuticals. They have been mainly studied in animals, although they are also present in plants, where they can be formed through enzymatic or non-enzymatic pathways [22]. The oxylipins formed depend on the FA composition (precursors) of the species and have recently been studied in macro- and microalgae, although to a lesser extent, both reservoir of a plethora of PUFA. Particularly, the presence of isoprostanoids in microalgae has been recently reported in species like *Chaetoceros gracilis*, *Chlamydomonas debaryana*, *Microchloropsis gaditana* (formerly *Nannochloropsis gaditana*), *Phaeodactylum tricornutum*, *Porphyridium cruentum*, *Rhodomonas salina*, and *Tisochrysis lutea* [23–25]. Regarding *Chlorella* one study seemingly reported the presence of the oxygenated derivatives *cis*-9,10-epoxyoctadecanoic acid and 7-methyl-*Z*-tetradecen-1-ol acetate in the *Chlorella* VKM Al-335 strain [26], while in another study we reported the presence of ALA oxygenated derivatives esterified to PL and GL, although the structural features of these oxylipins were not disclosed [27].

Oxylipins in microalgae can be generated via enzymatic and non-enzymatic pathways. Enzymatically-derived oxylipins are typically formed after FA are released from membrane complex lipids, facilitated by phospholipase A2, and catalyzed by enzymes such as cyclooxygenase (COX), cytochrome P450 (CYP) and lipoxygenase (LOX) [28]. These enzymes produce an array of structurally-diverse oxylipins including prostaglandins, thromboxanes, mono-hydroxylated (e.g. hydroxyeicosatetraenoic acid, HETE, and hydroxyeicosapentaenoic acid, HEPE) or dehydroxylated oxylipins (e.g. linotrins), epoxides (e.g. epoxyeicosatrienoic acids, EET), maresins, resolvins and protectins [15,28–30].

Isoprostanoids, in contrast, are formed during oxidative stress conditions when microalgae are exposed to distinct biotic and abiotic stressors leading to the generation of reactive oxygen species (ROS) [31,32], such as hydroxyl radical ($OH^{\bullet}$), superoxide anion radical ($O2^{\bullet-}$), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2). ROS are generally produced in chloroplasts' thylakoid membranes when the absorption of light by chlorophylls exceeds the energy utilization capacity of the photosynthetic apparatus. ROS are typically scavenged by a series of adaptation mechanisms including the production of natural antioxidants and activation of enzymes capable of maintaining reduction-oxidation (redox) status. Membrane lipids are generally exposed to environmental oscillations associated with external and environmental stressors and thus are major targets of oxidation. The exposure of these processes under stress conditions allows for the formation of oxylipins characterized by their structural features as phytoprostanes (PhytoPs) and phytofurans (PhytoF) derived from ALA, isoprostanes (IsoPs) and isofurans (IsoF) derived from arachidonic acid (ARA, C20:4 *n*-6) (series 2), and eicosapentaenoic acid (EPA, C20:5 *n*-3) (series 3), and neuroprostanes derived from docosapentaenoic acid (DPA, C22:5 *n*-6) and docosahexaenoic acid (DHA, C22:6 *n*-3) [22].

The biosynthesis of oxylipins can be affected by the microalgae cultivation conditions, nutrient availability and stressors [33]. Microalgae have a remarkable plasticity to environmental challenges allowing them to adapt and thrive under distinct biotic and abiotic stressors [34], allowing for manipulation of growth conditions to produce target-oxylipins. For instance, *Chlorella* sp. can be cultivated under autotrophic, heterotrophic and mixotrophic conditions. Under autotrophy *Chlorella* uses CO_2 and solar energy to generate biomass and valuable products, while the biomass is exposed to daily fluctuations of light and temperature [35]. In in heterotrophic growth this microalga is grown in the absence of light using external organic carbon as energy source, and is typically produced in a controlled environment in fermenters [36]. These conditions significantly influence the lipid profiles of *Chlorella* sp., for instance, autotrophic biomass is characterized by a higher content of PUFA (57.5 %) and GLs (47.5 %), while heterotrophic biomass possesses higher amounts of saturated FA (SFA, 35.0 %) and PLs (18.4 %) [9]. Despite the knowledge on the impact of auto- and heterotrophic growth on the lipid profile of *Chlorella* sp., their influence on the profile of oxylipins remains undisclosed hindering the understanding of this microalga as a possible source of bioactive oxygenated lipids. Given the high abundance of PUFA in *Chlorella* sp., it is hypothesized that these microalgae produce a diverse array of isoprostanoids. Furthermore, the abundance and composition of these oxylipins are expected to vary depending on the cultivation conditions, with autotrophic and heterotrophic growth. In this study, we performed the identification of isoprostanoids in *Chlorella sorokiniana* and assessed their differences between auto- and heterotrophically grown biomass using micro liquid-chromatography mass spectrometry (microLC-MS) analysis.

2. Materials and methods

2.1. Chemicals and reagents

Commercial standards such as the isoprostanoid (5-F_{2c}-IsoP), the prostaglandins ($PGF_{2\alpha}$, 15(*R*)-$PGF_{2\alpha}$, $PGF_{3\alpha}$) and the internal standard (ISTD) d4-15-F_{2t}-IsoP were obtained from Cayman Chemicals.

Other standards (d$_4$-10-F_{4t}-NeuroP, C19-16-F_{1t}-PhytoP, and C21-15-F_{2t}-IsoP) were synthesized following previous procedures [22], including phytoprostanes (9-F_{1t}-PhytoP, 9-*epi*-9-F_{1t}-PhytoP, *ent*-16-*epi*-16-F_{1t}-PhytoP, *ent*-16-F_{1t}-PhytoP, 16-B_1-PhytoP, and 9-L_1-PhytoP) and phytofurans (*ent*-16A-13-*epi*-ST-Δ^{14}-9-PhytoF, *ent*-16B-13-*epi*-ST-Δ^{14}-9-PhytoF, *ent*-16A-9-*epi*-ST-Δ^{14}-10-PhytoF, *ent*-16B-9-*epi*-ST-Δ^{14}-10-PhytoF, *ent*-9A-12-*epi*-ST-Δ^{10}-13-PhytoF, and *ent*-9B-12-*epi*-ST-Δ^{10}-13-PhytoF) obtained from the oxidation of α-linolenic acid (C18:3 n-3, ALA); isoprostanes derived from arachidonic acid (C20:4 n-6, ARA), (5-F_{2c}-IsoP, 5(*R*)-5-F_{2t}-IsoP, and 5(*S*)-5-F_{2t}-IsoP); isoprostanes derived from eicosapentaenoic acid (C20:5 n-3, EPA), (5(*R*)-5-F_{3t}-IsoP, 5(*S*)-5-F_{3t}-IsoP); and neuroprostanes (10(*R*)-10-F_{4t}-NeuroP, 10(*S*)-10-F_{4t}-NeuroP, 4(*R*)-4-F_{4t}-NeuroP,and 4(*S*)-4-F_{4t}-NeuroP from docosahexaenoic acid (C22:6 n-3, DHA); and, finally, 4(*RS*)-4-F_{3t}-NeuroP, obtained from the oxidation of docosapentaenoic acid (C22:5 n-3, DPA n-3). "A" and "B" designate the (R) or (S) configuration, but these were not determined.

2.2. Chlorella sorokiniana autotrophic and heterotrophic cultivation

The *C. sorokiniana* 0002CA strain was obtained from the Allmicroalgae (Rua 25 Abril, s/n, 2445–413 Pataias, Portugal) culture collection. The heterotrophic inoculum *of Chlorella* was stored cryopreserved in liquid nitrogen and was collected and transferred to 50 mL Erlenmeyer flasks when necessary.

Heterotrophic *Chlorella* was grown using the heterotrophic inorganic medium (IM-medium) described by Barros et al. [37], an altered version of Guillard's F2 culture medium, containing 20 g/L of glucose. Biomass was obtained by sequentially increasing the volumes, from 250 mL Erlenmeyer's to 5 L bench-top fermenter (New Brunswick BioFlo® CelliGen®115; Eppendorf AG, Hamburg, Germany), and later, to industrial fermenters of 200 L and 5000 L. All fermenters were operated in fed-batch under controlled temperature, pH and dissolved oxygen.

Industrial scale tubular photobioreactors with a working volume of 90 m^3 were inoculated with heterotrophically grown *Chlorella* cultures,

obtained from the 5000 L industrial fermenter. These outdoors photobioreators were grown autotrophically, by subjecting the microalga to a natural circadian light:dark cycle of approximately 16 h per day and ambient temperature. Autotrophic media described by Barros et al. [35] was used: Guillard's F2 culture medium, adapted to local water and using [N] = 10 mM considering nitrate as nitrogen source. The pH was kept constant at approximately 6 by pulse injections of CO_2.

2.3. Oxylipins extraction from Chlorella sorokiniana biomass

Oxylipins were extracted using the protocol previously established in the prior work [38]. Dry biomass (100 mg) were ground with 25 μL of a 1 % (*w/v*) BHT solution prepared in methanol (MeOH) in lysing matrix tubes (MP Biochemicals, Illkirch, France) with 1 mL of MeOH and 4 μL of ISTD mix (1 ng/μL) using a FastPrep-24 (MP Biochemicals) for 30 s at a speed of 6.5 m·s^{-1}. Technical replicates were performed accordingly (n = 3). Suspensions were transferred to centrifuge tubes with 1 mL of MeOH and 1.5 mL of phosphate buffer (50 mM, pH 2.1) saturated with NaCl, and tubes were stirred for 30 min at room temperature (100 rpm-45 s/90°-15 s/0°, Multi Rotator PTR-35 Grant bio). The mixture was then centrifuged at 5000 rpm for 5 min at room temperature and the supernatant was recovered. Subsequently, 4 mL of cold chloroform (4 °C) were added and the mixture was stirred for 30 s. Then, centrifugation at 2000 rpm for 5 min at 4 °C proceeded and the organic phase was collected to Pyrex® tubes and concentrated under N_2 for an average of 1 h20 at 60 °C in a system allowing centrifugation vacuum and heat (CentriVap Labconco®).

Hydrolysis was performed with 950 μL of 1 M KOH and stirred for 30 min at 40 °C. After incubation, 1 mL of 40 mM formic acid was added before starting the solid-phase extraction (SPE) on a Biotage® Extrahera™, an automatic sample preparation system. Samples were then loaded onto Oasis MAX cartridges and successively washed with 2 mL of NH_3 2 % (*v/v*), 2 mL of MeOH/20 mM formic acid (30:70; v/v), 2 mL of hexane, and 2 mL of hexane/ethyl acetate (70:30; v/v). The retained oxylipins were eluted by adding 2 mL of hexane/EtOH/acetic acid (70:29.4:0.6; v/*v*/v). Eluats containing oxylipins were concentrated for 1 h10 at 60 °C in a CentriVap system, then reconstituted in 100 μL of mobile-phase solvents - solvent A was composed of H_2O with 0.1 % (*v*/v) HCOOH, while solvent B was composed of ACN/MeOH (8/2 v/v) with 0.1 % (v/v) HCOOH – in a solvent mixture containing 83 % of solvent A and 17 % of solvent B and subjected to two ultrasonic cycles of 1 min with a 15 min interval. Finally, the reconstituted solution was transferred to a filtration tube (Pall Corporation Nanosep PTFE 0.45 μm), centrifuged (1 min, 10000 rpm, RT) and then placed to the vial for injection.

2.4. LC-MS/MS analysis of oxylipins from Chlorella sorokiniana

LC-MS/MS analyses were conducted using an Eksigent micro-High Performance Liquid Chromatography (HPLC) 200 Plus from Sciex Applied Biosystems, Framingham, MA, USA. The system was equipped with a HALO C18 analytical column (100 × 0.5 mm, 2.7 μm; Eksigent Technologies, CA, USA), which was maintained at a temperature of 40 °C. The mobile phase used consisted of a binary gradient of two solvents—solvent A was composed of H_2O with 0.1 % (*v*/v) HCOOH, while solvent B was composed of ACN/MeOH (8/2 v/v) with 0.1 % (v/v) HCOOH. The flow rate used was 0.03 mL·min^{-1}, and the injection volume was 5 μL in total loop. The elution gradient was as follows: 17 % B at 0 min, 17 % B at 2.6 min, 21 % B at 2.85 min, 25 % B at 7.3 min, 28.5 % B at 8.8 min, 33.3 % B at 11 min, 40 % B at 15 min, and 95 % B at 16.5 min for 1.5 min.

An AB SCIEX QTRAP 5500 (Sciex Applied Biosystems, Vaughan, ON, Canada) was used to perform mass spectrometry analyses. Electrospray (ESI) was employed as the ionization source, operating in negative ion mode. The source voltage was maintained at −4.5 kV, and N_2 was utilized as the curtain gas. Detection of the fragmentation ion products from each deprotonated molecule $[M - H]^-$ was carried out in multiple reaction monitoring mode (MRM), with individually optimized MS parameters for each compound. The mass spectrometer was operated using Analyst® software (Sciex Applied Biosystems) for the LC-MS/MS data acquisition. MultiQuant 3.0 software (Sciex Applied Biosystems) was used for peak integration and quantification of analytes.

The identification of isoprostanoids in *C. sorokiniana* biomass was performed based on an internal standards mixture of individual standards synthesized in our laboratory, as reported previously [39–42]. This identification was performed based on retention time (min), precursor ion accuracy (m/z) and the observed product ion (m/z), as reported elsewhere [23]. The quantification of each individual oxylipin identified in *C. sorokiniana* was performed through linear regression based on previously established calibration curves published elsewhere [23].

2.5. Validation of sample preparation

The global process efficiency (PE), matrix effect (ME), and extraction recovery (ER) [43–45], were determined to assess the efficiency of sample preparation and validity of the isoprostanoid profiles (Supplementary Table 1).

Briefly, we prepared four different sets at high concentrations for each compound (200 ng·mL^{-1}). The first set was obtained with a spike of 5 μL using an isoprostanoid mixture (4 μg·mL^{-1}) in freeze-dried microalgae biomass (100 mg) in technical replicates before extraction. Similarly, a second set was prepared with microalgal samples being spiked after extraction.

The last two sets were as follows: extraction without microalgal matrix following spiking with 5 μL of the isoprostanoid mixture (4 μg·mL^{-1}) before the extraction step (set 3) and prepared in 100 μL of a mixture of mobile phase A solvent/B solvent (83:17; *v*/v) spiked with 5 μL of the mixture (set 4). PE was determined based of the peak areas from set 1 (spike before extraction) and the set 3 peak areas (neat extraction solution). ME was assessed by comparing set 2 peak areas (spike after extraction) and set 3 peak areas (neat extraction solution). The ER was determined as the ratio of peak areas of set 1 to set 2.

2.6. Fatty acids analysis by gas chromatography–mass spectrometry analysis

The fatty acid methyl esters were prepared from total lipid extracts by alkaline transesterification as previously described [ref]. The gas chromatography–mass spectrometry (GC–MS) analyses were performed on an Agilent 6890 N gas chromatograph interfaced with an Agilent 5973 mass spectrometer (Agilent, Santa Clara, CA, USA) with electron impact ionization (70 eV). A DB-FFAP capillary column (30 m × 0.32 mm, 0.25 μm film thickness (J&W Scientific, Folsom, CA, USA)) was used. The following conditions were used: helium as carrier gas (constant flow 1.4 mL min^{-1}), inlet temperature 220 °C, detector temperature 280 °C, injection volume 2 μL (splitless). The oven temperature was programmed as follows: 80 °C for 3 min, 25 °C min^{-1} to 160 °C, 2 °C min^{-1} to 210 °C, 30 °C min^{-1} to 250 °C (held for 10 min). Three technical replicates of each lipid extract from each *Chlorella* were analysed. Fatty acids were identified by comparing their retention times with those of commercial standards (Supelco 37 Component Fame Mix, Sigma-Aldrich) and by comparison of their mass spectra fragmentation pattern to that of NIST Library. Results were expressed as relative abundance (%) and are described in Supplementary Table S1.

2.7. Statistical analysis

The statistical analysis was performed using the GraphPad Prism 8.0.1 software. Multiple *t*-test comparing each individual oxylipin were performed with a Holm-Sidak correction for multiple comparisons ($p <$ 0.05).

3. Results

3.1. Analysis of the extraction recovery and matrix effect

In this work we characterized the presence of isoprostanoids in the microalga *C. sorokiniana* cultivated under autotrophic and heterotrophic conditions using target lipidomics approaches.

Firstly, we evaluated the extraction of oxylipins from auto- and heterotrophic *Chlorella* to validate the obtained extracts for oxylipins analysis. For this, we determined the extraction recovery (ER) and matrix effect (ME), which are specific for each compound, to assess the efficiency of sample processing, as solid phase extraction (SPE) cartridges can significantly alter the quantitative values of these metabolites. These results are described in detail in Supplementary Tables S2. The ER evaluates product losses due to retention on the SPE cartridges and/or by partial elution during washing steps to separate other components. The ER values ranged between an average of 48 ± 19 % for hetero-*Chlorella* and 84 ± 10 % for auto-*Chlorella*. For most oxylipins identified in auto-*Chlorella* compound loss during SPE was low (<20 %), while in hetero-*Chlorella* some compound loss was observed (<50 %). While in hetero-*Chlorella* no specific trends were observed for the different types of compounds (PhytoPs, PhytoFs and IsoPs), in auto-*Chlorella* recovery of NeuroPs and enzymatic oxylipins was slightly lower. An ER above 100 % was observed for three identified oxylipins corresponding to a possible co-elution of a compound with the same MRM transition. Then, we assessed the ME which corresponds to an ion-suppression/enhancement of co-eluted matrix compounds. The ME was relatively low in auto-*Chlorella* extracts with an average of 23 ± 8 % and relatively high in hetero-*Chlorella* with an average of 61 ± 5 % indicating isoprostanoid retention in the matrix. A negative value was observed for one PhytoF (−14 %) in auto-*Chlorella* (Supplementary Table S1), indicating a possible enhancement of co-eluted matrix compounds. The SPE process and the matrix contributed to high overall process efficiency, with losses ranging from 14 ± 5 % in auto-*Chlorella* to 23 ± 17 %% in hetero-*Chlorella*.

3.2. Identification of the profile of isoprostanoids in Chlorella sorokiniana

The isoprostanoid profile of *C. sorokiniana* biomass cultivated under autotrophic and heterotrophic conditions was characterized using LC-MS/MS. This approach enabled the identification of a total of 22 non-enzymatic (Table S3), classified into four major classes, namely PhytoP (6), PhytoF (6), IsoP (6) and NeuroPs (4), while the enzymatic derivatives were identified as prostaglandins (3).

The variability of the 22 oxygenated metabolites identified in *C. sorokiniana* under auto- and heterotrophic conditions was inferred by quantification of the collected data. These results allowed to identify some variability between the oxylipin profile of *C. sorokiniana* grown under autotrophic and heterotrophic conditions. The amounts of each oxylipin varied according to the culturing conditions, observing an overall higher concentration of each identified species in auto-*Chlorella* when compared to hetero-*Chlorella* (Table S2). Also, one species was only detected in auto-*Chlorella*, namely 15-F_{2t}-IsoP, while the non-enzymatic 10(*R*)-10-F_{4t}-NeuroP was only observed in hetero-*Chlorella*. The following sections will provide a detailed description of the identified enzymatic and isoprostanoids, highlighting their respective concentrations and differences in abundance depending on the cultivation conditions.

3.2.1. Non-enzymatic alpha-linolenic acid derivatives in autotrophic and heterotrophic Chlorella sorokiniana

The oxylipin derived from omega-3 ALA derivatives identified d in *C. sorokiniana* belonged to the classes of phytoprostanes (6; PhytoP) and phytofurans (6; PhytoF). The PhytoP species included the metabolites 9-*epi*-9-F_{1t}-PhytoP, 9-F_{1t}-PhytoP, *ent*-16-*epi*-16-F_{1t}-PhytoP, *ent*-16-F_{1t}-PhytoP, 16-B_1-PhytoP and 9-L_1-PhytoP, and the PhytoF species were *ent*-16A-13-*epi*-ST-Δ^{14}-9-PhytoF, *ent*-16B-13-*epi*-ST-Δ^{14}-9-PhytoF, *ent*-16A-9-*epi*-ST-Δ^{14}-10-PhytoF, *ent*-16B-9-*epi*-ST-Δ^{14}-10-PhytoF, *ent*-9A-12-*epi*-ST-Δ^{10}-13-PhytoF and *ent*-9B-12-*epi*-ST-Δ^{10}-13-PhytoF.

In general, the total concentration of PhytoP and PhytoF was higher in auto-*Chlorella,* 5099 ± 131 and 2144 ± 50 pg/mg of biomass, respectively, when compared to hetero-*Chlorella* (1413 ± 78 and 437 ± 19 pg/mg, respectively). Each individual PhytoP and PhytoF had a significantly higher content in auto-*Chlorella* (Fig. 1A-B). The most abundant PhytoP in auto-*Chlorella* was 9-F_{1t}-PhytoP (1080 ± 35 pg/mg), while in hetero-*Chlorella* the most abundant PhytoP was 16-B_1-PhytoP (572 ± 38 pg/mg). The non-enzymatic *ent*-16B-9-*epi*-ST-Δ^{14}–10-PhytoF was the most abundant PhytoF in both auto-*Chlorella* (862 ± 25 pg/mg) and in hetero-*Chlorella* (165 ±8 pg/mg).

3.2.2. Non-enzymatic arachidonic acid and eicosapentaenoic acid derivatives in autotrophic and heterotrophic Chlorella sorokiniana

Four non-enzymatic ARA derivatives were identified in *C. sorokiniana*, namely the isoprostanes species 5-F_{2c}-IsoP, 5(*RS*)-5-F_{2t}-IsoP, 15-*epi*-15-F_{2t}-IsoP and 15-F_{2t}-IsoP. In addition, two non-enzymatic EPA derivatives were detected including 5(*R*)-5-F_{3t}-IsoP and 5(*S*)-5-F_{3t}-IsoP.

Total isoprostanes (IsoP) levels were higher in auto-*Chlorella* (153 ± 9 pg/mg) when compared to hetero-*Chlorella* (31 ± 6 pg/mg). The most abundant ARA-IsoP species was 5-F_{2c}-IsoP achieving 43 ± 1 pg/mg in auto-*Chlorella* and 3 ± 0 pg/mg in hetero-*Chlorella*, as observed in Fig. 2. On the other hand, the most abundant EPA-IsoP species was 5(*R*)-5-F_{3t}-IsoP achieving 50 ± 5 in auto-*Chlorella* and 18 ± 4 in hetero-*Chlorella*.

3.2.3. Non-enzymatic docosapentaenoic acid and docosahexaenoic acid derivatives in autotrophic and heterotrophic Chlorella sorokiniana

Regarding DPA and DHA non-enzymatic derivatives, we identified four species in *C. sorokiniana*, namely the DPA neuroprostane 4(*RS*)-4-F_{3t}-Neuro$P_{DPA\omega\text{-}3}$, and the three DHA neuroprostanes 4(*RS*)-4-F_{4t}-NeuroP, 10(*R*)-10-F_{4t}-NeuroP and 10(*S*)-10-F_{4t}-NeuroP. Similarly to the other derivatives, the total amount of neuroprostanes in auto-*Chlorella* was 23 ± 1 pg/mg of DWB and 5 ± 2 pg/mg in hetero-*Chlorella* (Fig. 3). The most abundant neuroprostane was the DPA derivative 4(*RS*)-4-F_{3t}-Neuro$P_{DPA\omega\text{-}3}$ in auto-*Chlorella* (17 ± 1 pg/mg of DWB) and the DHA derivative 4(*RS*)-4-F_{4t}-NeuroP in hetero-*Chlorella* (2 ± 1 pg/mg of DWB). Each individual neuroprostane was lower in the biomass grown under heterotrophic conditions, compared to autotrophic cultivated biomass.

4. Discussion

The presence of oxygenated FA metabolites with bioactive properties (e.g. anti-inflammatory) in microalgae has prompted research on their profiling, aiming to identify novel sources of these biocompounds. Several isoprostanoids, have been detected across different microalgae, such as *P. tricornutum*, *R. salina*, *T. lutea* and *C. gracilis* [23], however this represents a small pool of the available microalgae species. Furthermore, some studies have indicated that the oxylipins profile of these organisms is affected by different biotic and abiotic stresses [31,33], providing insights into the manipulation of microalgae cultivation to enhance production of these lipid mediators. However, very few studies have assessed the influence of cultivation conditions on the production of oxylipins in microalgae, leaving this topic of research severely understudied. *Chlorella* sp.is a Chlorophyte approved for consumption as food and food supplements, described as a source of high-value lipids [9], although its oxylipins profile is still unknown. Furthermore, this microalga can be grown under autotrophic and heterotrophic providing biomasses with different lipid compositions which in turn can translate into different oxylipins profiles. Thus, in the present study, we have identified isoprostanoids profile of *C. sorokiniana,* using technical replicates, and determined how autotrophic and heterotrophic growth influence their composition.

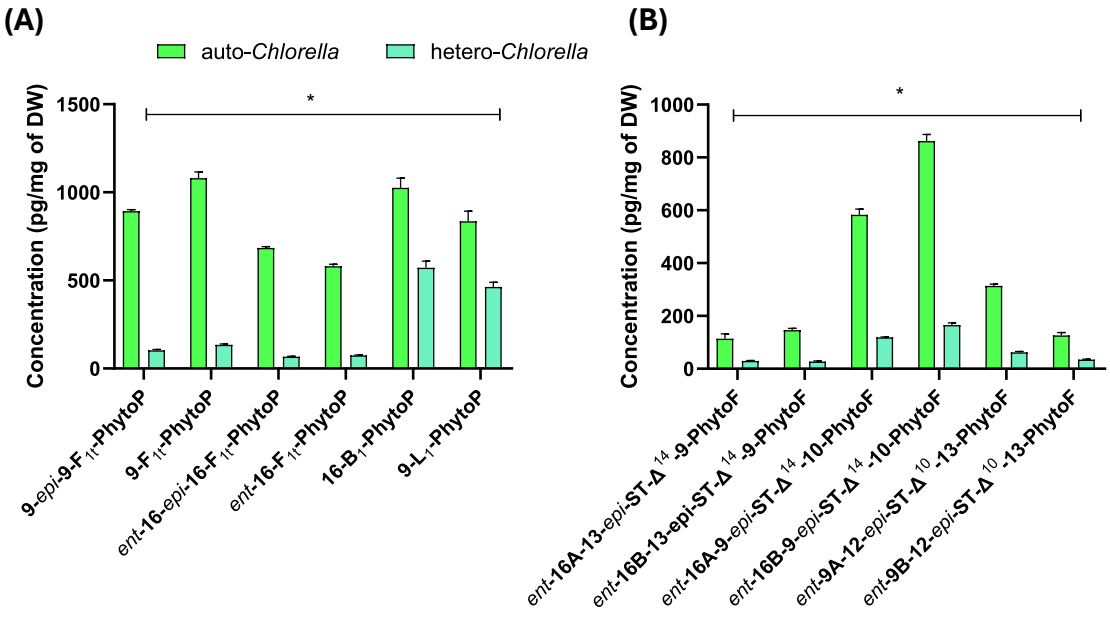

Fig. 1. Non-enzymatic oxygenation metabolites derived from α-linolenic acid identified in *Chlorella sorokiniana* grown under autotrophic and heterotrophic conditions. The identified (A) phytoprostanes (PhytoP) and (B) phytofurans (PhytoF) are expressed in pg/mg of dry weight (DW) biomass as mean ± SD of three technical replicates ($n = 3$). * indicates statistically significant differences between the two conditions ($p < 0.05$).

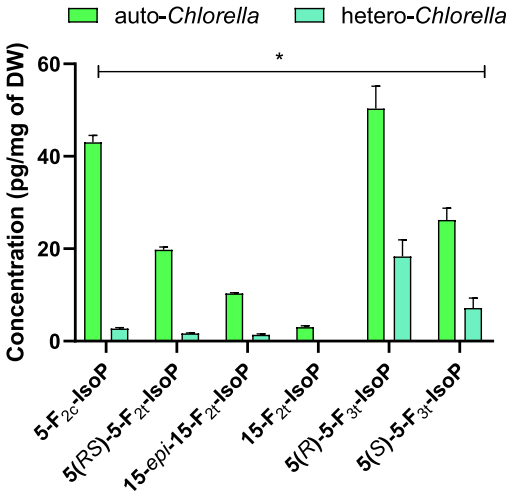

Fig. 2. Non-enzymatic oxygenation metabolites derived from arachidonic acid (ARA) and eicosapentaenoic acid (EPA) in *Chlorella* grown under autotrophic and heterotrophic conditions. The identified isoprostanes derived from ARA and EPA are expressed in pg/mg of dry weight (DW) biomass as mean ± SD of three technical replicates ($n = 3$). * indicates statistically significant differences between the two conditions ($p < 0.05$).

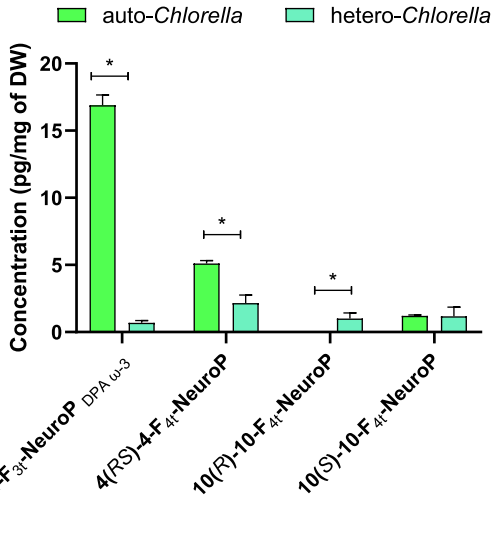

Fig. 3. Non-enzymatic oxygenation metabolites derived from docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA) in *Chlorella sorokiniana* grown under autotrophic and heterotrophic conditions. The identified neuroprostanes DPA and DHA derivatives are expressed in pg/mg of dry weight (DW) biomass as mean ± SD of three technical replicates ($n = 3$). * indicates statistically significant differences between the two conditions ($p < 0.05$).

Investigation on oxylipins from microalgae has defined the presence of both enzymatic and non-enzymatic derivatives in these organisms. Particularly, enzymatic-derived oxylipins have been well-documented in red macroalgae and more recently in diatoms [46,47]. Recently, many works have reported the presence of isoprostanoids in microalgae, typically distributed across the classes of PhytoP, PhytoF, IsoP and NeuroP [23,25,33]. In the present work, we have identified a total of 22 isoprostanoids in *C. sorokiniana*, comprising mainly of PhytoP and PhytoF species (ALA derivatives), IsoP from series 2 and 3 (ARA and EPA derivatives), and DPA and DHA derivatives (NeuroP). According to the GC–MS analysis performed in the present study, the most abundant PUFA species present in *C. sorokiniana* were ALA, 32.0 ± 0.8 and 8.4 ± 0.2 % in auto-*Chlorella* and hetero-*Chlorella*, respectively, and LA, 17.0 ± 0.2 and 34.3 ± 1.0 % in auto-*Chlorella* and hetero-*Chlorella*, respectively (Table S1). These results are in line with previous studies determining the FA composition of *C. sorokiniana* and indicating a high abundance of ALA and LA [3]. Moreover, this is consistent with the oxylipins results that revealed ALA oxygenated derivatives as the most abundant species (7.2 ng/mg of biomass) in *C. sorokiniana*, thus suggesting a correlation between the FA and the oxylipins profiles. This correlation with the FA profile is consistent with previous reports on the oxylipins profiling of *R. salina, T. lutea* and *C. gracilis* [23], which were characterized by a high abundance of oxylipins derived from their most abundant FA. Regarding LA this FA is present in high abundances in

C. sorokiniana however, due to the lack of commercially available internal standards for isoprostanoids, it was not possible to identify them in the present study.

Regarding previous reports on oxygenated FA derivatives, we have previously reported the presence of oxidized complex lipids with a prevalent presence of oxygenated ALA metabolites esterified to PL and GL in *Chlorella* sp. [27], although their structural features were not disclosed. Another this study identified two other oxylipins in the *Chlorella* VKM Al-335 strain, namely *cis*-9,10-epoxyoctadecanoic acid and 7-methyl-*Z*-tetradecen-1-ol acetate [26], although these oxidized metabolites were not detected in the present study. Compared to plant-based sources where only ALA-derived oxylipins such as PhytoP and PhytoF are typically present, namely palm fruit and pumpkin byproducts (seeds, pulp, oil) [48,49], and *Acacia cyanophylla* [50], the microalga *C. sorokiniana* demonstrates a higher yield and broader structural diversity of oxylipins, including long-chain derivatives from EPA and DHA. This unique profile supports its potential for high-value applications in health-related and nutraceutical sectors.

The oxylipin profile can be modulated using different cultivation strategies through the application of biotic and abiotic stressors [31,33]. External factors that induce oxidative stress such as saline stress, H_2O_2, copper stress and ethyl acetate alter PUFA metabolism and oxylipin production in microalgae as observed for species such as *Dunaliella salina*, *T. lutea, P. tricornutum* and *R. salina* [33,51]. These studies revealed an increase in non-enzymatic oxylipin production in cultures under stress conditions compared to control cultures. Our results similarly indicate that *Chlorella* cultivated under autotrophic conditions exhibited higher levels of isoprostanoids compared to heterotrophic cultures. As autotrophic *C. sorokiniana* was cultivated in outdoor photobioreactors where daily fluctuations in temperature and light intensity can subject biomass to increased oxidative stress, thus leading to an increase in ROS production and enhancing susceptibility for PUFA oxidation [32,36]. In contrast, heterotrophic cultivation was conducted in closed fermenters under controlled conditions, reducing the exposure to external environmental fluctuations such as light and temperature [36], thereby reducing oxidative stress conditions, ROS production and PUFA oxidation. However, ER and ME results were indeed lower in heterotrophic *Chlorella* compared to autotrophic *Chlorella* which should be taken into account. Interestingly, while most oxylipins were more abundant under autotrophic conditions, the neuroprostane 10(*R*)-10-F_{4t}-NeuroP was exclusively detected in heterotrophic *Chlorella*. Although present in low quantities, its selective occurrence suggests that it may serve as a potential marker for heterotrophic growth. Further investigation is needed to determine its biological significance and potential role in oxidative stress adaptation under heterotrophic growth.

Beyond oxidative stress biomarkers, oxylipins have intrinsic biological properties necessary for algae survival, namely generated against external pathogens and aggressors. Particularly, those derived from omega-6 FA are often associated with pro-inflammatory and adverse effects [52,53]. However, some oxylipins possess interesting bioactive properties with health-promoting benefits involving inflammation and immunomodulation. Although several studies on algae oxylipins have explored the bioactive impact of enzymatic-derived oxylipins, isolated studies have demonstrated the potential of isoprostanoids as bioactive compounds, in particular, omega-3-derived isoprostanoids [54], as the ones identified in *C. sorokiniana* in the present work. Particularly, PhytoP and PhytoF species, in concentrations ranging from 0.002 to 100 μM, have been associated with immunomodulatory, anti-inflammatory, apoptosis and neuroprotection properties [55]. 9-F_{1t}-PhytoP and 16-B_1-PhytoP were the most abundant PhytoP species observed in both auto- and hetero-*Chlorella*. B1-PhytoPs, for example, can inhibit NF-κB activation in human embryonic kidney cells and reduce NO production on RAW264.7 macrophages, while conferring neuroprotection against oxidant injury and promote myelination through PPAR-γ affecting immature neuroblasts and oligodendrocytes [56]. In another study,16-B_1-PhytoP and *ent*-16(*RS*)-9-*epi*-ST-Δ^{14}–10-PhytoF modulated LPS-induced prostaglandins production in THP-1 monocytes, namely decreasing levels of $PGF_{2\alpha}$, and increasing intracellular levels of 15-keto-$PGF_{2\alpha}$ [57]. Regarding 9-F_1-PhytoP little is known about its bioactive potential, namely extracts containing 9-F_{1t}-PhytoP, 9-L_1-PhytoP, and *ent*-16(*RS*)-13-*epi*-ST-Δ^{14}–9-PhytoF reduced expression of ICAM-1 and IL-6 in endothelial cells in the presence of the pro-inflammatory stimulus TNF-α, and vasorelaxation properties through induction of eNOS expression [58]. High levels of F_1-PhytoP species have been observed in healthy men supplemented with flaxseed oil [59], suggesting a contribution of these isoprostanoids to the overall well-being of individuals. Three PhytoP, including the 9-L_1-PhytoP, which was highly abundant in *C. sorokiniana*, combined with a sub-cytotoxic dose of the chemotherapy agent, Doxorubicin, exhibited cytotoxic effects on the MCF-7 breast cancer cell-line [60]. In this context, the presence of these oxylipins in *C. sorokiniana* emphasizes the benefits of this microalga as a source of high-value compounds with health-promoting benefits.

Other groups of bioactive oxylipins described in *C. sorokiniana* are the C20- and C22-derived metabolites IsoP and NeuroP. These oxylipins were less abundant in auto- (152.70 and 23.20 pg/mg of DWB) and in hetero-*Chlorella* (31.24 and 5.01 pg/mg of DWB), when compared to PhytoP and PhytoF. EPA and DHA-derived oxylipins have been recognized as immunomodulatory and anti-inflammatory compounds. These derivatives are capable of inhibiting LPS-induced activation of the transcription factor NF-kB and the consequent induction of mRNA for cell adhesion molecules in U937 monocytes [61]. Regarding was 5(*R*)-5-F_{3t}-IsoP and 4(*RS*)-4-F_{3t}-NeuroP, the most abundant IsoP and NeuroP identified in *C. sorokiniana*. F3-IsoP are considered useful biomarkers of oxidative stress, and have demonstrated interesting properties as modulators of neurotransmitters release, in particular epimers of 5-F_{3t}-IsoP [62], as these isoprostanoids have shown regulation of glutamate and dopamine release through mechanisms partially dependent on activation of pre-junctional prostanoid EP1-receptors [63,64]. This regulation can indirectly mitigate damage caused by oxidative stress in the retina, preventing the development of ocular neuropathies [62]. To the extent of our knowledge no study has explored the bioactive effects of the DPA-derived NeuroP. On the other hand, detected 4-F_{4t}-NeuroP in this microalga was described with significant anti-inflammatory effects against mRNA expression of pro-inflammatory markers, decreasing expression levels of TNF-α and IL-6 [65]. External administration of 4-F_{4t}-NeuroP provided neuroprotective effects in the neuroblastoma cell line, SH-SY5Y cells, and brain tissue from male Sprague Dawley rats and animal models by elevating levels of anti-inflammatory and pro-resolving lipid mediators and upregulating the transcriptional level of the antioxidant enzyme heme oxygenase-1 [66]. Neuroprotective effects were also observed on BV2 microglia cells through attenuation of ROS production after LPS stimulation, and suppression of NFκB-p65 pathway and the pro-inflammatory biomarkers, iNOS and TNF-α [67]. Other observations included suppression of LPS-induced mitochondrial dysfunction of BV2 cells, while simultaneously up-regulating Nrf2/HO-1 antioxidative pathway. Another study reported that administration of 4-F_{4t}-NeuroP before ischemia limits infarct size and reduces the occurrence of ventricular arrhythmias in ventricular cardiomyocytes and in post-myocardial infarcted mice [21].

5. Conclusion

This study provides a characterization of isoprostanoids present in *Chlorella sorokiniana* cultivated under autotrophic and heterotrophic conditions, revealing distinct oxylipin metabolic responses associated with each growth strategy. Our findings highlight the presence of mainly isoprostanoids, particularly PhytoP and PhytoF derived from ALA. Notably, autotrophic conditions led to a higher accumulation of isoprostanoids, likely due to increased oxidative stress from imbalance in the oxidative status during the onset and after photosynthesis fluctuating light and temperature conditions, whereas heterotrophic growth, under controlled conditions, resulted in lower overall oxylipin levels but

uniquely featuring the NeuroP 10(*R*)-10-F4t-NeuroP. These findings suggest that growth conditions can be strategically employed to influence oxylipin production, potentially enhancing generation of bioactive lipids in *Chlorella* sp. Given the known immunomodulatory, anti-inflammatory, and neuroprotective properties of several identified oxylipins, emphasizing *C. sorokiniana* as a valuable candidate for high-value applications. It is important to acknowledge the identification of several omega-6 derived oxylipins which are usually associated with pro-inflammatory and adverse effects, although these species were present in low abundances. Future studies should focus on elucidating the metabolic responses of *C. sorokiniana* to other growth conditions to further explore enhancement of bioactive oxylipins production, as well as the bioactivity and bioavailability of the identified oxylipins, as this microalga is an edible species.

CRediT authorship contribution statement

Tiago Conde: Validation, Investigation, Data curation, Conceptualization, Writing – review & editing, Writing – original draft. **Diana Lopes:** Investigation, Data curation, Conceptualization, Writing – review & editing. **Valérie Gros:** Investigation, Data curation. **Guillaume Reversat:** Investigation, Data curation. **Camille Oger:** Investigation, Data curation. **Jean-Marie Galano:** Investigation, Data curation. **Claire Vigor:** Investigation, Data curation. **Alexandre M.C. Rodrigues:** Validation, Writing – review & editing. **Natacha Coelho:** Investigation, Data curation, Writing – review & editing. **Helena Cardoso:** Validation, Writing – review & editing. **M. Rosário Domingues:** Validation, Supervision, Investigation, Funding acquisition, Data curation, Conceptualization, Writing – review & editing, Writing – original draft. **Thierry Durand:** Validation, Supervision, Investigation, Funding acquisition, Data curation, Writing – review & editing, Writing – original draft.

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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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2025.104424.

Data availability

Data will be made available on request.

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