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Chlorella vulgaris in a heterotrophic bioprocess: Study of the lipid bioaccessibility and oxidative stability

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ABSTRACT

Microalgal biomass is an emerging source of several health-related compounds, including polyunsaturated fatty acids. Herein, *Chlorella vulgaris* was cultivated heterotrophically in a 16-L stirred tank bioreactor. The lipid oxidative stability and lipid bioaccessibility of the biomass harvested during the exponential and stationary phases were evaluated. The biomass harvested during the stationary phase showed lower lipid oxidation than that harvested during the exponential phase, likely due to the higher content of antioxidants in the former. In both biomasses, the hexanal and propanal profiles showed only moderate increase over 12 weeks of storage at 40 °C, indicating good oxidative stability. Lipid bioaccessibility measured in an infant in vitro model was 0.66% ± 0.16% and 2.41% ± 0.61% for the biomass harvested during the exponential and late stationary phases, respectively. This study indicates that *C. vulgaris* biomass can be considered as a stable and nutritious (optimal ω3:ω6 profile) source of essential fatty acids. Our results suggested that regarding lipid stability and bioaccessibility, harvesting during stationary phase could be preferred choice. In general, treatment of the biomass to increase lipid bioaccessibility should be investigated.

1. Introduction

Microalgae are well known microorganisms for their high growth rate and limited competition with existing agriculture since they can be grown on non-arable land [1]. Microalgae can be grown in photoautotrophic mode: in presence of light and carbon dioxide, microalgae obtain their energy through photosynthesis. Certain microalgae species can grow also in heterotrophic conditions. In dark environments, microalgae need an additional organic carbon source (e.g., glucose, glycerol) as an energy supply [2,3]. Species can be strictly photoautotrophic or heterotrophic or able to grow in both modes (mixotrophic) (e.g., *Chlorella vulgaris* and *Auxenochlorella protothecoides*) [4]. Heterotrophic cultivation has been shown to lead to higher biomass productivity and can be thus considered to be more sustainable than photoautotrophic based production so far [1].

Microalgal biomass is an emerging source of several nutrients and health related compounds, such as polyunsaturated fatty acids (PUFAs), proteins, vitamins and antioxidants. Only a few species are allowed to be consumed as whole biomass in Europe according to EU Food regulation, including *Chlorella vulgaris* [5].

Microalgae lipids are divided into neutral lipids (triacylglycerides, diacylglycerides, and sterol esters), mainly located in lipid droplets in the cytoplasm or plastids, and polar lipids (phospho- and glycolipids), which build the cellular membranes [6]. Microalgae are the primary producers of PUFAs (ω3- and ω6-PUFAs), as microalgae contain the desaturation enzymes (acyl-CoA desaturases, acyl-ACP desaturases, and acyl-lipid desaturases) to produce these compounds [7]. *C. vulgaris* contains significant amounts of PUFA: up to 1.5% and 3.7% linoleic acid (LA, C18:2n6) and α-linolenic acid (ALA, C18:3n3), respectively, are present in dried biomass [8]. These essential fatty acids are well known to play a critical role in the growth and development of infants and young children [9]. Microalgae nutrients profile makes them interesting for infant nutrition applications.

One of the main issues related to PUFAs (ω3 and ω6) is that these compounds are very susceptible to oxidation due to their large number of double bonds. The oxidation process promotes the formation of off-flavours and the loss of nutritional value [10]. Natural endogenous antioxidants in microalgae, such as carotenoids and tocopherols, can protect PUFAs from oxidation [11]. Additionally, maintaining the integrity of the whole cell structure in microalgae has been shown to

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protect lipids from oxidation during wet storage post-harvest [12].

However, the presence of an intact and resistant cell wall may limit lipid bioaccessibility during digestion due to the presence of indigestible polysaccharides in the cell wall [13]. Only a few studies have investigated the bioaccessibility of microalgal lipids either by in vitro digestion model or by animal studies. An in vitro study on *Isochrysis galbana* showed that lipid bioaccessibility in adult digestion was less than 15% [14]. The in vitro bioaccessibilities of lutein and β-carotene (lipophilic compounds) were measured to be 7% and 0%, respectively, in *C. vulgaris* [15]. Lemahieu et al. studied ω3-PUFAs enrichment of eggs in laying hens fed with *Nannochloropsis* and *Isochrysis* [16]. These researchers showed that less than 44% ω3-PUFAs were effectively accumulated in the egg, with the highest enrichment obtained with microalgae biomass disrupted by high pressure homogenization at 100 MPa with 3-4 passes.

The cell wall mechanical stability, thickness and composition vary significantly within and between microalgae species and may depend on several process-related factors, such as the growth phase and harvesting time [17]. Reportedly, faster microbial growth leads to weaker cell wall [17]. Moreover, Safi et al. [13] reported that the nascent cell wall of *C. vulgaris* is usually thin and fragile, while the thickness increases progressively in the developed stage. During starvation or limited growth conditions, physiological signals may trigger the cells to reinforce the cell wall to prepare for survival [17]. In addition, the content of antioxidants may change over the growth, and therefore, the oxidative stability of the biomass may change. Enrichment of endogenous antioxidants was reported when harvesting a fermentation in the stationary phase instead of the exponential phase [18].

To date, no researchers have investigated the variation of lipid bioaccessibility and oxidative stability in microalgae biomass over the growth. Moreover, the lipid bioaccessibility of microalgae biomass in infants remains unknown. The aim of this study was to evaluate the lipid bioaccessibility and oxidative stability of *Chlorella vulgaris* biomass grown in heterotrophic conditions. In particular, this evaluation was carried out in different phases of the growth of a controlled cultivation process: the exponential, early stationary and late stationary phases. In this study, we aimed to provide further insights regarding the harvesting time during the growth process, with a focus on the nutritional and organoleptic aspects.

It was hypothesized that the oxidative stability of the microalgal biomass may be influenced by the presence of antioxidants. Therefore, biomass harvested in the stationary phase is expected to be more oxidatively stable than that harvested in exponential phase. Furthermore, it is hypothesized that the lipid bioaccessibility is higher in exponential phase biomass because of the weaker cell wall.

2. Material and methods

Materials were all standard analytical grade, except for the solvents used for methods 2.6 and 2.7, which were High Performance Liquid Chromatography (HPLC) grade. Chemicals and enzymes were purchased from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland). Pepsin (P7000, Sigma-Aldrich) and pancreatin (8XUSP, P7545, Sigma-Aldrich) were of porcine origin. Rabbit gastric extract (RGE) was provided by Lipolytech (Marseille, France). Egg phospholipid was provided by Cargill (Hamburg, Germany).

2.1. Microalgae and culture conditions

C. vulgaris (CCALA 256) was obtained from the Culture Collection of Autotrophic Organisms in the Czech Republic. Cells were maintained on sterile Bold's basal medium (BBM) at 25 °C, 150 rpm, and continuous illumination at 36 μmol photons m^{-2} s^{-1} in a shaking incubator (Infors HT Multitron, Bottmingen, Switzerland).

The cultures were inoculated with 10% (v v^{-1}) of an exponentially growing inoculum (50 mL in 250-mL shake flasks). The cultures were subcultured axenically every 3 weeks for maintenance. The inoculum of the *C. vulgaris* was prepared in BBM supplemented with 15 g L^{-1} glucose and grown in dark conditions at 25 °C for 48 h (log-phase growth) at 150 rpm.

2.2. Bioreactor cultivation

Batch cultivation was performed in a 16-L laboratory bioreactor (Bilfinger Industrial Technologies, Mannheim, Germany) with a working volume of 10 L. The culture was inoculated with 10% (v v^{-1}) exponentially grown inoculum. The temperature was set at 28 °C, the stirring speed was 300 rpm, the dissolved oxygen tension was kept above 75%, and aeration (4 L min^{-1}) was achieved with filter-sterilised air. The pH was kept constant at 7 by automatic addition of H_2SO_4 (0.5 M) and NaOH (0.5 M). The medium used for growth was a modified BBM (nitrate concentration of 1.5 g L^{-1}) enriched with 15 g L^{-1} glucose.

2.3. Experimental set-up

The medium composition and the cell growth were monitored with three samplings per day, with the exception of glucose (every 3 h). The fatty acid profile of the biomass was measured daily starting from day 2 due to biomass concentration limitations. Larger samples of biomass were harvested at the middle exponential phase (day 2) and analysed for lipid bioaccessibility, lipid oxidative stability, oxygen radical absorbance capacity (ORAC), pigments and lipid classes. An additional sample harvested at early stationary phase (day 3) was analysed for lipid bioaccessibility. Other analyses were not performed on this sample due to the limited available biomass. The cultivation was kept for 4 days in stationary phase to study the effect of nutrient starvation on lipid production. The lipid bioaccessibility, lipid oxidative stability, ORAC, pigments, and lipid classes were measured in the biomass harvested during the late stationary phase (day 7). All the analyses were performed on freeze-dried biomass (VirTis BenchTop Pro freeze-dryer, Gardiner, NY, USA).

2.4. Determination of cell dry weight and glucose, nitrate, and phosphate concentrations in the media

The performance of microalgae growth was monitored daily by measuring the cell dry weight concentration (CDW). CDW was measured in duplicate by centrifuging 2 mL of culture (10,000 × *g*, 10 min). Cells were washed with demineralised water. The weight of the dried biomass was measured in Eppendorf tubes after 24 h drying at 105 °C.

The specific growth rate (μ, h^{-1}) in the exponential phase was determined according to Eq. (1) [19], where CDW_2 (g L^{-1}) and CDW_1 (g L^{-1}) are the cell dry weight concentration at t_2 and t_1, respectively. t_2 and t_1 are expressed in hours.

$$\mu = \frac{\ln\left(\frac{CDW_2}{CDW_1}\right)}{t_2 - t_1} \quad (1)$$

Glucose content was determined by online measurements with HPLC (1200 system, Agilent Technologies, Basel, Switzerland). The separation column used was an Aminex HPX-87H (300 × 7.8 mm, Bio-Rad, Hercules, CA, USA). The sampling was performed every 3 h by using the automatic system NUMERA (Securecell, Switzerland).

The biomass yield on glucose ($Y_{CDW/G}$, g g^{-1}) was calculated using the following Eq. (2), [20]:

$$Y_{CDW/G} = \frac{CDW_2 - CDW_1}{G_1 - G_2} \quad (2)$$

where CDW_2, CDW_1, G_2, G_1 are the concentrations of the cell dry weight (g L^{-1}) at times 2 and 1 and the concentrations of glucose (g L^{-1}) at times 2 and 1, respectively.

The residual phosphate in the supernatant was measured according to Dick and Tabatabai [21]. The residual nitrate content in the supernatant was determined using the method proposed by Carpine et al. [22]. Phosphate and nitrate were measured in duplicates.

2.5. Microalgae biomass composition analysis

The fatty acid profile was determined following the method of Burja et al. [23], with certain adjustments. In brief, 30 mg of freeze-dried biomass was mixed with 1 mL internal standard solution (0.25 mg mL^{-1} methyl undecanoate (C11:0) and tritridecanoin (C13:0)). After adding 2 mL of 1,5 N methanolic hydrochloric acid solution, the tubes were closed hermetically and held at 100 °C for 60 min. After cooling, 2 mL of water was added. After centrifuging at 375 g per 5 min, the hexane phase, containing the fatty acid methyl esters (FAMEs), was analysed by gas chromatography (GC) equipped with a split-injection port and flame ionization detection (FID) (7890A, Agilent Technologies, Basel, Switzerland). The following temperature–time programme was used: 50 °C (0.2 min), 50 °C–180 °C (120 °C min^{-1}), 180 °C–220 °C (6.7 °C min^{-1}), 220 °C–250 °C (30 °C min^{-1}) on a 70% cyanopropyl polysilphenylene-siloxane column with a length of 10 m, i.d. of 0.1 mm, and film of 0.2 μm (BPX70, SGE Analytical Science, Milton Keynes, UK). Peak identification was performed by comparing the retention times with FAME standards (Nu-Chek Prep. Inc., Elysian, USA). The peak areas were quantified with OpenLab CDS VL software (Agilent Technologies, Basel, Switzerland). The amount of each fatty acid was calculated based on the amount and peak area of the internal standard. The determination of the fatty acid content was performed in duplicate for each time point. Blank controls (i.e., containing no sample) were used in each experiment. No fatty acids were recovered from any of the controls.

The protein content was measured in triplicate according to Lowry et al. [24] by pretreating samples at 100 °C for 10 min with 2 N NaOH. This method was chosen over others (e.g., Bradford and Kjeldahl) as no disruption pretreatment is required. Bovine serum albumin (BSA) was used as the standard for calibration.

The carbohydrate analysis was performed in triplicate by the anthrone method, according to Chen and Vaidyanathan [25].

2.6. Determination of lipid classes

2.6.1. Lipid extraction

A total of 100 mg freeze-dried microalgae biomass was mixed with 200 μL water for 30 s. Chloroform:methanol (2:1 v v^{-1}, 3.8 mL) was added, and the suspension was vortexed for 1 min at 530 ×*g* and sonicated for 15 min at 40 °C. After centrifugation (530 ×*g*, 10 min), 1 mL chloroform:methanol (2:1, v v^{-1}) was added to the supernatant and vortexed for 1 min. After centrifugation (530 ×*g*, 10 min), 1 mL potassium chloride solution (0.88% m v^{-1} in water) was added to the supernatant and vortexed for 1 min. After centrifugation (530 ×*g*, 10 min), the organic phase (lower) was collected and the supernatant was washed with 1 mL chloroform:methanol (2:1 v v^{-1}) and vortexed for 1 min. The organic phase was collected and pooled with the rest. A washing step with 1 mL chloroform:methanol (2:1 v v^{-1}) was repeated twice. The organic phase was dried at room temperature under nitrogen stream. The extraction was performed in duplicates.

2.6.2. High-performance liquid chromatography (HPLC) with evaporative light scattering detector (ELSD) analysis

The lipid class profile was determined following the method of Olsson et al. [26], with certain adjustments. The lipid extracts were dissolved in 50 μL toluene:methanol:acetic acid:triethylamin (600:400:0.02:0.001 v v^{-1}, mobile phase B), 950 μL n-heptane was added, and the suspension was vortexed and sonicated at 40 °C for 5 min. The suspension was vortexed for 1 min and centrifuged for 4 min at 10,000 ×*g*. The supernatant was injected in HPLC-ELSD (injection volume 50 μL). Chromatography was performed on a Reprosil-Pur UP5CN-250/046 250 × 4.6 mm i.d. with 5 μm particles with a 10 × 4.6 mm guard column of the same material (Interchim, Montluçon, France). The following binary gradient elution system was used at a flow rate of 1.0 mL min^{-1} and at 400 bar pressure: 100% solvent A (3 min), 60% solvent A, 40% solvent B (17 min), 50% solvent A, 50% solvent B (4 min), 100% solvent A (1.5 min). A 1290 Infinity II evaporative light scattering detector (Agilent Technologies, Basel, Switzerland) was used with an evaporator temperature of 40 °C, nebulizer temperature of 80 °C, and gas flow rate of 1.60 SLM. The analysis was performed in triplicates.

2.7. Lipid oxidation

2.7.1. Storage

The freeze-dried microalgae biomass harvested in the exponential and late stationary phases was stored in amber screw-cap vials (0.2 g), hermetically sealed and stored for 12 weeks at 40 °C. At this temperature, the lipid oxidation process is faster than that at room temperature without alterations in the oxidation pathways [11]. Ten different time points (weeks 0, 1, 2, 3, 4, 5, 6, 7, 8, and 12) were evaluated in terms of secondary oxidation. All the vials were stored at −20 °C until analysis to avoid further oxidation.

2.7.2. Determination of secondary oxidation

The secondary oxidation of the freeze-dried biomasses was evaluated according to the method of Rohfritsch et al. [27], with certain adjustments. The biomass (0.2 g) was dissolved in 2 mL chloroform/methanol (1:2, v v^{-1}). The sample weight was normalised according to the fat content of the cells in the exponential or stationary phase. The samples were mixed with a horizontal shaker (Sigma laboratory centrifuge 4K15C, Fisher Scientific, Reinach, Switzerland) at 2500 rpm for 10 min. The sample supernatant (100 μL) was combined with 100 μL 7-(diethylamino)-2-oxochromene-3-carbohydrazide (CHH) for 1.5 h of derivatization at 37 °C and 1400 rpm using a thermomixer (Comfort, Eppendorf, Schönenbuch, Switzerland). After derivatization, the sample was diluted with 100 μL acetonitrile and centrifuged at 2500 ×*g* (20 °C), and the supernatant was used for injection in ultra-performance liquid chromatography (UPLC) (Dionex UltiMate 3000 system, Thermo Scientific, Basel, Switzerland) - QExactive Plus (Thermo Scientific, Basel, Switzerland) system. The analysis was performed in duplicates. The identification of the volatile carbonyl compounds was based on the exact mass in full scan mode, the retention times, and the MS/MS fragmentation. The software Xcalibur (version 2.2) was employed for analysis. Volatiles originating from lipid oxidation were selected as indicator [27]: 3-hexenal, propanal, (*Z*,*Z*)-3,6-nonadienal, (*E*,*Z*)-2,4-heptadienal (from ω-3 fatty acids degradation pathways), hexanal and 2,4-decadienal (from ω-6 fatty acids degradation pathways), and (*E*)-2-decenal (from ω-9 fatty acids degradation pathways). The response factors of the identified volatiles were obtained by dividing the area of the volatiles by the area of the internal standard hexanal-d_{12}.

2.8. Pigment analysis

Chlorophyll (*a* and *b*) and carotenoids contents in the biomass were determined in triplicate using a spectrophotometric method, following the procedure described by Pruvost et al. [28].

2.9. Oxygen radical absorbance capacity

The hydrophilic oxygen radical absorbance capacity (H-ORAC) was measured according to Huang et al. [29]. The lipophilic ORAC (L-ORAC) was measured according to Huang et al. [30]. Both ORAC assays are based on the same principle; a fluorescent probe is oxidized by the addition of a free radical generator (APPH) that quenches the fluorescent probe over time. The antioxidants present in the sample block

the generation of free radicals until the antioxidant activity of the sample is depleted. The analysis was performed in duplicates on freeze-dried biomass.

2.10. Determination of lipid bioaccessibility

The lipid bioaccessibility was determined by an infant *in vitro* digestion model, as described in Ménard et al. [31], with certain slight modifications. Bile salt mix stock solution (65.7 mM) was prepared in Tris Buffer (2 mM, pH 6.8) with the following salts: sodium taurocholate (27.6 mM), sodium taurochenodeoxycholate (17.1 mM), sodium glycocholate hydrate (14.5 mM), and sodium glycohenodeoxycholate (6.6 mM). In brief, 1.22 g of freeze-dried microalgae biomass was suspended in 9.4 mL demineralised water. The suspension was then mixed with simulated gastric fluid (pH 5.5), containing pepsin (450 U m L^{-1}), rabbit gastric lipase (18 U m L^{-1}), bile salt mix (0.037 mM), and egg phospholipids (0.045 mM), and incubated for 1 h in a water bath at 37 °C with stirring at 400 rpm. Subsequently, simulated intestinal fluid (pH 6.5) containing pancreatin (84 USP m L^{-1}) and bile salt mix (1.22 mM) was added, and the sample was incubated for 2 h at 37 °C. The digesta was ultracentrifuged (12,500 ×*g*, 30 min, 4 °C, Optima XPN-80, Beckman Coulter, Fullerton, CA, USA), resulting in an aqueous phase, i.e., the micellar phase, and an insoluble pellet. All the samples (digest, micellar phase, and pellet) were lyophilized and analysed in terms of lipid composition as described earlier (see Section 2.5). The digestion was performed twice. The lipid bioaccessibility was determined according to the following equation [14]:

$$\textit{Lipid bioaccessibility}\ (\%) = \frac{\textit{Total fatty acids in micellar phase}}{\textit{Total fatty acids in sample before digestion}} \cdot 100 \quad (3)$$

3. Results and discussion

3.1. Heterotrophic cultivation of C. vulgaris under controlled conditions: biomass production and nutrient uptake

The biomass growth and nutrient uptake of *C. vulgaris* heterotrophic batch cultivation in a stirred tank bioreactor is shown in Fig. 1. The culture showed a one day lag phase, followed by a two-days exponential phase, when an 8.90 ± 0.07 g L^{-1} CDW was reached. After 4 days of starvation, the CDW decreased to 7.12 ± 0.32 g L^{-1}, indicating catabolic functions and probably partial cell death.

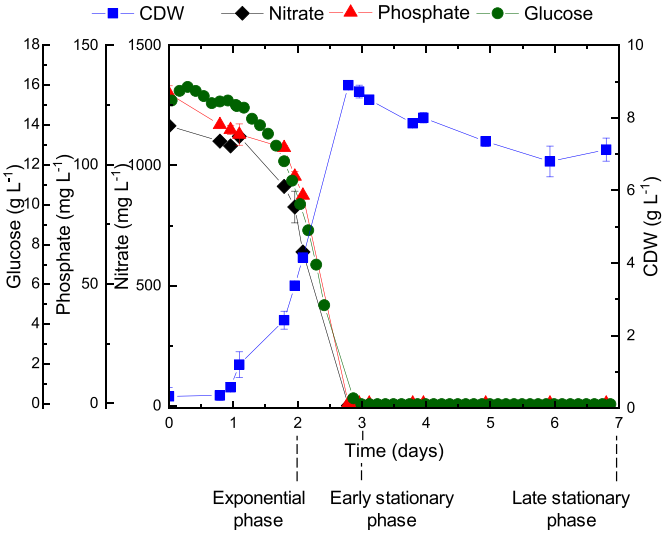

Fig. 1. CDW (■, g L^{-1}), nitrate (◆, mg L^{-1}), phosphate (▲, mg L^{-1}), and glucose (●, g L^{-1}) concentration over time in an industrially relevant 16-L pre-pilot scale batch cultivation of heterotrophic *C. vulgaris*. The harvesting time for the exponential (day 2), early stationary (day 3) and late stationary (day 7) phases is reported. Error bars represent the deviation between duplicate measurements (n = 2).

The glucose concentration in the growth medium was measured every 3 h. Such high resolution monitoring allowed unequivocal and accurate determination of the time when the glucose was depleted, which corresponded to the start of the stationary phase of the cultivation. The nitrate and phosphate showed similar uptake rates. Moreover, the nitrate and phosphate in the medium were simultaneously depleted with glucose after 2.8 days.

Based on the medium composition and CDW data from this strictly controlled process, samples for further analysis were taken after 2, 3 and 7 days, and identified as the exponential, early stationary and late stationary phases, respectively.

The specific growth rate during the exponential phase was calculated to be $\mu = 0.051\ h^{-1}$, which is in the upper range of that reviewed by Daliry et al. [32] for *C. vulgaris* optimal growth, ranging between $\mu = 0.036\ h^{-1}$ and $\mu = 0.051\ h^{-1}$. The specific growth rate obtained in this study was much higher than what reported by Morowvat et al. for *C. vulgaris* in heterotrophic conditions, going from $\mu = 0.001\ h^{-1}$ to $\mu = 0.004\ h^{-1}$ [33].

The biomass yield on glucose of the whole process was $Y_{CDW/G} = 0.47\ g\ g^{-1}$, which is comparable to that found by Singhasuwan et al. [20] for the heterotrophic cultivation of *C. vulgaris*, ranging between $Y_{CDW/G} = 0.47\ g\ g^{-1}$ and $Y_{CDW/G} = 0.65\ g\ g^{-1}$. The growth parameters showed a competitive process; however, cultivation conditions could be improved further to reach higher productivities, which was not in the scope of this study.

3.2. Biomass composition

3.2.1. Lipid, protein and carbohydrates

The dried biomass composition showed slight changes over growth (Table 1). These results are expressed as a percentage of dry matter, as data were corrected for the moisture content (5–6%).

In general, approximately 80% of the biomass composition can be analysed as lipid, carbohydrates and protein. The residual (ca. 20%) biomass consists of pigments, ashes, DNA and RNA [34].

The main constituent of the dried biomass was characterised by carbohydrates, which decreased over growth (from 63.7% ± 1.6% to 52.1% ± 1.6%). Usually, approximately 10% of the total carbohydrates comprises cell wall polysaccharides [35], while the remaining carbohydrates are storage polysaccharides, such as starch and chrysolaminarin [13]. The decrease in the carbohydrate content was probably due to conversion to other nutrients, such as lipid and protein and to catabolic functions during starvation.

The protein content increased from 15.5% ± 0.5% to 20.2% ± 0.4% at day 6. This result could be due to two reasons. First, cells might have synthetized protein even though the nitrate in the medium was depleted after 2.8 days, probably by converting the intracellular storage of nitrogen into protein [36]. Second, cells in

Table 1
Biomass composition expressed as percentage of dry matter from day 2 to day 7 of cultivation. Measured components were lipid, protein and carbohydrates. Errors represent the standard deviation between triplicate measurements (n = 3).

Day	Lipid (%)	Protein (%)	Carbohydrates (%)
2	6.9 ± 0.2	15.5 ± 0.5	63.7 ± 1.6
3	6.7 ± 0.1	14.5 ± 1.7	63.2 ± 2.9
4	7.4 ± 0.3	15.6 ± 0.6	58.1 ± 3.0
5	7.8 ± 0.1	18.8 ± 0.1	58.1 ± 1.3
6	8.3 ± 0.2	20.2 ± 0.4	56.7 ± 3.9
7	8.1 ± 0.1	19.2 ± 0.9	52.1 ± 1.6

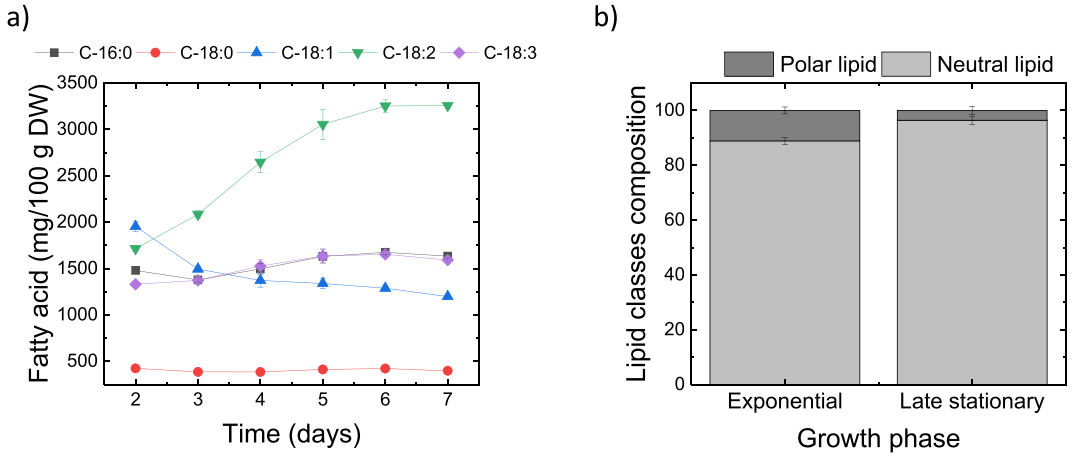

Fig. 2. Amounts of palmitic acid (C16:0, ■), stearic acid (C18:0, ●), oleic acid (C18:1, ▲), linoleic acid (C18:2, ▼), and linolenic acid (C18:3,n3, ◆), expressed as mg in 100 g dried biomass, measured over growth, are shown in a). Lipid classes compositions as neutral (light grey) and polar (dark grey) lipids in biomass harvested in exponential (day 2) and late stationary (day 7) phases are shown in b). Error bars represent the standard deviation between triplicate measurements (n = 3).

starvation may have started consuming intracellular carbohydrates depositories, which could have increased the relative fraction of protein in the dried biomass. This second theory agrees with the decrease in CDW between days 3 and 7.

The lipid content varied between 6.7% and 8.3%. In the literature, the lipid content of *C. vulgaris* significantly differs, ranging in values between 5% and 60% [35]. This variation is strongly dependent on the cultivation conditions, as the biomass composition can be modified by adjusting the growth conditions, such as temperature, nitrogen and salt concentration [6]. The lipid content increased from 6.7% ± 0.1% to 8.1% ± 0.1% after nutrient depletion (at day 2.8). Indeed, the microalgae continued to grow and explicate their function even after macronutrient (glucose, phosphate and nitrate) depletion. Nutrient limitations can trigger lipid metabolism by converting intracellular compounds. To obtain a biomass with higher lipid content, optimised carbon to nitrogen (C/N) could be investigated [32].

3.2.2. Fatty acid profile and lipid classes

The fatty acid composition of the biomass consisted of palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (LA, C18:2n6), and α-linolenic acid (ALA, C18:3n3) (Fig. 2a). The linoleic acid content almost doubled from day 2 of cultivation (1714 ± 34 mg/100 g) to day 7 (3256 ± 30 mg/100 g). The oleic acid concentration decreased from 1953 ± 54 mg/100 g to 1198 ± 54 mg/100 g. This result showed the conversion of oleic acid into linoleic acid, which is catalysed by a Δ12 desaturase (FAD2) [37]. The lipid fraction of biomass harvested in the stationary phase (day 7) contained 19.7% and 40.3% of ω3-PUFAs and ω6-PUFAs, respectively. These fractions yielded a ω3:ω6 ratio of approximately 1:2. The ω3:ω6 ratio in the biomass harvested in exponential phase was calculated to be 1:1.3. Both ω3:ω6 ratio values fit within the optimal range (from 1:1 to 1:4) according to nutrition studies regarding preventing effects towards chronic diseases [38].

The lipid classes were measured in samples harvested during the exponential (day 2) and late stationary phases (day 7) (Fig. 2b). Neutral lipids accounted for more than 88.0% of the total lipids in the exponential phase and increased up to 96.4% ± 1.4% at the end of stationary phase. In contrast, the polar lipids decreased from 11.1% ± 1.3% during the exponential phase to 3.6% ± 1.4% during the stationary phase. This result agrees with that of Gheysen et al. [11], where it is reported that heterotrophic grown microalgae contained mostly (more than 94%) neutral lipids, while in photoautotrophic algae, only 41–50% of the total lipids were neutral. The polar lipid (constituents of membranes) content was higher during the exponential phase, when the cells were growing and building biomass. Nitrogen starvation, which started after the end of the exponential phase, is known to induce neutral lipid accumulation in lipid droplets and degradation of photosynthetic membranes [39].

3.3. Oxidative stability

To evaluate the oxidative stability of the biomasses harvested at different growth stages, we determined the secondary oxidation in the biomasses subjected to an accelerated shelf life study. To better understand the difference in the secondary oxidation, the oxygen radical absorbance capacity (ORAC) and lipophilic pigments with the antioxidant activity (chlorophyll *a*nd carotenoids) were measured.

3.3.1. Lipid oxidation

The formation of secondary oxidation products was investigated after storage at 40 °C for 12 weeks (Fig. 3). Several volatiles were monitored (Section 2.7); however, here, only hexanal and propanal are shown, as targeted degradation products of LA (C18:2,n6) and ALA (C18:3,n3), respectively [40]. In Fig. 3, the hexanal and propanal profiles for the biomass harvested during the exponential and late stationary phases are shown. At week 0, the signal of the volatiles was clearly higher in the biomass harvested during the exponential phase than that during the stationary phase. This result may indicate that partial lipid oxidation could have already occurred in the biomass harvested during exponential phase before storage, e.g., in the drying step. Importantly, in both biomasses, the hexanal and propanal profiles showed only moderate increases over 12 weeks of biomass storage, indicating good oxidative stability. The presence of an intact cell wall likely contributed to lipid stabilization, as shown by Balduyck et al. [12].

Hexanal is characterised by a fishy and grassy flavour, while propanal has an earthy odour [27]. Technical blind sniffing was performed on the same samples by an untrained panel (4 members). Sensory assessment is generally considered the preferred method to measure the flavour changes in oils [41]. The sensory outcomes confirmed analytical data. Overall, neither the exponential nor the stationary biomasses showed major lipid oxidation over 12 weeks of accelerated shelf-life, confirming the oxidative stability. The exponential biomass was described by a slightly stronger cheese-like flavour than that of the stationary biomass after 12 weeks of storage. This result is consistent with the greater signal intensities of hexanal and propanal found in the exponential biomass after 12 weeks of storage (1.616 area hexanal/area IS and 1.422 area propanal/area IS) than that of the stationary phase (0.820 ± 0.049 area hexanal/area IS and 0.558 ± 0.042 area propanal/area IS). In future research, a powder (e.g., maltodextrin) system

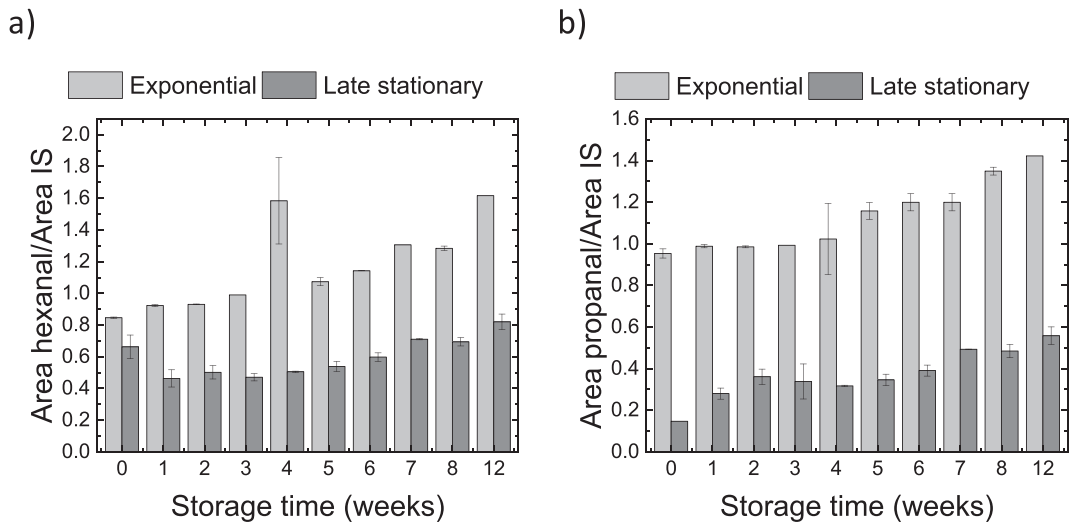

Fig. 3. Evolution of secondary oxidation products - a) hexanal and b) propanal - during 12 weeks of storage at 40 °C for *C. vulgaris* biomass harvested during the exponential (day 2, light grey) and late stationary phases (day 7, dark grey). Error bars represent the deviation between duplicate measurements (n = 2).

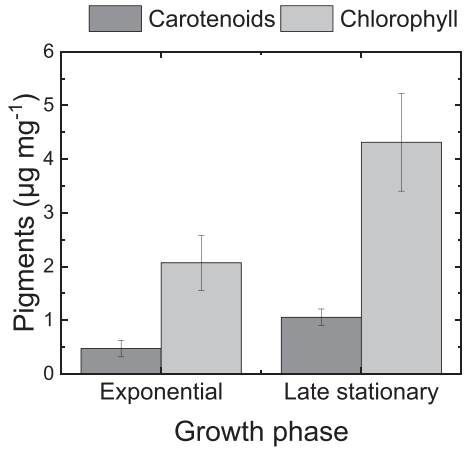

Fig. 4. Content (μg mg^{-1}) of total carotenoids (dark grey) and total chlorophyll (light grey) in dried biomasses harvested in exponential (day 2) and late stationary (day 7) phases. Error bars represent the standard deviation between triplicate measurements (n = 3).

containing ω3-PUFAs and ω6-PUFAs could be included as a reference. Additionally, the disrupted biomass could be included as positive control, as greater and faster lipid oxidation is expected in such a system.

The lipid class distribution may impact the oxidative stability of the whole system. The role played by polar lipids in oxidative stability is debatable and depends on several factors: type of polar lipid (e.g., glycol- vs phospholipids), type of side chain moieties [42], and matrix [43]. The higher fraction of polar lipids, mainly represented by phospholipids, during the exponential phase could have already contributed to the higher signal of the volatiles at time 0, compared with the biomass harvested during the stationary phase. Phospholipids have been reported to act as prooxidants, as phospholipids can serve as oxidation substrates themselves (especially in foods containing significant amounts of biological membranes, such as meats). Oxidation is triggered because of the significant unsaturation of phospholipids (to preserve membrane fluidity) and the presence of negative charges that attract prooxidant metals [44]. On the other hand, phospholipids may have antioxidant capacity due to their ability to chelate prooxidative metals, regenerating primary antioxidants (e.g., carotenoids and tocopherols) and changing the location of the primary antioxidants [43]. Therefore, the fatty acid distribution in the lipid classes may have affected the different secondary oxidations of the exponential and stationary biomasses at time 0, although it cannot be clearly established in which direction.

To obtain a deeper understanding of the difference between the secondary oxidations of the exponential and stationary biomasses, we investigated other factors that may have played roles, such as the ORAC and chlorophyll and carotenoids contents.

3.3.2. Pigments

Carotenoids are among the most efficient natural scavengers of oxygen and can retard oxidation by scavenging free radicals [44]. In a previous study reported by Gheysen et al. [45], endogenous carotenoids were shown to play a predominant role in maintaining the lipid oxidative stability of microalgae biomass, compared with the roles of phenolic compounds, tocopherols and ascorbic acid.

Chlorophyll is a pigment present in microalgae in two forms, *a* and *b*. Chlorophyll has been associated with either pro- or antioxidant activity depending on the matrix and conditions. Chlorophyll *a* and the degradation products of chlorophyll *a*, being photosensitive, are well-known prooxidants under light conditions. However, chlorophyll can act as an antioxidant in dark conditions, as reported by Lanfer-Marquez et al. [46]. The antioxidant activity of chlorophyll was explained by the ability to protect linoleic acid against oxidation and/or preventing decomposition of hydroperoxides [46].

Fig. 4 shows the amounts of carotenoids and total chlorophyll (*a* + *b*) expressed as μg per mg of dried biomass.

The carotenoids content increased from 0.47 ± 0.15 μg mg^{-1} during the exponential phase to 1.05 ± 0.15 μg mg^{-1} during the stationary phase. The same trend was observed for the total amount of chlorophyll. The exponential biomass contained 2.07 ± 0.51 μg mg^{-1} chlorophyll, while the stationary biomass showed 4.31 ± 0.91 μg mg^{-1}. The increases in the amounts of carotenoids and chlorophyll from the exponential to stationary phase may have been triggered by stress conditions due to nutrient depletion [47].

The higher content of carotenoids may have contributed to the lower secondary oxidation of the biomass harvested during the stationary phase compared with that harvested during the exponential phase. Even though chlorophyll was reported to have lower capacity to maintain oxidative stability than carotenoids [46], chlorophyll might have played a protective role against lipid oxidation, as our study was performed in dark conditions.

3.3.3. Oxygen radical absorbance capacity

The oxygen radical absorbance capacity (ORAC) has been widely

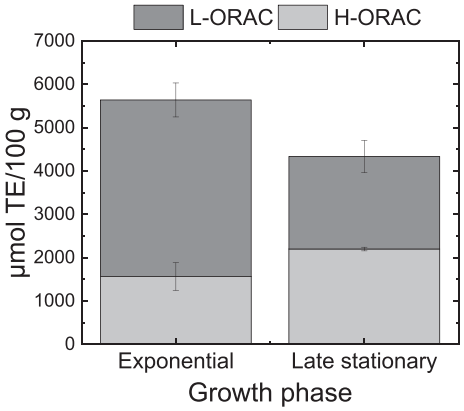

Fig. 5. Lipophilic (L-, dark grey) and hydrophilic (H-, light grey) oxygen radical absorbance capacity (ORAC) of dried biomasses harvested during the exponential (day 2) and late stationary (day 7) phases. The values are expressed as µmol trolox equivalent (TE) per 100 g dried biomass. Error bars represent the deviation between duplicate measurements (n = 2).

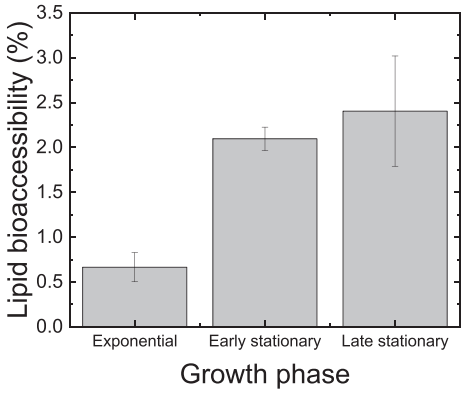

Fig. 6. Lipid bioaccessibility (%) in dried biomasses harvested in exponential (day 2), early stationary (day 3) and late stationary (day 7) phase. Error bars represent the standard deviation between digestion replicates (n = 4).

applied to characterise the antioxidant capacity of pure or complex compounds [48]. The ORAC assay measures the antioxidant scavenging activity against oxygen radicals. The ORAC consists of the L-ORAC and H-ORAC, based on the lipophilic and hydrophilic antioxidant compounds, respectively. Fig. 5 shows the H-ORAC and L-ORAC of the biomasses harvested during the exponential and late stationary phases.

The H-ORAC was higher in the late stationary phase (2200 ± 43 µmol TE/100 g) than in the exponential phase (1564 ± 320 µmol TE/100 g). In contrast, the late stationary phase showed lower L-ORAC (2134 ± 373 µmol TE/100 g) than the exponential phase (4076 ± 391 µmol TE/100 g), which led to a lower total ORAC in the late stationary phase. The measured ORAC in *C. vulgaris* biomass in this study is comparable with that found in *Dunaliella salina* and *Tetraselmin chui* by Ahmed et al. [49]. The carotenoids, as lipophilic oxygen scavenging compounds, probably contributed to the L-ORAC [50]. However, a greater content of carotenoids was found in the stationary phase, when the L-ORAC was lower. This result suggests that other compounds, such as tocopherols, might have significantly contributed to the L-ORAC [51]. The increase in the H-ORAC from the exponential to stationary phase might contribute to the lesser oxidation of the biomass harvested in the stationary phase.

In general, the ORAC does not seem to be the only factor that could explain the oxidative stability, as discussed by Lv et al. [48]. A synergistic effect of different antioxidant compounds likely contributed to the great stability of microalgae biomass powder over 12 weeks of accelerated shelf-life and to the different degrees of volatile formation of the biomasses harvested during different growth phases.

3.4. Lipid bioaccessibility

Only a handful of studies are available in the literature on lipid bioaccessibility in microalgae. Moreover, no studies have investigated the lipid bioaccessibility in microalgae by using infant *in vitro* digestion. In our study, the lipid bioaccessibility was found to be lower than 2.5%, indicating a poor accessibility of lipids during digestion (Fig. 6). Our results are lower than that of Bonfanti et al. [14] by using an adult *in vitro* digestion model for another microalgae, *Isochrysis galbana*, where lipid bioaccessibility was measured as between 7% and 15%. This result could be due to the lower concentration of digestive enzymes and more neutral pH used in infant digestion [31].

The lipid bioaccessibility was higher in the biomass harvested in the stationary phase compared with that of the exponential phase. In the exponential phase biomass, 0.66% ± 0.16% of the total lipids was bioaccessible, while 2.10% ± 0.13% and 2.41% ± 0.61% were bioaccessible in early and late stationary biomasses, respectively. From our results, factors other than the cell wall thickness, which is expected to be thicker in the stationary phase [6], affected the bioaccessibility of the lipids.

Older cells, being in the stationary phase, may be more damaged and therefore more prone to breakage and release of intracellular compounds during digestion. Bensalem et al. [52] studied lipid extraction in *Chlamydomonas reinhardtii* over growth. These researchers found that cells that were stressed for longer times had cell walls with more layers but also a higher lipid extraction efficiency. Additionally, greater cell lysis upon solvent extraction was observed in older cultures, which agrees with our results. The limited lipid bioaccessibility suggests the need for pre-treatment of the biomass to enhance the release of nutrients during digestion.

4. Conclusions

C. vulgaris was cultivated heterotrophically in an industrially relevant 16-L pre-pilot scale stirred tank bioreactor. The biomass showed an ideal oxidative stability over 12 weeks of accelerated shelf-life. This study indicates that *C. vulgaris* biomass can be considered to be a stable and nutritious (optimal ω3:ω6 profile) source of essential fatty acids. Our results suggested that regarding lipid stability and bioaccessibility, harvesting during the stationary phase could be preferable, which also agrees with the economic feasibility and sustainability of the fermentation. In general, biomass treatment to increase the lipid bioaccessibility should be considered and investigated.

CRediT authorship contribution statement

Greta Canelli: Conceptualization, Methodology, Formal analysis, Project administration, Writing - original draft, Writing - review & editing. **Lukas Neutsch:** Conceptualization, Methodology, Writing - review & editing. **Roberta Carpine:** Conceptualization, Methodology, Formal analysis, Writing - review & editing. **Sabrina Tevere:** Formal analysis. **Francesca Giuffrida:** Conceptualization, Methodology, Writing - review & editing. **Zhen Rohfritsch:** Methodology, Formal analysis, Writing - review & editing. **Fabiola Dionisi:** Conceptualization, Methodology, Writing - review & editing. **Christoph J. Bolten:** Conceptualization, Methodology, Writing - review & editing. **Alexander Mathys:** Funding acquisition, Conceptualization, Methodology, Writing - review & editing.

Declaration of competing interest

The authors declare that Nestlé Research Center supported the research conducted for this manuscript. The authors declare that the

research was conducted in the absence of any commercial or financial relationships that could be constructed as a potential conflict of interest.

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Declaration of authors' agreement

All authors agreed to the authorship and submission of the manuscript to Algal research for peer review.

Statement of informed consent, human/animal rights

No conflicts, informed consent, human or animal rights applicable.

References

[1] S. Smetana, M. Sandmann, S. Rohn, D. Pleissner, V. Heinz, Autotrophic and heterotrophic microalgae and cyanobacteria cultivation for food and feed: life cycle assessment, Bioresour. Technol. 245 (2017) 162–170, https://doi.org/10.1016/j.biortech.2017.08.113.

[2] J. Mu, S. Li, D. Chen, H. Xu, F. Han, B. Feng, Y. Li, Enhanced biomass and oil production from sugarcane bagasse hydrolysate (SBH) by heterotrophic oleaginous microalga *Chlorella protothecoides*, Bioresour. Technol. 185 (2015) 99–105, https://doi.org/10.1016/j.biortech.2015.02.082.

[3] H. Xu, X. Miao, Q. Wu, High quality biodiesel production from a microalga *Chlorella protothecoides* by heterotrophic growth in fermenters, J. Biotechnol. 126 (2006) 499–507, https://doi.org/10.1016/j.jbiotec.2006.05.002.

[4] M.P. Caporgno, I. Haberkorn, L. Böcker, A. Mathys, Cultivation of *Chlorella protothecoides* under different growth modes and its utilisation in oil/water emulsions, Bioresour. Technol. 288 (2019) 121476, , https://doi.org/10.1016/j.biortech.2019.121476.

[5] M.P. Caporgno, A. Mathys, Trends in microalgae incorporation into innovative food products with potential health benefits, Front. Nutr. 5 (2018) 1–10, https://doi.org/10.3389/fnut.2018.00058.

[6] E.G. Grima, F.G. Acién Fernàndez, A. Robles Medina, Handbook of Microalgal Culture, Wiley Blackwell, New Jersey, United States, 2013, https://doi.org/10.1002/9781118567166.

[7] D. Morales-Sánchez, O.A. Martinez-Rodriguez, A. Martinez, Heterotrophic cultivation of microalgae: production of metabolites of commercial interest, J. Chem. Technol. Biotechnol. 92 (2017) 925–936, https://doi.org/10.1002/jctb.5115.

[8] A.P. Batista, L. Gouveia, N.M. Bandarra, J.M. Franco, A. Raymundo, Comparison of microalgal biomass profiles as novel functional ingredient for food products, Algal Res. 2 (2013) 164–173, https://doi.org/10.1016/j.algal.2013.01.004.

[9] Z. Yang, S.L. Huffman, Modelling linoleic acid and α-linolenic acid requirements for infants and young children in developing countries, Matern. Child Nutr. 9 (2013) 72–77, https://doi.org/10.1111/j.1740-8709.2012.00448.x.

[10] E.N. Frankel, T. Satue Ä-Gracia, A.S. Meyer, J.B. German, Oxidative Stability of Fish and Algae Oils Containing Long-chain Polyunsaturated Fatty Acids in Bulk and in Oil-in-water Emulsions, (2002), pp. 2094–2099, https://doi.org/10.1021/jf0111458.

[11] L. Gheysen, T. Bernaerts, C. Bruneel, K. Goiris, J. Van Durme, A. Van Loey, L. De Cooman, I. Foubert, Impact of processing on n-3 LC-PUFA in model systems enriched with microalgae, Food Chem. 268 (2018) 441–450, https://doi.org/10.1016/j.foodchem.2018.06.112.

[12] L. Balduyck, T. Stock, S. Bijttebier, C. Bruneel, G. Jacobs, S. Voorspoels, K. Muylaert, I. Foubert, Integrity of the microalgal cell plays a major role in the lipolytic stability during wet storage, Algal Res. 25 (2017) 516–524, https://doi.org/10.1016/j.algal.2017.06.013.

[13] C. Safi, B. Zebib, O. Merah, P.Y. Pontalier, C. Vaca-Garcia, Morphology, composition, production, processing and applications of *Chlorella vulgaris*: a review, Renew. Sust. Energ. Rev. 35 (2014) 265–278, https://doi.org/10.1016/j.rser.2014.04.007.

[14] C. Bonfanti, C. Cardoso, C. Afonso, J. Matos, T. Garcia, S. Tanni, N.M. Bandarra, Potential of microalga *Isochrysis galbana*: bioactivity and bioaccessibility, Algal Res. 29 (2018) 242–248, https://doi.org/10.1016/j.algal.2017.11.035.

[15] A. Gille, A. Trautmann, C. Posten, K. Briviba, Bioaccessibility of carotenoids from *Chlorella vulgaris* and *Chlamydomonas reinhardtii*, Int. J. Food Sci. Nutr. 67 (2016) 507–513, https://doi.org/10.1080/09637486.2016.1181158.

[16] C. Lemahieu, C. Bruneel, C. Dejonghe, J. Buyse, I. Foubert, The cell wall of autotrophic microalgae influences the enrichment of long chain omega-3 fatty acids in the egg, Algal Res. 16 (2016) 209–215, https://doi.org/10.1016/j.algal.2016.03.015.

[17] C. Ratledge, H. Streekstra, Z. Cohen, J. Fichtali, Downstream Processing, Extraction, and Purification of Single Cell Oils, Second edi., AOCS Press, 2010, https://doi.org/10.1016/B978-1-893997-73-8.50013-X.

[18] W.R. Barclay, Process for the heterotrophic production of microbial products with high concentrations of omega-3 highly unsaturated fatty acids, Patent US005130242A, 1992.

[19] J. Doucha, K. Lívanský, Production of high-density *Chlorella* culture grown in fermenters, J. Appl. Phycol. 24 (2012) 35–43, https://doi.org/10.1007/s10811-010-9643-2.

[20] S. Singhasuwan, W. Choorit, S. Sirisansaneeyakul, N. Kokkaew, Y. Chisti, Carbon-to-nitrogen ratio affects the biomass composition and the fatty acid profile of heterotrophically grown *Chlorella* sp. TISTR 8990 for biodiesel production, J. Biotechnol. 216 (2015) 169–177, https://doi.org/10.1016/j.jbiotec.2015.10.003.

[21] W.A. Dick, M.A. Tabatabai, Determination of orthophosphate in aqueous solutions containing labile organic and inorganic phosphorus compounds, J. Environ. Qual. 6 (2010) 82, https://doi.org/10.2134/jeq1977.00472425000600010018x.

[22] R. Carpine, K.J. Hellingwerf, G. Olivieri, A. Marzocchella, F. Branco dos Santos, W. Du, A. Pollio, Genetic engineering of *Synechocystis* sp. PCC6803 for poly-β-hydroxybutyrate overproduction, Algal Res. 25 (2017) 117–127, https://doi.org/10.1016/j.algal.2017.05.013.

[23] A.M. Burja, R.E. Armenta, H. Radianingtyas, C.J. Barrow, Evaluation of fatty acid extraction methods for *Thraustochytrium* sp. ONC-T18, J. Agric. Food Chem. 55 (2007) 4795–4801, https://doi.org/10.1021/jf070412s.

[24] O. Lowry, N.J. Rosenbrough, R.J. Randall, A.L. Farr, Protein measurment with the folin phenol reagent, Anal. Biochem. 217 (1951) 220–230, https://doi.org/10.1016/0304-3894(92)87011-4.

[25] Y. Chen, S. Vaidyanathan, Simultaneous assay of pigments, carbohydrates, proteins and lipids in microalgae, Anal. Chim. Acta 776 (2013) 31–40, https://doi.org/10.1016/j.aca.2013.03.005.

[26] P. Olsson, J. Holmbäck, B. Herslöf, Separation of lipid classes by HPLC on a cyanopropyl column, Lipids 47 (2012) 93–99, https://doi.org/10.1007/s11745-011-3627-0.

[27] Z. Rohfritsch, O. Schafer, F. Giuffrida, Analysis of oxidative carbonyl compounds by UPLC-High-Resolution Mass Spectrometry in milk powder, J. Agric. Food Chem. (2019), https://doi.org/10.1021/acs.jafc.9b00674 (acs.jafc.9b00674).

[28] J. Pruvost, G. Van Vooren, B. Le Gouic, A. Couzinet-mossion, J. Legrand, Bioresource technology systematic investigation of biomass and lipid productivity by microalgae in photobioreactors for biodiesel application, Bioresour. Technol. 102 (2011) 150–158, https://doi.org/10.1016/j.biortech.2010.06.153.

[29] D. Huang, B. Ou, M. Hampsch-Woodill, J.A. Flanagan, R.L. Prior, High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format, J. Agric. Food Chem. 50 (2002) 4437–4444, https://doi.org/10.1021/jf0201529.

[30] D. Huang, B. Ou, M. Hampsch-Woodill, J.A. Flanagan, E.K. Deemer, Development and validation of oxygen radical absorbance capacity assay for lipophilic antioxidants using randomly methylated β-cyclodextrin as the solubility enhancer, J. Agric. Food Chem. 50 (2002) 1815–1821, https://doi.org/10.1021/jf0113732.

[31] O. Ménard, C. Bourlieu, S.C. De Oliveira, N. Dellarosa, L. Laghi, F. Carrière, F. Capozzi, D. Dupont, A. Deglaire, A first step towards a consensus static in vitro model for simulating full-term infant digestion, Food Chem. 240 (2018) 338–345, https://doi.org/10.1016/j.foodchem.2017.07.145.

[32] S. Daliry, A. Hallajisani, J.M. Roshandeh, H. Nouri, A. Golzary, Investigation of optimal condition for *Chlorella vulgaris* microalgae growth, Glob. J. Environ. Sci. Manag. 3 (2017) 217–230, https://doi.org/10.22034/gjesm.2017.03.02.010.

[33] M.H. Morowvat, Y. Ghasemi, Maximizing biomass and lipid production in heterotrophic culture of *Chlorella vulgaris*: techno-economic assessment, Recent Pat. Food. Nutr. Agric. 10 (2018) 115–123, https://doi.org/10.2174/2212798410666180911100034.

[34] J.H. Janssen, J. Kastenhofer, J.A. de Hoop, P.P. Lamers, R.H. Wijffels, M.J. Barbosa, Effect of nitrogen addition on lipid productivity of nitrogen starved *Nannochloropsis gaditana*, Algal Res. 33 (2018) 125–132, https://doi.org/10.1016/j.algal.2018.05.009.

[35] T.M.M. Bernaerts, L. Gheysen, C. Kyomugasho, Z. Jamsazzadeh Kermani, S. Vandionant, I. Foubert, M.E. Hendrickx, A.M. Van Loey, Comparison of microalgal biomasses as functional food ingredients: focus on the composition of cell wall related polysaccharides, Algal Res. 32 (2018) 150–161, https://doi.org/10.1016/j.algal.2018.03.017.

[36] Y. Li, M. Horsman, B. Wang, N. Wu, C.Q. Lan, Effects of nitrogen sources on cell growth and lipid accumulation of green alga *Neochloris oleoabundans*, Appl. Microbiol. Biotechnol. 81 (2008) 629–636, https://doi.org/10.1007/s00253-008-1681-1.

[37] N.N. Zulu, K. Zienkiewicz, K. Vollheyde, I. Feussner, Current trends to comprehend lipid metabolism in diatoms, Prog. Lipid Res. 70 (2018) 1–16, https://doi.org/10.1016/j.plipres.2018.03.001.

[38] A.P. Simopoulos, Importance of the ratio of omega-6/omega-3 essential fatty acids: evolutionary aspects, World Rev. Nutr. Diet. 92 (2003) 1–22 http://www.ncbi.nlm.nih.gov/pubmed/14579680.

[39] J.H. Janssen, P.P. Lamers, R.C.H. de Vos, R.H. Wijffels, M.J. Barbosa, Translocation and de novo synthesis of eicosapentaenoic acid (EPA) during nitrogen starvation in *Nannochloropsis gaditana*, Algal Res. 37 (2019) 138–144, https://doi.org/10.1016/j.algal.2018.11.025.

[40] F. Shahidi, Headspace volatile aldehydes as indicators of lipid oxidation in foods, in: R.L. Rouseff, K.R. Cadwallader (Eds.), Headsp. Anal. Food Flavors Theory Pract, Kluwer Academic/Plenum Publishers, New York, 2001, pp. 113–123, , https://doi.org/10.1007/978-1-4615-1247-9_9.

[41] K.D. Petersen, K.K. Kleeberg, G. Jahreis, M. Busch-Stockfisch, J. Fritsche, Comparison of analytical and sensory lipid oxidation parameters in conventional

and high-oleic rapeseed oil, Eur. J. Lipid Sci. Technol. 114 (2012) 1193–1203, https://doi.org/10.1002/ejlt.201200112.
[42] H. Saito, K. Ishihara, Antioxidant activity and active sites of phospholipids as antioxidants, J. Am. Oil Chem. Soc. 74 (1997) 1531–1536, https://doi.org/10.1007/s11746-997-0072-6.
[43] L. Cui, E.A. Decker, Phospholipids in foods: prooxidants or antioxidants? J. Sci. Food Agric. 96 (2016) 18–31, https://doi.org/10.1002/jsfa.7320.
[44] F. Shahidi, Y. Zhong, Lipid oxidation and improving the oxidative stability, Chem. Soc. Rev. 39 (2010) 4067–4079, https://doi.org/10.1039/b922183m.
[45] L. Gheysen, N. Lagae, J. Devaere, K. Goiris, P. Goos, T. Bernaerts, A. Van Loey, L. De Cooman, I. Foubert, Impact of microalgal species on the oxidative stability of n-3 LC-PUFA enriched tomato puree, Food Chem. 279 (2019) 389–400, https://doi.org/10.1016/j.foodchem.2018.12.026.
[46] U.M. Lanfer-Marquez, R.M.C. Barros, P. Sinnecker, Antioxidant activity of chlorophylls and their derivatives, Food Res. Int. 38 (2005) 885–891, https://doi.org/10.1016/j.foodres.2005.02.012.
[47] X.M. Sun, L.J. Ren, Q.Y. Zhao, X.J. Ji, H. Huang, Microalgae for the production of lipid and carotenoids: a review with focus on stress regulation and adaptation, Biotechnol. Biofuels 11 (2018) 1–16, https://doi.org/10.1186/s13068-018-1275-9.
[48] J. Lv, X. Yang, H. Ma, X. Hu, Y. Wei, W. Zhou, L. Li, The oxidative stability of microalgae oil (*Schizochytrium aggregatum*) and its antioxidant activity after simulated gastrointestinal digestion: relationship with constituents, Eur. J. Lipid Sci. Technol. (2015) 1–12, https://doi.org/10.1002/ejlt.201400588.
[49] F. Ahmed, K. Fanning, M. Netzel, W. Turner, Y. Li, P.M. Schenk, Profiling of carotenoids and antioxidant capacity of microalgae from subtropical coastal and brackish waters, Food Chem. 165 (2014) 300–306, https://doi.org/10.1016/j.foodchem.2014.05.107.
[50] C. Galasso, A. Gentile, I. Orefice, A. Ianora, A. Bruno, D.M. Noonan, C. Sansone, A. Albini, C. Brunet, Microalgal derivatives as potential nutraceutical and food supplements for human health: a focus on cancer prevention and interception, Nutrients 11 (2019) 1226, https://doi.org/10.3390/nu11061226.
[51] C.R. Brown, D. Culley, M. Bonierbale, W. Amorós, Anthocyanin, carotenoid content, and antioxidant values in native South American potato cultivars, HortScience 42 (2007) 1733–1736.
[52] S. Bensalem, F. Lopes, P. Bodénès, D. Pareau, O. Français, B. Le Pioufle, Understanding the mechanisms of lipid extraction from microalga *Chlamydomonas reinhardtii* after electrical field solicitations and mechanical stress within a microfluidic device, Bioresour. Technol. 257 (2018) 129–136, https://doi.org/10.1016/j.biortech.2018.01.139.