Bioprospecting and characterization of temperature tolerant microalgae from Bonaire

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Abstract

Control of temperature is a major challenge for industrial microalgal production in photobioreactors outdoors. Strains with tolerance for high temperatures can reduce the cost of production as active temperature control is not required. In this study, marine photoautotrophic microorganisms were isolated to reduce the need for control of high temperature.

Twenty-two samples were taken from different saline waters on the Caribbean island Bonaire. During strain enrichment, a temperature of 40 °C was used as selective pressure and strains with the highest growth rate were selected. We isolated and identified 59 strains, after which 5 were selected for characterization on growth rate and biomass composition. Picoclorum sp. and Leptolyngbya sp. showed optimal growth at 40 °C and 35 °C with a growth rate of 0.12 h\textsuperscript{-1} during daytime, respectively. The strains contain 62.1% and 68.2% of protein and have varying fatty acid compositions suitable for application as edible oil and biofuel.

1. Introduction

Microalgae are recognised as a promising alternative to traditional crops. They are unicellular photosynthetic microorganisms which can be grown in photobioreactors. Applications are found in food, feed, biofuel, bioplastics, cosmetics and nutraceuticals industries [1,2]. Cultivation of microalgae can be done anywhere, as long as basic requirements for growth, such as sufficient light and nutrients, and tolerable temperatures are met. The ability to grow microalgae in photobioreactors and open ponds removes the requirement for arable land and creates opportunities for production in desert regions [3,4]. Sustainable and cost-effective production of microalgae should be done in places offering high solar irradiance, constant climatological conditions and seawater availability [5]. The Caribbean island Bonaire has been presented as a location which meets all these requirements [6].

The culture inside photobioreactors, when not controlled, can reach peak temperatures up to 50 °C due to high irradiation levels [7,8]. The optimal growth temperature for most industrial microalgal strains is commonly between 20 °C and 30 °C [9,10]. At temperatures higher than their optimum growth temperature, microalgae suffer from enzyme degradation and failure of the photosynthetic system as a result of heat stress [10,11]. One major cost factor for microalgae cultivation in photobioreactors is cooling during day-time to avoid losses of productivity and culture collapses [12]. Currently applied methods for cooling involve bioreactor shading, spraying with water, use of a heat exchanger or reactor submersion in water [13]. Each of these methods has the disadvantage of either increasing process costs or decreasing productivity. Removing the requirement for bioreactor cooling would result in a significant decrease in production costs. Microalgae species which can tolerate peak temperature of 45 °C instead of 30 °C when grown in flat panel photobioreactors would decrease production costs by 31%, as calculated by the techno-economic model of [14].

The microalgal biodiversity around the world is large, with 72,500 species known and an estimated 1 million species still to be discovered [15]. This significant untapped potential harbour organsisms with beneficial traits for growth and production in regions around the equator. Such regions commonly harbour a constant climate all year round, high light intensities, and high temperatures. Bioprospecting of new microalgae species for both research and industrial applications has been performed in many regions of the world [16–20]. In most of these studies, the aim was to isolate strains for production of specific compounds, such as lipids, protein or carbohydrates, without considering process conditions. As a result, process control is currently more expensive than it could be. More attention must be given to process-oriented bioprospecting to reduce temperature control requirements. In this study, the tropical island of Bonaire is presented as a blueprint for microalgal production in other regions with comparable climatological conditions. The temperature of a photobioreactor on
Bonaire will fluctuate between 27 °C and 50 °C in a diel pattern, depending on the time of the day and weather conditions [7,8]. To reach the highest possible productivity under these conditions, the ideal strain must have a high growth rate over this temperature range.

The focus of the present study was to isolate robust microalgae strains which grow at temperatures higher than current industrial strains. Water bodies on Bonaire were sampled to isolate indigenous strains. We selected strains after a strain enrichment to find strains with high growth rates at 40 °C. After this initial selection, the growth kinetics were studied for 5 strains at different temperatures and a light intensity of 300 μmol·m⁻²·s⁻¹. These strains were then screened for their chemical composition to identify potential commercial applications.

2. Materials and methods

2.1. Bioprospecting and strain identification

2.1.1. Collection, enrichment, isolation of microalgae

Water samples were collected from two coastal areas and inland saline water bodies at 16 different locations on Bonaire (Fig. 1). Sampling was performed on March 2018 and October 2018. Samples were transferred into pH 7.4 HEPES buffer (C₈H₁₈N₂O₄S) buffered artificial seawater enriched with nutrients, resulting in the following concentrations (in mM): NaCl, 419.5; Na₂SO₄, 22.5; CaCl₂·2H₂O, 5.4; K₂SO₄, 4.9; MgCl₂·6H₂O, 48.2; Ca₃H₈O₇NO₃S, 20; NaN₃, 24.94; KH₂PO₄, 1.69; Na₂EDTA·2H₂O, 2.810⁻¹; FeSO₄·7H₂O, 1.110⁻¹; MnCl₂·2H₂O, 1.110⁻²; ZnSO₄·7H₂O, 2.310⁻³; Co(NO₃)₂·6H₂O, 2.410⁻⁴; CuSO₄·5H₂O, 9.610⁻⁵; Na₂MoO₄·2H₂O, 1.010⁻⁵. The cultures were incubated in a 2% CO₂ enriched shaking incubator (Multitron, Infors HT, Switzerland) at a rotational speed of 125 rpm and relative humidity of 60%. The circadian cycle comprised 12 h of day-time at 40 °C and 12 h of night-time at 30 °C. Wide spectrum white fluorescent light (Philips master TL-D reflex, color temperature 840) was set at an intensity of 140 μmol·m⁻²·s⁻¹ during the daytime. An enrichment phase was used to increase the number of cells which were able to grow under the enforced growth conditions. The cultures were diluted twice weekly in fresh culture medium at 3% v/v for 2–4 weeks until only a few species remained in the culture. Isolation of single species was performed by serial dilution on agar enriched growth medium. Colonies were selected for transfer to liquid medium based on colony size and microscopic observations.

2.1.2. Phylogenetic analysis of isolated strains

Isolated microalgae were identified by PCR and sequencing of the 16S rRNA gene (rDNA) in cyanobacteria and 18S rRNA gene (rDNA) for microalgae. DNA was extracted from pelleted microalgae and prepared for direct PCR according to recommendations of the manufacturer of the Phire Plant Direct PCR Kit (Finnzymes, Woburn, MA, USA). Primers specific for cyanobacterial 16S rRNA gene (rDNA) amplification were used as described by Nübel et al. [21]. A DNA product of approximately 700 bp was generated using the CYA106F and CYA781R (a + b) primers. 18S rRNA gene (rDNA) amplification was performed using FW primer 5’ CTC GGT TGA TCC TGG CAG 3’ and RV primer 5’ (A/T)TG ATC CTT CT/C/G CAC GTT CA 3’ with a melting temperature of 64.9 °C [22]. A DNA product of approximately 1500 bp was generated using these 18 s RNA gene (rDNA) primers. PCR products were sequenced using Sanger sequencing. The sequence ends were trimmed off manually, after which identification of the microalgae was performed through the NCBI BLAST. The closest named species is presented as determined using NCBI BLAST. The isolated strains are presented at a genus level in this article as more detailed identification is necessary to identify at a species level.

2.2. Characterization

2.2.1. Maximum growth rate

Cells were grown in a stirred incubator (Algaebator, ‘ontwikkelwerkplaats’ Wageningen University, The Netherlands, Wageningen) and a daily sequencing batch mode was applied for cultivation, in which 100 ml microalgae cultures were diluted daily to a set cell density to avoid light limitation. Warm white LED light (BXRA W1200, Bridgelux, USA, Livermore) was provided at the bottom of a 250 ml erlenmeyer flask and controlled at 300 μmol·m⁻²·s⁻¹ in a 12/12 h day/night cycle. A 2% v/v CO₂ enriched headspace was created to ensure a non-limiting carbon concentration. Optical density measurements at 750 nm (OD750) were used to determine the dilution factor for starting the daily sequencing batch culture. Cultures were diluted daily to an OD750 of 0.1 directly after the onset of the dark period. Duplicate cultures were grown in sequencing batch mode at 20, 25, 30, 35, 40 and 45 °C, until the growth rate between days stabilized, at which the culture was considered to be at steady-state.

Data from minimally 3 consecutive days at steady-state was used to calculate the maximum growth rate under each culture condition studied. Cultures were considered in steady state when the deviation of the growth rate between days was < 10%. The growth rate was calculated as seen in Eq. (1). N₀ and Nₓ represents the OD750 value directly after dilution (t₀ in h⁻¹) and 24 h after dilution (tₓ in h⁻¹). The growth rate (ux in h⁻¹) was calculated only considering the illuminated hours.

\[ u_x = \frac{\ln(N_x) - \ln(N_0)}{t_x - t_0} \]  

2.2.2. Biomass and composition

Biomass samples for compositional analysis were taken in a separate batch cultivation experiment. The growth conditions were as described in ‘2.2.1. Maximum growth rate’ except for the dilution and harvesting procedure. Algae were harvested during the linear growth phase, either when a biomass concentration of 2.5 g·l⁻¹ was reached, or after 14 days of growth. The biomass was centrifuged at 4000 g for 5 min, washed twice with 0.5 M ammonium formate, and stored at −20 °C. Afterwards, samples were lyophilised for 24 h.

2.2.3. Fatty acid composition and content

Total fatty acid content was determined through gas
3. Results and discussion

3.1. Bioprospecting and strain identification

The waters of Bonaire represent a wide range of environmental conditions. Isolated saline water bodies of different pH levels (pH 6.8–10), salinities (15–150 ppt) and temperatures (26.3–35.5 °C) harbour a rich variety of microorganisms. Twenty-two water samples were taken on Bonaire. The biodiversity was high in all bioprospected samples, independent of sampling location. Every culture contained a wide variety of species at the very beginning of the enrichment phase. The waters of Bonaire represent a wide range of environmental conditions. Isolated saline water bodies of different pH levels (pH 6.8–10), salinities (15–150 ppt) and temperatures (26.3–35.5 °C) harbour a rich variety of microorganisms. Twenty-two water samples were taken on Bonaire. The biodiversity was high in all bioprospected samples, independent of sampling location. Every culture contained a wide variety of species at the very beginning of the enrichment phase.

3.2. Growth kinetics at varying temperatures

Five isolated species were selected from the list of species provided in Table 1, based on visual observations of the cell cultures for two weeks. The criteria for selection were based on the potential for upscaling. Considering the ability to grow to a high density in cell suspension without forming biofilm or flocs. Phycobiliprotein productivity decreases due to biofilm formation, as a large fraction of the incident light is absorbed by this oversaturated layer of microalgae. Cleaning of biofilms is time-consuming and therefore undesirable, which makes it an important selection criterion. The ability to grow at high cell densities was observed for most species that were also able to grow without forming biofilms.

A high growth rate is important to start photobioreactors quickly and to prevent contamination. In addition, a high growth rate will lead to a faster upscaling trajectory and will result in high bioreactor productivity [9,26]. To determine the growth rate of the selected microalgae, they were cultured in repeated batch mode at a daily starting cell density of 0.03 g L⁻¹. The low cell density was chosen to assess their growth rate without the effect of cell shading. The cultures required five days under repeated batch cultivation to reach a stable growth rate, which was considered a steady-state. Reproducible growth rates were then observed between replicate cultures. Growth rates of the selected species at temperatures from 20 to 45 °C are presented in Fig. 2. The relevance of these data is not only found in the maximum rate of growth but especially in the temperature at which the maximum growth is reached.

As previously mentioned, The strains of the genus Picochlorum were well represented among the identified strains. To reduce the number of strains before performing elaborated growth experiments 6 strains were chosen for a preliminary short growth study. These subspecies of Picochlorum sp. were subjected to the repeated batch growth experiment at 35 °C. Picochlorum sp. CTM20019 (BPE23) was found to possess the highest growth rate (data not shown). Accordingly, this strain was selected for the more detailed growth studies, shown in Fig. 2.

This study aimed to find strains with a high growth rate from 25 °C to 45 °C in combination with a light intensity of 300 μmol m⁻² s⁻¹. Based on these criteria, Leptolyngbya sp. (BPE12) and Picochlorum sp. (BPE23) were the best performers. A maximum growth rate up to an observed 0.12 h⁻¹ was observed for these strains at 35 °C and 40 °C. Picochlorum species have recently gained attention because of their high growth rates, sometimes in combination with high thermo- and halotolerance [20,27].

Tetraselmis sp. (BPE14), showed growth up to 40 °C with an observed growth rate of 0.08 h⁻¹ at 35 °C. Most of the other reported Tetraselmis species have a temperature optimum around 25 °C [28]. Tetraselmis strains with a temperature tolerance up to 40 °C have been reported before [29]. However, no growth rates were reported which complicates good comparison.

Dunaliella sp. (BPE46) and Tetraselmis sp. (BPE14) grew under temperatures up to 35 °C and 40 °C, respectively. Picochlorum sp. (BPE23), Synecochococcus sp. (BPE37) and Leptolyngbya sp. (BPE12) showed growth up to 45 °C, which was the maximum temperature studied. While for Picochlorum sp. (BPE23) a decrease in growth rate was observed when the temperature was increased from 40 °C to 45 °C, this was not observed for Synecochoccus sp. (BPE37) and Leptolyngbya sp. (BPE12). It is, therefore, speculated that the optimum temperature of these strains may be beyond 45 °C. This is demonstrated by Synecochoccus elongatus, which is closely related to Synecochoccus sp. (BPE37). This species is capable of active growth up to 60 °C with an optimum at 55 °C [30].

For Synecochoccus sp. (BPE37) it was found that growth was not optimal due to excessive light stress as under dense culture conditions the observed growth rate of Synecochoccus sp. (BPE37) was comparable to the growth rate of Leptolyngbya sp. (BPE12) and Picochlorum sp. (BPE23) (data not shown) [31].
Table 1
Isolated species as found through NCBI online database using nucleotide BLAST. Date accessed: 11.01.2019. Origin corresponds to the sampling location as shown in Fig. 1: sampling locations on Bonaire.

<table>
<thead>
<tr>
<th>Division</th>
<th>Closest named species (Genbank)</th>
<th>Isolate</th>
<th>Origin (location)</th>
<th>GenBank accession no.</th>
<th>Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillariophyta</td>
<td>Halimeda subtrigona (MG027330.1)</td>
<td>BPE42</td>
<td>Saline puddle (4)</td>
<td>MN907400</td>
<td>100.00%</td>
</tr>
<tr>
<td>Bacillariophyta</td>
<td>Stauroeis latissimae (KY054994.1)</td>
<td>BPE48</td>
<td>Saltwater lake (15)</td>
<td>MN907403</td>
<td>96.59%</td>
</tr>
<tr>
<td>Chlorophyta</td>
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<td>Salt plains (5)</td>
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<td>Salt plains (5)</td>
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</tr>
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<td>Estuary (14)</td>
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</tr>
<tr>
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<td>Saltwater lake (16)</td>
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<td>98.96%</td>
</tr>
<tr>
<td>Chlorophyta</td>
<td>Picochlorum sp. (KF495093.1)</td>
<td>BPE23</td>
<td>Ray (13)</td>
<td>MN907399</td>
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</tr>
<tr>
<td>Chlorophyta</td>
<td>Tetraselmis sp. (KCR20794.1)</td>
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<td>Cyanobacteria</td>
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<td>Cyanobacteria</td>
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<td>Cyanobacteria</td>
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<tr>
<td>Cyanobacteria</td>
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<tr>
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<td>Cyanobacteria</td>
<td>Synechococcus elongatus (AY946243.1)</td>
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<td>94.56%</td>
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</tbody>
</table>

3.3. Biomass composition

Industrial microalgal production strains must produce components with economical applications and value. The selected strains were analysed for protein, carbohydrate and fatty acid levels during the linear growth phase, shown in Fig. 3. These three major groups comprise up to 86.5% of microalgal biomass in our study and are currently the most relevant bulk products. *Synechococcus sp.* (BPE12) and *Leptolyngbya sp.* (BPE12) show a protein content of 62.1% and 68.2% of total biomass, respectively. Compared to the microalgal strains *Tetraselmis sp.* (BPE46) this is significantly higher. This is partly because the microalgal strains *Tetraselmis sp.* (BPE14) and *Dunaliella sp.* (BPE46) have a higher carbohydrate content 25.9 and 24.5%, respectively. Microalgae accumulate storage compounds as a stress response to nutrient depletion [32,33]. The cells within our research were grown under nutrient replete conditions. Due to the absence of a nutrient starvation phase, a low fatty acid content was expected. Lipid bodies are absent under these conditions. Therefore the majority of measured fatty acids must be in a membrane-bound state.

The composition of fatty acids largely determines their economic value. This gives relevance to the analysis of fatty acid composition for our species [32,33]. Microalgae and cyanobacteria found in high-temperature regions are commonly composed of short-chain saturated fatty acids (SFAs). This is the result of a lower need for high membrane fluidity which is provided by unsaturated fatty acids. It was found that fatty acid composition varied significantly among species. Both cyanobacteria, *Leptolyngbya sp.* (BPE12) and *Synechococcus sp.* (BPE37) show low saturation levels for C16 and C18 fatty acids, which is comparable to palm oil.

*P. battersii* (BPE14) and *Synechococcus sp.* (BPE37) report variable fatty acid compositions with different fatty acids, including the same fatty acid (C16:0), linoleic acid (C18:2), and **α**-linoleic acid (C18:3). The level of fatty acid unsaturation is high compared to algae strains in literature and commercial oils (Table 2) [32]. Research papers on other strains of *P. battersii* report variable fatty acid compositions with 20–29% SFA, 34–62%MUFA and 36–51%PUFA [34,35]. One commercial oil with fatty acid chain lengths comparable to *P. battersii* (BPE23), is rapeseed oil, with the exception that the level of fatty acid unsaturation is higher in *P. battersii* (BPE23). *Dunaliella sp.* (BPE46) registered a very comparable fatty acid profile as *P. battersii* (BPE23), with a higher C18:3 content at the cost of C18:2. *Tetraselmis sp.* (BPE12) shows a large number of different fatty acids, including the

![Fig. 2. Maximal growth rate (u<sub>t</sub> in h<sup>-1</sup>) at temperatures ranging from 20 °C to 45 °C with 5 °C intervals of five isolated microalgae and cyanobacteria. Cultures were grown in repeated batch mode at temperatures ranging from 20 °C to 45 °C with 5 °C intervals. Incident light intensity was 300 μmol m<sup>−2</sup> s<sup>−1</sup>. Data points represent the average ± SD of n ≥ 6 biological replicates. *, no growth observed.](image-url)
omega 3 fatty acids eicosatrienoic acid (C20:3-N3) and eicosapentaenoic acid (C20:5-N3) which can be used as a fish oil replacement. Other major fatty acid groups include C16:0, C18:2, and C18:3.

The high level of fatty acid unsaturation of the Tetraselmis sp. (BPE14), Picochlorum sp. (BPE23) and Dunaliella sp. (BPE46) make these strains suitable for edible oil production. Especially the unsaturated Omega-3 and Omega-6 fatty acids are of interest within this group. The beneficial fatty acid composition combined with the microalgae's high protein content makes these strains an interesting food and feed production platform.

4. Conclusions

To reduce process costs and improve yields when cultivating microalgae in tropical regions there is a need for thermo-tolerant species. Process-oriented strain selection was performed to isolate photoautotrophic microorganisms with a temperature optimum between 30 °C and 45 °C. Two ideal species were found, Picochlorum sp. (BPE23) and Leptolyngbya sp. (BPE12), which showed growth up to 45 °C, with a maximum growth rate of 0.12 h⁻¹ between 35 °C and 40 °C. Measurements on biomass composition indicate applicability for food because of the high protein content and as a replacement for palm tree oil, fish oil and rapeseed oil.

Data availability

Sequencing data of the 18s gene identification can be found in the NCBI nucleotide sequence repository under the reference of MN907396:MN907403[accn]. Sequencing data of the 16s gene identification can be found in the NCBI nucleotide sequence repository under the reference of MN909716:MN909723[accn] and MN906016:MN906017[accn]. Other data used to support the findings of this study are available from the corresponding author upon request. Microalgae strains were cryopreserved and stored at Wageningen University facilities. These are available from the corresponding author upon request.

Table 2

Fatty acid composition of five isolated microalgal and cyanobacterial strains from Bonaire. The fatty acid content was calculated and expressed as a percentage of total fatty acid in the cell. Cultures were grown in batch mode at 35 °C. Incident light intensity was 300 μmol·m⁻²·s⁻¹. Cells were harvested in the linear growth phase. Fatty acid compositions for Rapeseed and palm oil were obtained from literature [36]. Data points represent the average of n = 2 biological replicates. The average relative error for all tests was 4.16%.

<table>
<thead>
<tr>
<th></th>
<th>Picoclorum sp. (BPE23)</th>
<th>Tetraselmis sp. (BPE14)</th>
<th>Dunaliella sp. (BPE46)</th>
<th>Synechococcus sp. (BPE37)</th>
<th>Leptolyngbya sp. (BPE12)</th>
<th>Rapeseed oil</th>
<th>Palm oil</th>
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<td>C13:0</td>
<td>1.38</td>
<td>1.29</td>
<td>1.10</td>
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<td>7-19</td>
<td>51</td>
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<tr>
<td>C14:0</td>
<td>1.67</td>
<td>4.50</td>
<td>1.66</td>
<td>2.49</td>
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<tr>
<td>C14:1 cis-9</td>
<td>1.59</td>
<td>1.80</td>
<td>1.10</td>
<td>1.06</td>
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<tr>
<td>C16:0</td>
<td>24.82</td>
<td>23.80</td>
<td>29.15</td>
<td>46.56</td>
<td>41.83</td>
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<td>C16:1</td>
<td>1.39</td>
<td>2.47</td>
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<td>10.45</td>
<td>10.55</td>
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<td>C16:2</td>
<td>1.32</td>
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<td>1.72</td>
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<td>C18:0</td>
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<td>2.16</td>
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<td>1.73</td>
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<td>2.83</td>
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<td>Saturated fatty acids</td>
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<td>Monounsaturated fatty acids</td>
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<td>Polyunsaturated fatty acids</td>
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CRediT authorship contribution statement

Robin J.P. Barten: Investigation, Data curation, Formal analysis, Methodology, Writing - original draft. Rence H. Wijffels: Conceptualization, Writing - review & editing, Supervision, Funding acquisition. Maria J. Barbosa: Conceptualization, Writing - review & editing, Methodology, Supervision, Funding acquisition.

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. No conflicts, informed consent, or human or animal rights are applicable to this study.

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