LWT - Food Science and Technology 143 (2021) 111115

Contents lists available at ScienceDirect

LWT

journal homepage: www.elsevier.com/locate/lwt

Protein enrichment of wheat bread with the marine green microalgae *Tetraselmis chuii* – Impact on dough rheology and bread quality

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ARTICLE INFO

Keywords:
Microalgae
Bread quality
Protein enrichment
Dough rheology
Amino acid composition

ABSTRACT

Marine green microalgae are a sustainable source of high-quality protein. However, due to their green pigmentation and composition of volatiles, their incorporation into food products is a challenge. Incorporation of cell-wall disrupted *Tetraselmis chuii* (Tc) into bread (0, 4, 8, 12 and 16% wheat flour substitution) affected dough rheology and bread quality negatively. These effects were more pronounced at addition levels necessary for a EFSA nutrition claim on protein enrichment (12 and 16%). Treatment of Tc with ethanol not only removed much of the green pigmentation and volatiles, but also reduced the negative impact on dough rheology and bread quality. Doughs prepared with ethanol treated Tc (TcEt) showed a clear improvement in dough rheology evident as increased dough-stability-time (DST), resistance to extension (Rmax) and elastic-recovery-compliance (Je) particularly at substitution levels >4%. This was accompanied by an increase in bread quality e.g. at 12% substitution level specific volume increased from 2.1 to 2.69 mL/g, crumb firmness decreased from 1358 to 297 g and slice brightness increased from 25.2 to 49.0. Ethanol treatment of algae may be a feasible strategy to address the sensory and structural challenges that hinder incorporation of algae into foods at levels that can potentially confer nutritional benefits.

1. Introduction

Certain green microalgae (*Chlorophyta*) are potential sustainable sources of high-quality protein (40–65 g/100g in dry matter) for human nutrition (Becker, 2007). *Tetraselmis chuii*, a marine species, cultured in a photobioreactor and harvested in the stationary growth phase contained 46.5 g/100g dry weight (DW) protein with an essential amino acid (EAA) index of 0.9 (Tibbetts et al., 2015). *T. chuii* has enormous potential as a dietary source of sustainable protein of high nutritional quality. For a real impact on nutrition high enough amounts need to be incorporated into food products, which is linked to several challenges such as palatability, sensory-acceptance, and regulatory approval as a general food ingredient. Wheat bread is a prime candidate food for evaluating *T. chuii* suitability for protein fortification since it is a staple food with a low protein content and protein quality (Hafsa, Amel, Samia, & Sidahmed, 2014).

To date there are no definitive scientific studies on the incorporation of *T. chuii,* or its protein, into wheat bread, or any other wheat flour based baked products. Current commercial food applications of *T. chuii* in the EU through novel food approval have been limited to sauces, condiments and salts albeit at a very limited maximum permissible addition level of 250 mg/serving/d (AECOSAN, 2017). Several other genera of dried green and blue-green (cyanobacteria) microalgae, and even an unidentified species of *Tetraselmis* (Lafarga, 2019), have been evaluated as experimental ingredients in wheat bread making with a proliferation of interest in the last five years (Ak et al., 2016; Finney, Pomeranz, & Bruinsma, 1984; García-Segovia, Pagán-Moreno, Lara, & Martínez-Monzó, 2017; Graça, Fradinho, Sousa, & Raymundo, 2018; Lafarga, 2019; Sanjari, Sarhadi, & Shahdadi, 2018; Tertychnaya, Manzhesov, Andrianov, & Yakovleva, 2019). Wheat flour replacement with microalgae up to 10% w/w have been investigated (Ak et al., 2016; Finney et al., 1984) although most studies tended to add less (1–5%) and often as a direct addition rather than a substitution of wheat flour. Various types of bread have been investigated including soft white

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https://doi.org/10.1016/j.lwt.2021.111115
Received 10 November 2020; Received in revised form 16 January 2021; Accepted 11 February 2021
Available online 14 February 2021

bread, baguettes, and sourdough breads. At low doses of addition (≤3%) the functional properties of bread (specific volume and crumb density) are only slightly impacted (García-Segovia et al., 2017; Graça et al., 2018; Hafsa et al., 2014; Lafarga, 2019), while at higher microalgae doses (5–10%) such effects can be quite substantial (Finney et al., 1984; Graça et al., 2018). It appears that larger addition levels of microalgae can negatively impact the gluten network during dough development leading to inferior bread quality. Even at low addition levels (García--Segovia et al., 2017), the chlorophyll in microalgae can impart a green-brown colouration of the crumb and crust which does not always appeal to the consumer especially in light-coloured breads (Finney et al., 1984). A further obstacle is the sulphurous algal smell (Becker, 2007; Graça et al., 2018; Lafarga, 2019) that can be imparted to the bread that probably also impacts taste.

Organic solvents can be used to extract/remove fats, colour- or flavour compounds from food raw materials. Ethanol is commonly used in specialist food ingredient manufacture due to its benign toxicity and its regulative acceptability as a food processing aid (Stevenson & Inglett, 2011). Solvent extraction has been widely used experimentally on microalgae for the purpose of defatting and decolouration (Tibbetts et al., 2015), but few examples exist on use of such microalgal material in food applications. A study, were ethanol treated *Spirulina platensis* and *Oscillatoria amphibia* with a protein content of approximately 70 g/100g were added to wheat bread (7% wheat flour substitution corresponding to 5 g algae protein per 100 g wheat flour), reported a positive effect on dough rheology (increased resistance to extension) and bread quality (increased volume) compared to the wheat control without algae (Saleh, 1987).

The aim of this work was to investigate the effect of increasing *T. chuii* flour replacement levels (0–16%) on the rheological properties of wheat-based dough and the quality of final bread. The use of ethanol extraction as suitable pre-treatment of *T. chuii* was investigated and compared to cell wall disrupted *T. chuii* with the goal of producing breads of improved sensory properties and higher nutritional value (enriched in protein). An implicit aim was to make and assess algae fortified doughs and breads that met European Food Safety Authority (EFSA) specific guidelines for the nutrition claim "increased protein content", which can be used for products providing a minimum of 12% of its energy from protein ("source of protein") and containing at least 30% more protein compared to a similar product (non-enriched bread). With a protein content of 40–50 g/100g in the algae biomass a wheat flour substitution of 12% is required to achieve this protein level in bread. Finally, we discuss the regulatory hurdles that are a prerequisite to overcome should *T. chuii* fortified bread reach the market.

2. Materials and methods

2.1. Materials

Wheat flour of high protein strength was purchased from Lantmannen (hvetemel standard, Lantmannen Cerealia, Oslo, Norway). *T. chuii* (UTEX LB232) was cultured in the National Algae pilot plant, Mongstad, Norway, in a fed-batch process using four 800L photobioreactors (GemTube MK2-750, LGem, Rotterdam, Netherlands) with chemically sterilized seawater. The photobioreactors were located in a greenhouse exposed to natural light and additionally equipped with artificial illumination (EAX 170W LED lights, Evolys AS, Norway) with an average incident artificial light of 200 μmol m-2 s −1. Further details on the cultivation can be found elsewhere (Kokkali et al., 2020).

The microalgae cultures were harvested and concentrated by centrifugation (Evodos 50). The concentrated paste (dry matter content ca.35 g/100g) was vacuum packed and frozen at −20 °C until further use. Taxonomic identification was confirmed by molecular nuclear marker analysis of the 18S rRNA gene. A 100% match in the Basic Local Alignment Search Tool (BLAST) was obtained for 18S rDNA oligonucleotide primers ss5 (F) and ss3 (R) and primers 18SF Forward and 18SR Reverse (Khaw, Khong, Shaharuddin, & Yusoff, 2020) with 651 base pairs sequenced for each. Cell wall disruption was performed by bead milling using 0.25–0.4 mm diameter glass beads at 80% chamber filling rate in a Dyno-Mill Multi Lab (Willy A. Bachofen, Muttenz, Switzerland). The bead milling conditions were chosen based on previous optimisation trials for this species (Kokkali M.E., 2018). In brief, the *T. chuii* paste was diluted to ca. 21 g/100g dry matter analysed before and after dilution in a moisture analyser. Bead milling was realised by single pass in a 1.4 L bead mill chamber at 2865 rpm (12 m/s) tip speed and 9.4 kg/h biomass flow rate at processing temperature of ca. 26 °C. Cell wall disintegration degree was estimated in freshly thawed aliquots to ca. 97% by cytometry in a Neubauer counting chamber, observed in a Nikon eclipse Ci optical microscope. The biomass was freeze-dried and used 'as is' for ethanol extraction, but milled with a laboratory hammer mill (ZM 200, Retsch, 0.5 mm screen) prior to use in dough.

2.2. Reflux in ethanol

Cell wall disintegrated and freeze-dried *T. chuii* ~~biomass~~, termed Tc, was treated with 96% (v/v) ethanol using a Soxhlet extractor. Approximately 50–70 g Tc was added to a cellulose thimble and then placed inside the extraction chamber. A condenser was attached to the top of the extraction chamber and cooled with water (at 9 °C). 500 mL of ethanol was placed in the boiling flask and heated with a stirred silicon oil bath set to 130 °C. Each batch of Tc in the thimble was continuously extracted at ~45 °C for ~48–72h until the ethanol in the extractor was colourless. The ethanol extracted biomass, termed TcEt, was spread on a tray and placed at 60 °C for 6 h in a ventilated oven to remove traces of remaining ethanol. The dried TcEt was milled as described above.

2.3. Particle size measurement

The particle size distribution of milled Tc and TcEt was determined using a Sympatec HELOS laser diffraction sensor with a ROSOS dry dispersing unit fitted with a R6 lens (0.5–1750 μm).

2.4. Macronutrient composition of Tc and TcEt

Moisture content was measured by drying at 105 °C to constant weight (ICC 109/01). The ash content was determined gravimetrically as residue after combustion in a muffle furnace (AACC 08–01). Protein content was determined by combustion (Kirsten, 1979) using a Vario EL elemental analyser (Elementar, Langenselbold, Germany). A nitrogen to protein conversion factor of 4.78 was used for microalgae (Tibbetts et al., 2015). Total amino acids (AA) were analysed using HPLC with fluorescence detection (Cohen & Michaud, 1993), after the samples were hydrolysed in 6 N HCl for 22 h at 110 °C. Free amino acids (FAA) were determined by homogenizing the sample in an internal standard solution, derivatization with phenyl isothiocyanate, separation with reverse-phase HPLC and UV detection (Brekken, 1989). Crude fat content was estimated by a gravimetric method using solvent extraction (Bligh & Dyer, 1959). The total dietary fibre (TDF) was measured gravimetrically (AOAC 985.29) using an Ankom dietary fibre analyser. Total starch content was measured according AOAC 996.11.

2.5. Dough rheology

2.5.1. Farinograph mixing properties

Farinograph mixing properties for wheat substitution levels of 0, 4, 8, 12 and 16% Tc or TcEt were determined using a Newport Scientific DoughLab (Perten Instruments, Stockholm, Sweden) with a 50g bowl. Farinograph water absorption (WA) and dough stability time (DST) were determined according to ISO 5530–1 (63 rpm, 30 °C, 500 FU) and based on a flour moisture content of 14 g/100g, while the moisture content of Tc and TcEt were not considered (n ≥ 2).

2.5.2. Extensional measurements

Extensional tests were performed using an extensograph-E (Brabender, Duisburg, Germany) equipped with a Micro-extensograph unit for 20 g dough pieces. Doughs were prepared with a DoughLab in a 50 g bowl according to Farinograph WA using 126 rpm to a total energy input of 12 Wh/kg. Bowl temperature was set at 24 °C to ensure a final dough temperature of 30 °C. Tests were otherwise performed according to AACC method 54–10. Extensibility and resistance to extension were recorded (n = 3 independent doughs).

2.5.3. Creep recovery and small amplitude oscillatory shear (SAOS) measurements

Creep recovery and small amplitude oscillatory shear (SAOS) measurements were performed with an MCR 301 Rheometer (Anton Paar, Stuttgart, Germany) using a 25 mm parallel plate at 30 °C on subsamples of doughs prepared for extensograph measurements. Dough pieces were rested for 30 and 130 min respectively in a controlled heating chamber at 30 °C and 80%RH before sheeting with a pasta machine to ~2.3 mm thickness. Disks of 2.5 cm diameter were cut out and placed on a corrugated lower plate (used to prevent slippage). Sample loading was normal force controlled with a maximum of 5N. Samples were trimmed at a position of 2.025 mm using a dough cutter. The measurement gap was set at 2 mm. To prevent drying, silicon grease was applied to the outside of the trimmed sample. SAOS tests were performed immediately after loading without rest to monitor the time dependent behaviour of dough. SAOS were performed at 6.28 rad/s and strain 0.1%, within the linear viscoelastic region as determined previously, for a total of 420s. A linear regression ($y = mx + c$) was applied on each data set (n = 3), were c equals the G' intercept ($G'_{t=0}$).

Creep recovery test were performed immediately after SAOS using the same loaded dough piece. The sample was subjected to a constant 100 Pa stress for 300 s. After removal of the stress, the samples recovered the elastic part of their deformation for 1120 s. The stress level and stress time were chosen based on initial experiments to achieve steady state (linear increase in the creep compliance with time). Data were fitted to the Burgers model (Mezgar, 2011) to obtain the maximum creep compliance (J_{max}), the elastic recovery compliance (J_e) and the viscous recovery compliance (J_v).

All rheological data were processed in the rheometer software, Rheoplus Ver. 3.2. All the rheological measurements were replicated at least three times.

2.6. Baking

A small scale, straight dough process was used to study the baking properties of Tc and TcEt at wheat flour replacement levels of 4, 8, 12 and 16% w/w. Doughs were mixed in a DoughLab using a 300g bowl with 1 g/100g dry yeast and 1.5 g/100g salt (based on flour weight). Water addition was according to Farinograph water absorption (58.6–63.7 mL/100g flour). All doughs were prepared twice and in randomized order. Mixing was performed at 126 rpm to a total energy input of 12 Wh/kg. The bowl temperature was set to 22 °C to achieve a final dough temperature of 27 ± 1 °C. The doughs prepared were rested at 27 °C, RH 70% for 1hr (Lillinord, Odder, Denmark). After resting, 3 dough pieces of 150 g each were shaped (Dough rounder R10, FriulCo, Maniago, Italy) and placed into greased pans before proving at 35 °C and RH 70% for 1 h. Breads were baked for 20 min in a rotating oven (Revent type 626 G EL IAC, Revent international, Vesby, Sweden) set at 240 °C which was reduced to 220 °C while steam (2L) was injected. After baking, breads were immediately de-paned and cooled for 1h before measurement of bread weight and volume using a TexVol BVM-6630 Series analyser (Perten, Stockholm, Sweden). A 1.25 cm slice was removed from each bread before a 2.5 cm thick slice was cut with a spacer and knife. The crumb characteristics of this slice were determined by using a C-cell imaging system (Calibre control international, Warrington, UK). The crumb firmness was measured according to AACC Method 74–09 using a TA.XT2 Texture Analyser equipped with a 10 kg load cell (Stable Micro Systems, Surrey, UK).

2.7. Colour measurements

The colour of Tc and TcEt biomass and breadcrumbs were measured with a colorimeter, Minolta CR-400, Japan (n = 7). The results acquired are presented following CIELAB system: L* - lightness (0%–100%); a* - green to red (−60 to 60) and b* -blue to yellow (−60 to 60).

2.8. Volatile organic compounds

Volatile compounds were analysed using an Gerstel DHS-TDU-MPS automated dynamic headspace thermal desorption unit integrated with an Agilent 7890B gas chromatograph (GC) and 5977 B mass spectrometer. One gram of Tc, TcEt or unprocessed *T.chuii* biomass were weighed into 20 mL headspace test tubes, flushed with nitrogen and sealed. Volatile compounds were collected automatically after incubation at 70 °C for 5 min on activated carbon adsorbent tubes (Tenax GR, grain size 60/80 mesh, Alltech) for 2 min at 100 mL/min. Volatiles were separated on an Agilent DB-WAXETR GC column (30 m x 0.25 mm x 0.5 μm) with helium as carrier gas using the following temperature gradient: 30 °C (10 min), 1 °C/min to 40 °C (0 min), 3 °C/min to 70 °C (0 min), 6.5 °C/min to 230 °C (5 min). The mass spectrometer was operated in electron ionization (EI) mode at 70 eV ionization energy and positive ion fragments were recorded with scan range m/z 50–500. Peak area integration and compound identification were performed using Agilent MassHunter software (Ver. 10.0) and comparison of measured mass spectra of the GC peaks with the pure standards according to the NIST014 mass spectral library.

2.9. Statistical analysis

Analysis of Variance (ANOVA) was conducted *via* a general linear model (GLM) using Minitab 19 software. One factor (treatment), or were appropriate a second factor (resting time) with interaction, was used for each set of response data in the GLM. Prior to ANOVA, and where necessary, a Box-Cox transformation was applied to all raw data to achieve a near normal distribution. Equal variance was confirmed by conducting the test for equal variances. The pair-wise comparison information presented in figures/tables were acquired using a Tukey test at 95% confidence interval. Pearson correlation was performed to find correlation between rheological measurements and the bread crumb properties using Microsoft Excel.

3. Results

3.1. Nutrient composition, colour and volatile compounds

Tc had a high protein content of approximately 40g/100g with a good correlation between the two different methods (combustion with N x 4.78 and sum of detected amino acids) for raw Tc (Table 1).

Treatment of Tc biomass with ethanol increased the protein content to 60 g/100g for sum of detected amino acids and combustion using N x 6.25, but not N x 4.78. A conversion factor of 6.25 may be more appropriate where ethanol might have removed low molecular weight non-proteinaceous nitrogen containing compounds. The amino acid composition of Tc did not change upon ethanol treatment (Fig. 1) and a high protein quality with contents of all essential AA above the reference scoring pattern for adults (FAO, 2007) was retained.

Ethanol treatment of Tc removed nearly all the fat (Table 1) with a corresponding increase in protein and reduced content of FAA's by 50%. There was no difference in ash before and after ethanol treatment. The mineral content of Tetraselmis sp. consists to a considerable degree of calcium carbonate (Martignier et al., 2018) and a calcium content of 2.6 g/100 g was determined in Tc (Table 1). The cumulative distribution of

Table 1
Nutrient composition of Tc and TcEt in g/100g (dry basis), particle size distribution (μm) and colour. Values are presented as the mean with standard deviation. n.a = not analysed.

	Tc	TcEt
Protein (as total amino acids)	*42.1 ± 0.1*	*59.5 ± 0.2*
Protein (as combustion N x 4.78)	*38.2 ± 0.1*	*46.0 ± 0.1 (60.1 ± 0.1)*[x]
Free amino acids	*5.4*	*2.9*
Crude fat	*13.8*	*0.3*
Dietary fibre	*8.9 ± 0.8*	*15.1 ± 2.2*
Starch	*2.1 ± 0.1*	*2.2 ± 0.2*
Ash	*16.0 ± 0.1*	*16.7 ± 0.1*
Calcium	*2.6*	*n.a*
Moisture	*7.6 ± 0.6*	*5.0 ± 0.3*
Colour		
L*	*20.91 ± 1.36*	*51.65 ± 3.57*
a*	*−10.15 ± 1.02*	*−5.65 ± 0.25*
b*	*15.41 ± 2.01*	*17.62 ± 0.76*
Cumulative particle size distribution % (μm)		
x10	*9.9 ± 0.2*	*18.2 ± 0.7*
x50	*50.9 ± 1*	*69.3 ± 1.7*
x90	*137.2 ± 2.7*	*162.3 ± 4.5*

[x] Using conversion factor N x 6.25.

particle size increased slightly with ethanol treatment, but 90% of particles remained below 162 μm (Table 1). Treatment of Tc with ethanol reduced the green colour (Table 1). The TcEt powder was significantly brighter (L*), less green (a*) and blue (b*).

The volatile profile of Tc is shown in Fig. 2A. The algae volatiles were characterized by 4 sulphur compounds including dimethyl sulphide, dimethyl disulphide, dimethyl sulfoxide (peak 7) and dimethyl sulfone, 4 alcohols, including 1-pentanol, 2-penten-1-ol, 1-penten-3-ol (peak 3) and 1-octen-3-ol, 8 ketones, including 6-methyl-5-hepten-2-one (peak 5), tr,tr,3,5-octadien-2-one, 9 aldehydes, including hexanal, and benzaldehyde, 2 aromatics including benzene and benzaldehyde, heterocyclic compounds, 6 pyridines including 2-ethyl-pyridine (peak 4) and 8 pyrazines including 2,3-dimethyl-pyrazine and tetramethyl-pyrazine (peak 6). Pyridines and pyrazines were absent from the volatile profile of unprocessed *T.chuii* (results not shown). The volatile profile of TcEt is shown in Fig. 2B. The ethanol treatment removed 78–85% of the volatile compounds, mainly the aldehydes, alcohols, pyridines and pyrazines (Fig. 2B). The volatile profile of TcEt was characterized by 59 hydrocarbons, including 3,6-dimethyl-decane (peak 9) and 4-methyl-tetradecane (peak 10), 5 aldehydes including 2- and 3-methyl-butanal, 2 aromatics, including benzene and benzaldehyde. Also low amounts of the sulphur compounds and 2 pyrazines including methyl-pyrazine and 2,5-dimethyl-pyrazine were found.

3.2. Dough mixing properties

Farinograph DST and WA of wheat flour replaced by 0, 4, 8, 12 and 16% w/w Tc or TcEt is shown in Fig. 3. DST decreased significantly compared to the control for all substitution levels. The decrease was more pronounced for higher substitution levels (significant difference between 4 and 16%), but no significant differences were noticed between DST for Tc or TcEt at each addition level. Water absorption (WA in %) increased dose dependently with the addition of Tc (Fig. 3). The increase in water absorption was significant for wheat flour substitution levels of 12% or higher in comparison to the control. There was a general trend of higher WA for TcEt substituted doughs compared to Tc substituted doughs. When comparing all substitution levels, this difference between TcEt and Tc was significant, but not for each individual substitution level.

3.3. Extensional measurements

The control dough (0% substitution) had both the highest extensibility and maximum resistance to extension (Rmax) (Fig. 4). Extensibility decreased and Rmax increased for all doughs with resting time. Substitution of wheat flour dose-dependently decreased both extensibility and Rmax. From a substitution level of 12% and higher, this decrease was significant compared to the control dough. For Tc, substitution levels above 4% resulted in very sticky doughs, that could not be rolled in the extensograph and hence doughs with 8, 12 and 16% Tc could not be measured. A direct comparison of Tc and TcEt was therefore only possible at the substitution level of 4%, where the Rmax was higher for the TcEt compared to the Tc (not significant) on all the three resting times 45, 90 and 135min.

3.4. Creep recovery and SAOS

The effect of resting time (30 or 130 min) was not significant for any of the measured parameters and therefore only results from 30 min resting were reported (Table 2). Doughs prepared with 12 and 16% Tc were too sticky to measure. The control dough showed the highest J_{max} and highest J_e (Table 2). Substitution of wheat flour with Tc or TcEt

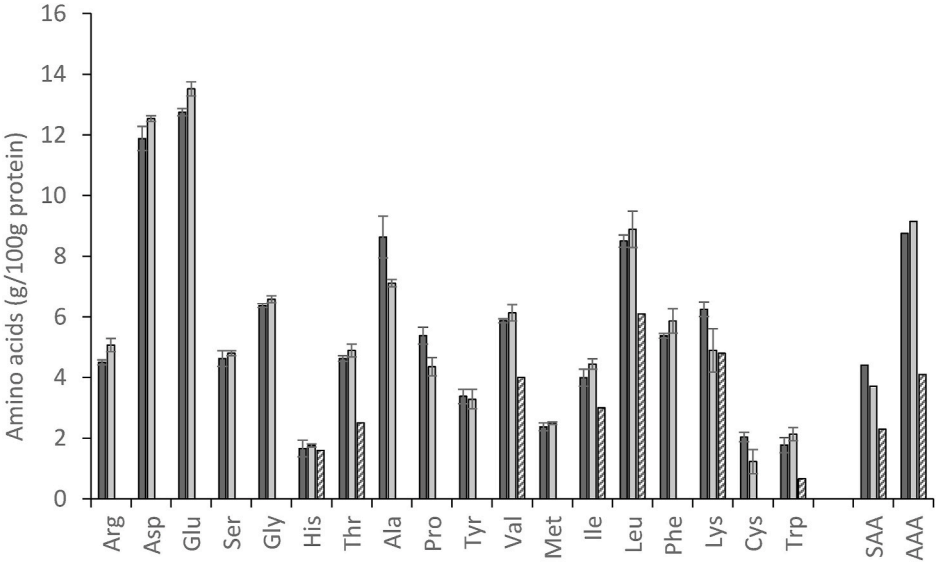

Fig. 1. Amino acid profile of Tc (dark grey) and TcEt (light grey) (g/100g protein) compared to the recommended amino acid scoring pattern (striped) (FAO, 2007) SAA, sulphur amino acids (cysteine + methionine), AAA, aromatic amino acids (phenylalanine + tyrosine).

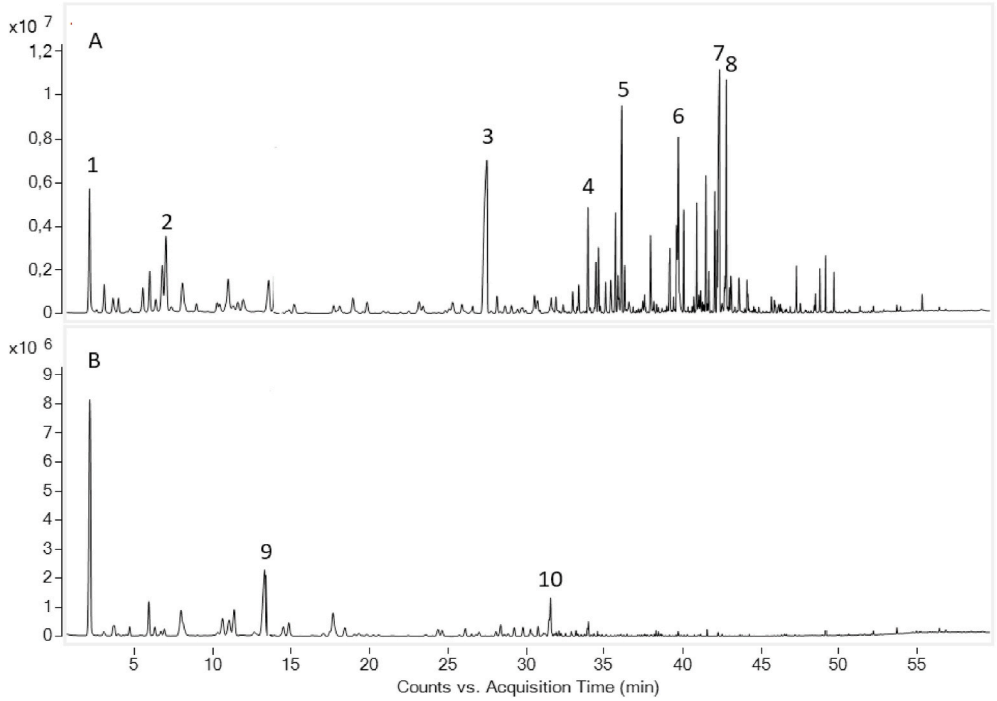

Fig. 2. Total ion chromatograms of volatile compounds of raw algae (A) and ethanol treated algae (B). Major compounds: n-hexane (1), 3-methyl-butanal (2), 1-penten-3-ol (3), 2-ethylpyridine (4), 6-methyl-5-hepten-2-one (5), tetramethyl-pyrazine (6), dimethyl sulfoxide (7), 2,6-dimethyl-cyclohexanol (8) 3,6-dimethyldecane (9), 4-methyl-tetradecane (10).

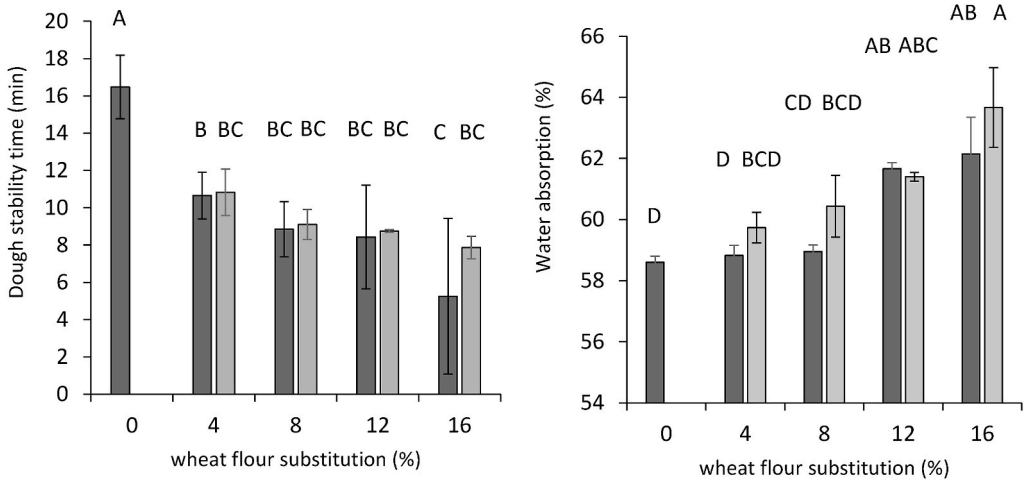

Fig. 3. Dough stability time (left) and water absorption (right) of doughs prepared with Tc (dark grey) and TcEt (light grey) at different levels of wheat flour substitution. The results are mean ± standard deviation of triplicate measurements. Bars sharing the same letters are not significantly different ($p > 0.05$).

decreased J_{max}, J_v and J_e (Table 2). The decrease of J_{max} was significant for a substitution level of 8% Tc and for 12 and 16% TcEt. Doughs prepared with either 16% TcEt or 8% Tc had J_e values that were significantly lower than the control. For J_v, significant differences were observed already at lower substitution levels and only doughs prepared with 4% Tc were not significantly different form the control. Doughs prepared with 8% Tc had the lowest J_{max}, J_v and J_e of all doughs and were significantly different from the control. There was a clear tendency towards higher J_{max}, J_v and J_e for TcEt compared to Tc at the same substitution level, especially at the highest level of 8% (12 and 16% Tc doughs were too sticky). However, the differences between Tc and TcEt at 4 and 8% substitution level were not significant.

The G' remained constant during the measurement period of 420s. The control dough had the lowest $G'_{t=0}$ (15.2 kPa). For increasing levels of wheat flour substitution, the $G'_{t=0}$ increased and was significantly different from the control for 16% TcEt (20.0 kPa). No significant difference in $G'_{t=0}$ was observed between Tc and TcEt at the same substation level even if $G'_{t=0}$ for 8% Tc was higher (19.0 kPa) compared to 8% TcEt (17.3 kPa).

3.5. Bread properties

Wheat flour substitution with Tc or TcEt had a profound effect on bread appearance, crumb structure, specific volume, and crumb firmness (Figs. 5 and 6). Wheat flour substitution dose-dependently decreased specific volume (significant from 4% substitution). At substitution levels of 8% and higher, there was a significant difference in specific volume between breads prepared with Tc or TcEt. Similarly,

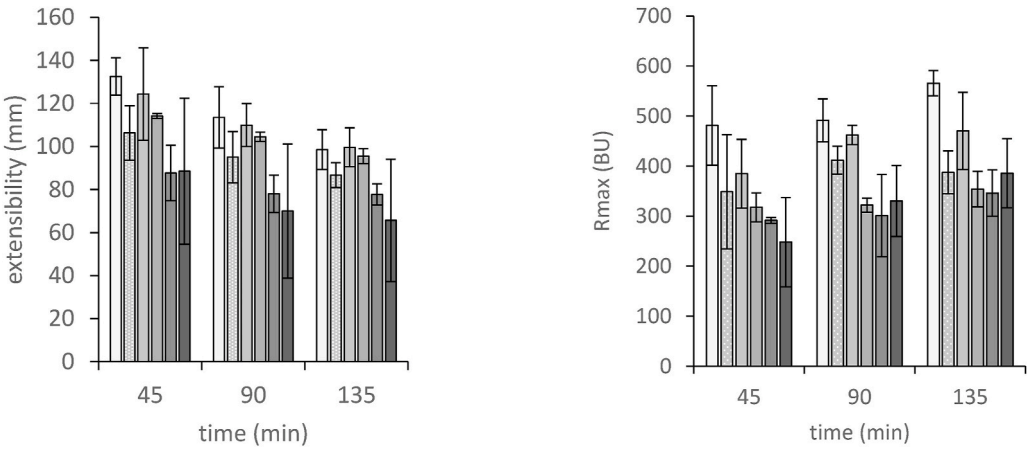

Fig. 4. Effect of wheat flour substitution with Tc (stippling) and TcEt (no pattern) at 0 (white), 4 (light grey), 8 (dark grey), 12 and 16% substitution level (increasing darkness with increasing substitution level) on extensibility and maximum resistance to extension (Rmax) at different relaxation times of 45, 90, and 135 min. Mean ± SD (n = 3). Note: Wheat flour substitution with Tc above 4% resulted in sticky dough, that could not be rolled in the extensograph and was hence not measured.

Table 2
Maximum creep compliance (J_{max}), elastic recovery compliance (J_e), viscous recovery compliance (J_v) and storage moduli (G') of doughs prepared with Tc and TcEt at different levels of wheat flour substitution after 30 min rest.

	J_{max} (10^{-4} Pa^{-1})	J_e	J_v	$G'_{t=0}$ (10^3 Pa)
Control	11.4 ± 3.7^{c}	5.9 ± 2.5^{c}	5.5 ± 1.7^{a}	15.2 ± 1.8^{b}
4% Tc	6.9 ± 1.2^{bc}	3.9 ± 0.8^{abc}	3.0 ± 1.2^{ab}	16.5 ± 1.2^{ab}
4% TcEt	6.5 ± 1.1^{abc}	4.0 ± 0.4^{bc}	2.4 ± 0.7^{b}	16.7 ± 1.8^{ab}
8% Tc	$3.9 \pm 0.^{a}$	2.4 ± 0.2^{a}	1.5 ± 0.4^{b}	19.0 ± 0.4^{ab}
8% TcEt	6.1 ± 1.2^{abc}	3.7 ± 0.6^{abc}	2.3 ± 0.6^{b}	17.3 ± 2.0^{ab}
12% TcEt	4.7 ± 0.4^{ab}	3.0 ± 0.3^{abc}	1.7 ± 0.3^{b}	19.5 ± 0.8^{ab}
16% TcEt	4.6 ± 1.0^{ab}	3.0 ± 0.7^{ab}	1.6 ± 0.4^{b}	20.0 ± 2.4^{a}

The results are mean ± standard deviation (n = 3). Values sharing a letter in the same column are not significantly different ($p > 0.05$).

wheat flour substitution dose-dependently increased crumb firmness. Already at 4% substitution with Tc, breads had a significantly firmer crumb compared to the control. With higher substitution levels the difference between Tc and TcEt increased, i.e. Tc giving significantly higher crumb firmness from 8% substitution and higher. At 12 and 16%, breads with Tc had very high crumb firmness, reflecting a very dense crumb, which showed a high number of very small gas-cells (Fig. 5).

The colour of bread baked with different levels of Tc and TcEt was visibly different (Fig. 5) and a similar difference in crumb colour was observed. Crumb colour darkened (L*values decreased) with increasing wheat flour substitution levels (Supplementary Table 1). However, this was much more pronounced for Tc than TcEt and breads prepared with 16% TcEt had a brighter crumb than breads prepared with only 4% Tc. The crumb colour of breads containing TcEt and the control breads was yellow (positive b* value) and slightly green (negative a* value). Breads containing 4% Tc had the greenest crumb. At higher concentrations of Tc, the crumb colour changed to dark blue (negative b* value).

4. Discussion

The nutrient content of cell-wall disrupted Tc was similar to that found in earlier studies of non-processed *T.chuii* (Tibbetts et al., 2015). Ethanol treatment enriched protein in the algae by extracting fat as expected. The favourable AA composition of Tc was not affected by ethanol treatment and Tc and TcEt contained all essential AA above the recommended reference values (FAO, 2007). The removal of green chlorophyl-type pigments by ethanol treatment gives a product with higher application potential as the dark brown-green breads resulting from use of unprocessed microalgae are not favoured by all consumers (Finney et al., 1984).

Many of the volatile compounds identified in Tc are typical for algae (seaweeds and microalgae) and have previously been identified in *T. chuii* (Lafarga, 2019; Van Durme, Goiris, De Winne, De Cooman, & Muylaert, 2013). One of the major compounds found in the cell-wall disrupted Tc, dimethyl sulfoxide, has no odour. Another major compound is 2,6-dimethyl-cyclohexanol, which has an earthy odour (Polak, Trotier, & Baliguet, 1978), but no odour threshold has been reported for this compound. Other major volatile compounds found in the cell-wall disrupted Tc were secondary lipid oxidation products (1-penten-3-ol, 6-methyl-5-hepten-2-one, 1-octen-3-ol), non-enzymatic browning products (pyrazines and pyridines) and protein Strecker degradation products (2- and 3- methyl-butanal, dimethyl disulphide). Some of these compounds such as 1-penten-3-ol, which has a green flavour, 6-methyl-5-hepten-2-one, which gives a typical seafood aroma and 1-octen-3-ol imparting an earthy mushroom odour have previously been shown to contribute to algae flavour (Garicano Vilar, O'Sullivan, Kerry, & Kilcawley, 2020; Lafarga, 2019; Van Durme et al., 2013). A high number of pyridines (6 different) and pyrazines (8 different) were present in Tc. Even though 2-ethyl-pyridine, and tetramethyl-pyrazine, have previously been reported in dried seaweeds (López-Pérez, Picon, & Nuñez, 2017), the high content of pyridines and pyrazines in this study is associated with processing (e.g. cell wall disruption followed by freeze-drying) as these compounds were absent in the unprocessed *T. chuii* (results not shown). Lipid oxidation products, pyridines and pyrazines were largely removed by ethanol treatment. Instead, TcEt volatile compounds were dominated by hydrocarbons, which have a high odour threshold and are therefore unlikely to contribute to the perceived flavour of TcEt and TcEt containing breads. However, low levels of some of the sulphur compounds, aldehydes and aromatics were retained in TcEt albeit at lower concentrations than in Tc. In total this indicates a significant reduction of odour contributing volatile compounds by ethanol treatment.

Incorporation of Tc and TcEt into wheat flour dough influenced mixing properties, dough rheology and bread quality. Wheat flour substitution with Tc or TcEt increased WA in a dose dependent manner. Similar results have been obtained in previous studies when incorporating *Chlorella vulgaris* (Graça et al., 2018) or a protein concentrate from blue green algae *Spirulina platensis* and *Oscillatorio amphibia* (Saleh, 1987) into wheat flour dough. Other forms of protein enrichment of wheat flour with chickpea (Mohammed, Ahmed, & Senge, 2012) or soybean flour (Lazo-Vélez, Chuck-Hernandez, & Serna-Saldívar, 2015) also led to an increased Farinograph WA.

Besides WA, substitution of wheat flour with Tc and TcEt profoundly

Fig. 5. Overall bread appearance (upper) and gas cell distribution in the crumb (lower) of breads prepared with Tc or TcEt at different levels of wheat flour substitution.

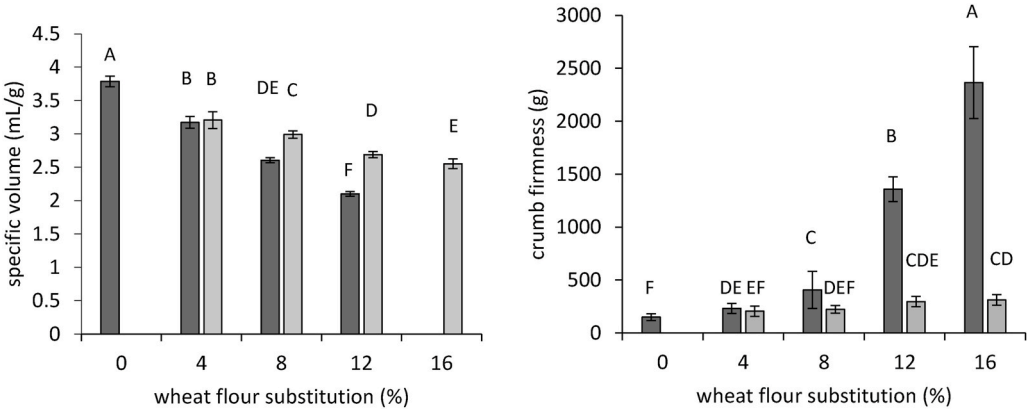

Fig. 6. Specific volume (left) and crumb firmness (right) of breads prepared with Tc (dark grey) or TcEt (light grey) at different wheat flour substitution levels. The volume of breads prepared with 16% Tc could not be measured due to reflection of the laser beam by the shiny and dark surface.

affected dough rheology. The different methods applied to study different aspects of dough rheology showed the same tendencies of a weakening effect with increasing substitution levels. Even the lowest substitution level (4%) significantly reduced DST during mixing, while the observed reduction of Rmax and extensibility was only significant for substitution levels of 12% or higher. Similarly, significant reductions in J_{max} and J_e were observed for 8% Tc and for TcEt from 12 to 16% substitution. A substitution of wheat flour with other ingredients inevitably leads to gluten dilution and therefore a weakening of the dough structure (Rieder, Holtekjølen, Sahlstrøm, & Moldestad, 2012). However, the observed dramatic effect of low substitution levels (4%), which is also in accordance with previous studies on *Chlorella vulgaris* (Graça et al., 2018), points towards an additional destabilizing effect of Tc apart from gluten dilution. Doughs prepared with Tc at higher substitution levels were too sticky to measure (>8% by extensograph, >12% by rheometer). Compared to TcEt, doughs prepared with Tc at the same substitution level had a tendency towards weaker dough structure (lower Rmax, lower extensibility, lower J_{max} and J_e) even at the low substitution levels of 4 and 8% where a direct comparison was possible. It is likely that different factors contributed to the observed difference between Tc and TcEt substituted wheat flour doughs and the stickiness of Tc at higher substitution levels. The FAA's were nearly halved by the ethanol treatment. FAA's and small peptides have been shown to act as plasticizers during extrusion by screening of intermolecular attractive forces (Oterhals & Samuelsen, 2015). In a dough system this could possibly contribute to an interference with gluten network development resulting in a weaker dough structure and stickier dough. Lipids in dough may also act as plasticizers and liquid oil has been shown to have a negative effect on gas cell stabilization compared to solid fat (Pareyt, Finnie, Putseys, & Delcour, 2011). The high content of unsaturated fatty

acids in Tc compared to TcEt ~~(fat removed)~~ may therefore have negatively affected dough structure. Since wheat flour dough is a complex system were the balance of reducing and oxidizing actions of dough improvers such as ascorbic acid (present in the commercial wheat flour applied here) is important for the correct development of disulphide bonds from SH groups in glutenin subunits (Bushuk, 1985), the presence of high amounts of antioxidants from Tc (but not TcEt) may also potentially influence gluten network development and dough rheology. Removal of salt from the microalgae *Dunaliella* has been shown to improve baking performance and increase loaf volume of composite wheat breads (10% wheat flour replacement) (Finney et al., 1984). Ethanol treatment of Tc biomass did not influence the ash content but might have changed the mineral composition (not analysed). In a previous study with protein concentrate from *Spirulina platensis* and *Oscillatoria amphibia* a positive effect of wheat flour substitution with algae (6–7%) on dough rheology (DST, Rmax) and bread quality (volume) was reported (Saleh, 1987). The employed algae protein concentrates were produced by ethanol extraction. Together, these results indicate that purification of protein from algae biomass may improve the technological performance in bread and enable higher incorporation levels.

There was a tendency of increased $G'_{t=0}$ (significant from 16% substitution) with higher substitution levels of wheat flour even though these doughs were prepared with higher amounts of water (according to Farinograph WA). Higher water addition in wheat flour doughs (without microalgae) results in lower G' and G'' values (Faubion, 1985). It is therefore possible that the increased water absorption capacity of the composite doughs due to the microalgae was not sufficiently compensated for by using Farinograph WA as optimal water addition as this resulted in stiffer doughs for high addition levels of Tc and TcEt. The 12 and 16% Tc doughs did not expand much during baking and had a very compact crumb with very small gas cells. Higher water addition levels might therefore improve baking performance of Tc and TcEt substituted doughs, however this will further increase stickiness and needs to be investigated.

The different rheological measurements of dough correlated well with baking performance as there was a clear effect of wheat flour substitution level and ethanol treatment of Tc on bread quality. Wheat flour substitution dose dependently decreased specific volume and increased crumb firmness of breads, which is in accordance with previous studies on *Chlorella vulgaris* (Graça et al., 2018), *Nannocloropsis sp.* and *Tetraselmis sp.* (Lafarga, 2019) incorporation at low substitution levels (1–5%). The effect on loaf volume and crumb firmness in our study was more pronounced for Tc compared to TcEt (significant from 8% substitution). Although not verified in the breads, the reduction of volatile compounds by ethanol treatment is expected to give breads with much lower levels of compounds associated with typical microalgae smell (e.g. fishy, green, oily, earthy, mushroom), which is extremely important. In a sensory analysis of breads fortified with 1% *Nannochloropsis sp.* or 2% *Tetraselmis sp.* by a group of semi-trained panellists, who were willing to buy microalgae enriched products, the aroma of the fortified breads was scored low, while flavour, texture and visual appearance were much closer to the wheat control (Lafarga, 2019). It seems therefore, that the different aroma of microalgae containing breads is the biggest challenge in terms of consumer acceptance at least for breads with low addition of microalgae.

Breads enriched with 12% Tc qualify for the nutrition claim, "enriched in protein" and ethanol treatment of Tc seems to be a promising approach to achieve such high incorporation levels in bread. Not only did ethanol treatment of Tc improve dough structure and physical bread properties (volume & crumb firmness), it also had a positive impact on colour and aroma profile of microalgae fortified breads, two aspects which have previously been shown to hinder microalgae incorporation into bread at higher levels (Becker, 2007; Lafarga, 2019). Improvement of the ethanol extraction procedure and or a combination with further or alternative purification techniques of Tc protein (Pereira, Lisboa, & Costa, 2018; Schwenzfeier, Wierenga, & Gruppen, 2011) may help reduce colour, undesirable aroma compounds as well as negative effects on dough structure even further. Recipe (e.g. water addition, dough improvers) and baking processing optimization will be another important strategy to further improve bread containing Tc microalgae. A comprehensive safety assessment with following application to extend the novel food approval of Tc (currently 250 mg per d) to higher doses and to other food categories by EFSA is another hurdle before Tc fortified foods with high enough protein contents can be commercialized.

CRediT authorship contribution statement

Waqas Muhammad Qazi: Writing - original draft, Formal analysis, Investigation, Methodology. **Simon Ballance:** Writing - review & editing, Validation, Methodology, Funding acquisition. **Anne Kjersti Uhlen:** Project administration, Funding acquisition, Supervision. **Katerina Kousoulaki:** Project administration, Funding acquisition, Investigation, Resources, Writing - review & editing. **John-Erik Haugen:** Formal analysis, writing, Investigation. **Anne Rieder:** Supervision, Validation, Writing - review & editing, Visualization, Methodology, Conceptualization.

Declaration of competing interest

We certify that there is no conflict of interest by the authors.

Acknowledgements

The work was supported by the Norwegian Research Council project A2F (NFR 267872), FoodPilotPlant Norway (208674/F50) and FoodPilotPlant Norway phase II (296083/F50). It was also supported by the Fund for Research Fees for Agricultural Products (FFL) through the strategic research project SunnMat (NFR 262300). We acknowledge Hanne Zobel, Charlotte Nilsen, Ida Rud, Marialena Kokkali for their technical assistance. Dorinde Kleinegris is thanked for providing the algae biomass and production information.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2021.111115.

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