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Mechanical processing of *Phaeodactylum tricornutum* and *Tetraselmis chui* biomass affects phenolic and antioxidant compound availability, nutrient digestibility and deposition of carotenoids in Atlantic salmon

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ABSTRACT

Cell walls of microalgae differ both in terms of chemical composition and architecture. Certain characteristics of cell walls limit both the effectiveness of extraction of valuable constituents and nutrient bioavailability and bioaccessibility in fish. We first investigated the potential of bead milling to disrupt cell walls to improve extractability of bioactive compounds from *Phaeodactylum tricornutum* and *Tetraselmis chui*. We also evaluated the effect of the mechanical treatment on i) digestibility of nutrients in the two microalgae and ii) fatty acid and pigment content of fillet of Atlantic salmon fed the algae diets.

Cell wall disruption facilitated efficient release of total phenolic compounds (TPC), chlorophyll A, chlorophyll B and total carotenoids from both microalgae species, but increased only the total antioxidant capacity (TAC) of *T. chuii*. The polyphenol profile varied between the two species; we identified 2 and 6 polyphenols from *P. tricornutum* and *T. chui*, respectively.

For the feeding experiment, microalgae biomass, intact or cell wall disrupted (90.7 and 76.4% disruption degree for *T. chui* and *P. tricornutum*, respectively), were mixed with the other feed ingredients. Atlantic salmon (315 g start weight / 55 fish per tank) were fed one of the five extruded experimental diets (a fishmeal-based reference diet and four feeds with 30% of the whole or broken microalgae biomass added to the reference diet). The digestibility of protein in the *P. tricornutum* was significantly higher compared to that of *T. chui*. Breaking the cell walls by bead milling caused a significant improvement in digestibility of protein in *T. chui*. The apparent digestibility coefficient of lipid was higher in *P. tricornutum* and bead milling improved the digestibility of the two algae. However, bead milling did not favourably change the fatty acid profile of Atlantic salmon. At the end of the feeding trial, *P. tricornutum*-fed fish had significantly improved pigmentation, probably due to the higher content of fucoxanthin and fucoxanthinol compared to the fish fed the reference diet. The main conclusion from the mechanical processing of the microalgae is that bead milling can increase i) the extractability of polyphenols and pigments, ii) the digestibility of lipid in both algae while the effect on protein was improved only for *T. chui*. Furthermore, *P. tricornutum* can be considered a natural pigment source in the feeds of Atlantic salmon.

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1. Introduction

The growth of the global aquaculture industry will increase the demand for high-quality feed ingredients. This demand necessitates the identification of alternative protein and lipid sources for the farmed fish species. Microalgae are considered sustainable alternatives to certain feed components and these microorganisms have the potential to reduce the dependence on marine ingredients as well as the conventional plant ingredients currently used in aquafeeds (Beal et al., 2018; Shah et al., 2018). Although the chemical composition of different strains of microalgae varies considerably, their amino acid profile is similar to fishmeal (Tibbetts, 2018). Microalgae are the primary producers of long-chain polyunsaturated fatty acids in the marine environment and some strains may have the potential to supply fish diets with EPA and DHA (Barba et al., 2015), as well as bioactive components such as polyphenols, pigments and chlorophylls that are known to have antioxidant properties (Goiris et al., 2012; Kokkali et al., 2020; Safafar et al., 2015).

The utilization of microalgae by carnivorous fishes depends on the bioaccessibility and bioavailability of nutrients, mainly by breaking their rigid cell walls. Practical use of whole cells of microalgae such as *Phaeodactylum*, *Nannochloropsis, Scenedesmus* and *Spirulina* in diets for salmonids is limited to incorporation levels <10%, to avoid negative effects on growth (Gong et al., 2019; Gong et al., 2020; Sørensen et al., 2016; Sørensen et al., 2017). Other microalgae products such as lipid extracted *Nanofrustulum* and *Desmodesmus* can be incorporated at 17% and 20% without adversely affecting the growth of Atlantic salmon (Kiron et al., 2012; Kiron et al., 2016). The cell walls of the microalgae, in the latter experiment, were broken to improve lipid extraction, and hence, processed microalgae were incorporated into the salmon diets. Disruption of cell walls is proven efficient to improve the extraction and bioavailability of nutrients from microbial biomass (Kokkali et al., 2020; Storebakken et al., 2004b; Storebakken et al., 2004a; Teuling et al., 2019). Cell walls of microalgae belonging to different phyla and genera differ both in terms of chemical composition and architecture (Bernaerts et al., 2018), and therefore, nutrient bioavailability differ in response to pre-treatment methods used to break cell walls (Teuling et al., 2017).

An overview of different methods that can be used to break cell walls of microalgae is given in previous studies (Barba et al., 2015; Passos et al., 2014). Extrusion processing, a thermo-mechanical treatment, was chosen in our previous studies (Gong et al., 2018; Gong et al., 2019) because this method is scalable and is already in use in commercial production of fish feed. Gong et al. (2018) reported that extrusion processing can improve the digestibility of nutrients in microalgae diets compared to the cold-pelleting method. However, double extrusion did not further increase the digestibility of nutrients in microalgae (Gong et al., 2019). Bead milling is another scalable, mechanical process used for grinding and disrupting cells. To our knowledge, previous studies have not gathered information about the use of bead mills for microalgae cell wall disruption and then demonstrate the efficacy of the method by conducting biological studies in fish. The aim of the present study was thus to investigate i) the potential of bead milling as a means to disrupt cell walls and consequently release bioactive compounds including pigments, ii) the digestibility of nutrients in intact and broken *Phaeodactylum tricornutum* (*P. tricornutum*) and *Tetraselmis chui* (*T. chui*) fed to Atlantic salmon and iii) fatty acids and pigments in fillets of Atlantic salmon fed intact and broken microalgae.

2. Material and methods

We obtained an approval from the National Animal Research Authority in Norway to conduct the feeding trials. The reference number is FDU: Forsøksdyrutvalget ID-5887.

A feeding trial was designed to investigate the apparent digestibility coefficients (ADCs) of dry matter (DM), protein, ash, and energy of whole or broken microalgae-derived biomass in the feeds for Atlantic salmon. Skin and flesh color were also evaluated as the study groups could be differentiated based on their skin color (visual inspection).

Table 1
Ingredient composition (%) of the reference and test diets.

Diet	Reference diet	Test diet
Fishmeal	66.38	46.47
Microalgae*	–	30.00
Fishoil	15.00	10.50
Wheat	15.00	10.50
Vitamin premix	2.86	2.00
Mineral premix	0.74	0.52
Yttrium oxide	0.020	0.014

* *P. tricornutum* or *T. chui;* whole or broken cells.

2.1. Microalgae biomass

The microalgae biomass *P. tricornutum* B58 and *T. chui* UTEX LB232 were produced at the National Algae Pilot Mongstad (NAM; Mongstad, Norway) employing a fed-batch process in four 800 L photobioreactor systems (GemTube MK2–750, LGem, Rotterdam, The Netherlands). The microalgae were harvested twice a week, concentrated to a paste by centrifugation (Evodos 50, Evodos, Raamsdonksveer, The Netherlands), vacuum packed, and directly frozen at −20 °C before further downstream processing. Production and harvesting of the microalgae biomass were described by Kokkali et al. (2020). The microalgae paste, of both *P. tricornutum* and *T. chui*, was diluted to graded dry matter (DM) levels (13.65–24.12% for *P. tricornutum* and 10.0–22.5% for *T. chui*) and processed by single passes through a Dyno-Mill Multi Lab bead mill (WAB, Muttenz, Switzerland). The bead mill had a 0.6 l grinding chamber and small glass beads (0.25–0.4 mm) filled at 85% of the chamber volume. The processing conditions employed were agitator tip speed of 8–12 m/s (2391–3586 rpm for *P. tricornutum* and 1970–2852 rpm for *T. chui*) and biomass flow rate of 4–18 kg/h. Samples of both the processed microalgae species having graded cell wall disruption degrees as evaluated by cell cytometry (48%, 55%, 61%, 65%, 72%, 73% for *P. tricornutum* and 61%, 67%, 77%, 83%, 90%, 96%, 99% for *T. chui*) were freeze-dried and kept at 5 °C until further analysis. For the fish trial, larger batches of *P. tricornutum* and *T. chui* paste (biomass with 76.4 and 90.7 disrupted cell walls, respectively), were processed under conditions found optimal through initial trials of cell wall disruption (biomass was diluted with tap water until DM was 22.9 and 18.7% respectively, agitator tip speed was set at 12 m/s and biomass flow rate at 6 kg/h). The cell wall was disrupted and whole microalgae biomasses were spray dried to fine powders and used for feed production.

2.2. Amino acid and peptide profiles of the algal biomass

To profile the total amino acids, samples were hydrolysed in 6 M HCl for 22 h at 110 °C and analyzed by HPLC, using a fluorescence technique for detection (Cohen and Michaud, 1993).

Peptide size distribution was analyzed by combining results from HPLC size exclusion chromatography using a Superdex™ Increase 10/300 GL (I.D. 10 mm × 300 mm) (GE Healthcare, Upsala, Sweden) column (measuring range 200–15,000 Da); detection was based on UV adsorption at 214 nm. The water soluble SWC extract was diluted to approximately 150 mg/ml with the eluent (30% acetonitrile, 0.1% trifluoroacetic acid in water) and filtered through a 0.2 μm syringe filter. The following peptides were used to define the standard curve: Carbonic anhydrase (MW 29000), lysozyme (MW 14300), cytochrome C (MW 12400), aprotinin (MW 6500), insulin A (MW 2532), gastrin I (MW 2126), polymyxin (MW 1470), [Val 4]-Ang III (MW 917), leucine*3* (MW 357), glycine (MW 75) and Alberta standards (MW 4057, 3249, 2442, 1634 and 826).

Table 2
Chemical composition (%) of the experimental diets.

	PI	PB	TI	TB	RD
Moisture	7.1	7.8	7.9	8.0	7.2
Dry basis					
Crude protein	48.4	48.4	50.1	49.3	50.1
Crude lipid	18.4	19.0	19.0	18.5	23.2
Ash	14.6	14.1	14.3	14.4	13.1

PI – *P. tricornutum* whole; PB – *P. tricornutum* broken; TI – *T. chui* whole; TB - *T. chui* broken; RD – Reference diet.

Table 3a
Chemical composition of feed ingredients (%) employed in the study.

	PI	PB	TI	TB	FM
Moisture	2.8	4.6	3.8	5.6	8.8
Crude protein	41.8	44.6	46.4	45.2	70.2
Crude lipid	11.8	16.7	12.8	11.8	7
Ash	17.9	14.1	16.1	16	16.7
Salt	5.4	5	3.3	3.3	

PI – *P. tricornutum* whole; PB – *P. tricornutum* broken; TI – *T. chui* whole; TB - *T. chui* broken; FM – Fishmeal.

2.3. Diet formulation and preparation

The reference diet was formulated based on marine ingredients and the test diets were formulated by incorporating 30% of the processed or unprocessed microalgae into the reference diet, diluting all other ingredients (Table 1). Chemical composition of the experimental diets is given in Table 2. Proximate composition of the feed ingredients is presented in Table 3a. Fishmeal and fish oil were used as the main protein and lipid sources in the reference diet. Four test diets ("Tetra whole", "Tetra broken", "Phaeo whole" and "Phaeo broken") were formulated by mixing 70% of the reference diet and 30% of microalgae. Apparent digestibility coefficients of DM, protein, lipid, and ash in the two microalgae species *P. tricornutum* and *T. chui*, for both whole and broken cells) were determined by performing a digestibility study.

The diets for this study were produced at the Feed Technology Center of Nofima in Bergen, Norway. The feed mixes were conditioned with steam and water in an atmospheric double differential preconditioner before sending through a TX-52 co-rotating, fully intermeshing twin-screw extruder (Wenger Manufacturing Inc., Sabetha, KS, USA). The temperature of the feed mash entering the extruder was 84–87 °C. The temperature in the extruder outlet ranged from 109 °C for "Tetra whole", "Tetra broken" and "Phaeo whole" to 113 and 117 °C for the "Reference diet" and "Phaeo broken", respectively. The wet extrudates, passing through the 24 circular 2.5 mm die holes at the extruder outlet, were cut at the die surface with a rotating knife. After achieving the steady-state conditions in the preconditioner and extruder, pellet samples that came out of the die were collected. These extrudates were dried in a hot air dual-layer carousel dryer (Paul Klockner, Nistertal, Germany) at a constant air temperature (80 °C) to obtain approximately 7–8% moist pellets. Then each of the diets was coated with fish oil in a vacuum coater (Pegasus PG-10VC LAB, Dinnissen BV, Sevenum, The Netherlands).

2.4. Digestibility trial

The digestibility study was conducted at the Research Station, Nord University, Bodø, Norway. Atlantic salmon post-smolts (Aquagen strain, Aquagen AS, Trondheim, Norway) were purchased from a commercial producer (Cermaq Norway AS, Hopen, Norway) and maintained at the Research Station until they were used for the feeding trials. The fish were fed Spirit Supreme 150 and Spirit Supreme 300 (Skretting, Stavanger, Norway) during the holding period. The trials were conducted according to the procedures approved by the National Animal Research Authority (Forsøksdyrutvalget, Norway).

Atlantic salmon with an average body weight of approximately 315 g were stocked into 800 L tanks (55 fish per tank). The fish were randomly assigned to 3 replicate tanks of the different dietary treatments. These tanks were part of a flow-through system with seawater drawn from a depth of 250 m from Saltenfjorden. The water was filtered and aerated before use. Throughout the experimental period, the average water temperature in the tanks was 7.7 °C and oxygen saturation was above 85%. The fish were reared under 24 h of continuous light conditions and were fed by automatic feeders - approximately 1.4% of the fish biomass daily - for 35 days i.e., until the first feces collection by stripping. Fish were starved for two days, after the feces collection. Thereafter, feeding continued for another 9 days and a second stripping was performed to collect samples for analysis.

2.5. Sampling

Before handling, the fish were anesthetized with 140 mg l^{-1} of tricaine methanesulfonate (MS-222, Argent Chemical Laboratories, Redmond, USA). The ventral caudal area of the anesthetized fish was dried before feces collection (Austreng, 1978). The fecal samples from individual fish from a tank were pooled and freeze-dried for 72 h. Samples for analysis of carotenoids and fatty acids were taken from 18 fish per diet group. Small fillet samples were collected from the posterior part of the fish (Fig. 1). Skin and bones from these samples were removed, vacuum packed, and immediately frozen. All the samples (feces and fillets) were kept at −40 °C, prior to chemical analyses.

The skin color of samples collected from both dorsal and ventral regions of the left side of the fish was measured in triplicate using a portable spectrophotometer (CM-700d, Konica Minolta Sensing Singapore Pte Ltd., Singapore). The spectrophotometer was set to both SCI (specular component included) and SCE (specular component excluded) modes, and the average of these values was used in the downstream analyses. The flesh color of the left side samples from the dorsal part of the fish was measured in triplicate using both the spectrophotometer and the SalmonFan™ lineal (DSM, Basel, Switzerland). The SalmoFan evaluation was carried out under standardized conditions in a light cabinet (Ra >90, color temperature > 5000 K) according to a

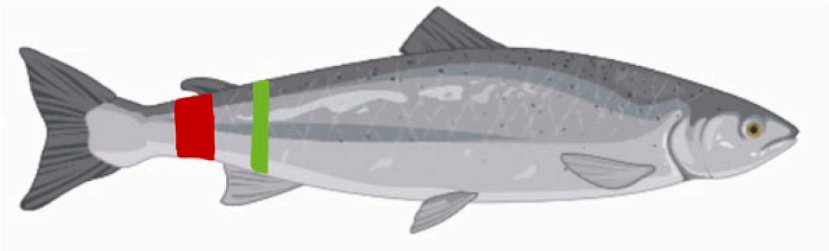

Fig. 1. Illustration indicating the locations from where the tissues were collected for the fatty acid (red area) and carotenoid (green area) assessment. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Norwegian standard (NS-9402E 1994).

2.6. Chemical analyses

Microalgae-incorporated experimental diets and freeze-dried feces were finely ground and homogenized prior to analyzing the dry matter (105 °C for 20 h), crude protein (N × 6.25; Kjeldahl Auto System, Tecator Systems, Höganäs, Sweden), crude lipid (Soxtec HT6, Tecator, Höganäs, Sweden), ash (incineration in a muffle furnace at 540 °C for 16 h), and energy (IKA C200 bomb calorimeter, Staufen, Germany). Yttrium oxide was analyzed by inductive coupled plasma mass spectroscopy (ICP-MS) at Eurofins (Moss, Norway). All the samples were analyzed in duplicate.

2.7. Carotenoids in flesh

Carotenoid analysis was performed on 18 individual fish samples. Approximately 10–15 g of fish fillets without the skin and bone, were cut into 8–10 mm square cubes. Carotenoids were extracted in acetone, flushed with nitrogen gas, and kept at −30 °C for at least 22 h. Fifty ml benzene and 25 ml absolute ethanol were added to the extract and evaporated under reduced pressure at max 30 °C until the extract was dried. The procedure was performed four times until all the pigments were extracted. The combined extracts were evaporated under reduced pressure, diluted with acetone up to 5 ml in a volumetric flask, and then filtered through a syringe filter (GHP membrane, 13 mm diameter, 0.2 μm pore size, Acrodisc®). In the next step, the extracts were flushed with a slow rate of nitrogen, capped and placed in the HPLC (1100 series, Agilent, Santa Clara, California, USA). The samples were analyzed in HPLC with continuous addition of 0.1% hexane, following the procedures described by Egeland (2012). Two identical C18 columns were used (ACE 5 C18 part no. ACE-121-2546, 4.6 × 350 mm each, with a separate 5 μm packing) with separate guard column (ACE).

2.8. Fatty acids in microalgae, feeds and flesh

Analysis of fatty acids was performed on lipids in Bligh and Dyer extracts (Bligh and Dyer, 1959). Preparation of fatty acid methyl esters (FAME) was done according to AOCS Official Method Ce 1b-89. The fatty acid composition in microalgae was analyzed at BioLab (Nofima, Bergen, Norway), while fatty acids in feed and flesh was analyzed at Nord University (Bodø, Norway), with a slightly different gas chromatograph (GC). The GC analyses of the microalgae lipid was conducted on a GC (TRACE GC, Thermo Fisher Scientific, Waltham, MA USA) with a flame ionization detector (GC–FID), equipped with a 60 m × 0.25 mm BPX-70 cyanopropyl column with 0.25 μm film thickness (SGE Analytical Science, Ringwood, VIC, Australia). He 4.6 was used as the mobile phase under the pressure of 2.60 bar. The injector temperature was 250 °C and the detector temperature was 260 °C. The oven was programmed as follows: 60 °C for 4 min, 30 °C/min to 164 °C, and then 1 °C/min to 213 °C, and 100 °C/min to 250 °C where the temperature was held for 10 min. The sample solution (3.0 μl) was injected splitless and the split was opened after 2 min. The FAME were identified by comparing the elution pattern and relative retention time with the reference FAME mixture (GLC-793; Nu-Chek Prep Inc., Elysian, MN, United States). Chromatographic peak areas were corrected by empirical response factors calculated from the areas of the GLC-793 mixture. Fatty acid composition was calculated by using 23:0 FAME as the internal standard and reported on a sample basis as g/100 g FAME. All analyses were performed in duplicate. If differences between parallels exceeded standardized values, new duplicate analyses were carried out according to accredited procedures.

The FAME analyses for the feed and freeze-dried fillet (100 mg/sample) were performed in duplicate in a GC (SCION 436-GC, Scion Instruments, Goes, Netherlands) fitted with a flame ionization detector at 250 °C. The separation was achieved using a wax embedded column of 25 m length, 0.25 mm internal diameter and 0.2 μm film thickness (Agilent Technologies, Middelburg, Netherland), with H_2 as carrier gas. Individual FAME was identified and quantified by comparing with known standard mixtures of common fatty acids (FAME MIX 2/GLC-473, Nu-Chek Prep) and the results are expressed as relative area percentage of the total fatty acid using a software Compass CDS, Bruker Cooperation.

2.9. Bioactive compounds-release kinetics

Extracts from *P. tricornutum* and *T. chui* samples with different cell wall disruption degrees were prepared at a concentration of 0.1 g/ml, by mixing in a vortex for 5 min. The extracts used for the determination of total phenolic content (TPC) and total antioxidant capacity (TAC) were prepared with absolute ethanol (99.9%; Baker, Deventer, The Netherlands), while the extracts destined for pigment analysis were prepared with methanol (95%; Baker, Deventer, The Netherlands). After mixing with a vortex, the samples were filtered (paper filter, pore size 10–20 μm), and the extracts were stored at −20 °C for further analyses.

2.10. Polyphenols

Folin-Ciocalteu method was used to determine TPC, as previously described by Kokkali et al. (2020). Briefly, 100 μl of the sample extract was mixed with 3 ml of Na_2CO_3 2% (p/v) and 100 μl of Folin-Ciocalteu reagent (previously diluted at 50% (*v*/v)). The samples were then incubated for 1 h at room temperature and in darkness. Finally, the absorbance was measured at 750 nm (Perkin Elmer Lambda2*s* spectrophotometer, Markham, Ontario, Canada). A TripleTOF™ 5600 (AB SCIEX) LC-MS/MS system equipped with Agilent 1260 Infinity (Agilent, Waldbronn, Germany) was used to determine the phenolic profile of microalgae biomasses. MS acquisition was carried out using the negative mode in the range 80 to 1200 *m/z*, following the experimental procedure and methodology previously described by Alcántara et al. (2020).

2.11. Total carotenoids (Total car), chlorophyll A (Chl A) and chlorophyll B (Chl B)

Pigment concentration was estimated spectrophotometrically according to the method previously described by Lichtenthaler and Buschmann (2001). The extracts were measured at 470 (A_{470}), 652.4 ($A_{652.4}$), and 665.2 ($A_{665.2}$) nm wavelength. The concentration of each pigment was calculated using Lichtenthaler equations and the results were expressed as "mg of pigment/g DW":

$$C_{Chl\ A} = 16.82A_{665} - 9.28A_{653} \quad (1)$$

$$C_{Chl\ B} = 36.92A_{653} - 16.54A_{665} \quad (2)$$

$$C_{Total\ car} = (1000A_{470} - 1.91C_{Chl\ A} - 95.15C_{Chl\ B})/225 \quad (3)$$

2.12. Total antioxidant capacity

Total antioxidant capacity was measured by the ABTS/TEAC method, following the procedure reported by Kokkali et al. (2020). ABTS radical cation ($ABTS^{\cdot+}$) was produced by reacting ABTS 7 mM stock solution with 140 mM potassium persulfate ($K_2S_2O_8$) at room temperature for 12–16 h. The solution was then diluted 1/100 with ethanol (Baker, Deventer, The Netherlands) to obtain an absorbance of 0.700 ± 0.020 at 734 nm. Then, 2 ml of ABTS+ was mixed with 100 μl of the extract, and the sample was incubated for 20 min at 20 °C. Finally, the absorbance was measured again at 734 nm (Perkin Elmer Lambda2 spectrophotometer). All analyses were performed in triplicate. If differences between parallels exceeded 5%, new duplicate analyses were carried out. A standard curve was established using Trolox (Sigma-Aldrich, Steinheim, Germany), and the percentage of inhibition of the

samples was interpolated. The results are expressed as micromolar Trolox equivalent (μM TE).

2.13. Calculations and statistical analysis

The apparent digestibility coefficients (ADCs) of nutrients or dry matter of the reference diet and test diets were determined using the following equation (Cho & Slinger 1979):

$$ADC_{nutrient} = \left[1 - \frac{(Marker_{diet} \times Nutrient_{faeces})}{(Nutrient_{diet} \times Marker_{faeces})}\right] \times 100$$

$$ADC_{drymatter} = \left[1 - \frac{(Marker_{diet})}{(Marker_{faeces})}\right] \times 100$$

where $Marker_{diet}$ and $Marker_{faeces}$ represent the marker content (% dry matter) in the diet and feces, respectively, and $Nutrient_{diet}$ and $Nutrient_{faeces}$ represent the nutrient contents (% dry matter) in the diet and feces, respectively.

The apparent digestibility coefficients of the test ingredients were calculated according to the equation of Bureau and Hua (2006) as follows:

$$ADC_{ingredient} = ADC_{testdiet} + \left[\left(ADC_{testdiet} - ADC_{ref.diet}\right) \times \left(\frac{0.7 \times Nutrient_{ref.}}{0.3 \times Nutrient_{ingredient}}\right)\right]$$

Where $Nutrient_{ref.}$ represents the nutrient content (% dry matter) in the reference diet and $Nutrient_{ingredient}$ is the nutrient content (% dry matter) in the test ingredient.

Statistical analyses were carried out using RStudio 1.3.959. Normality and equal variance of the data were tested before performing one-way ANOVA (weight gain, growth data as well as whole diet digestibility). Tukey's multiple comparisons test was employed to detect the significant differences between the means of interest. Kruskal-Wallis test followed by Dunn's multiple comparisons test was employed in the case of non-parametric data, to understand the differences between the study groups. The digestibility data were analyzed using a two-way ANOVA employing a function from ARTool package; here aligned rank transformation was performed before ANOVA. The differences between groups were considered significant at $P < 0.05$, and differences at $0.10 > P > 0.05$ suggest a trend.

3. Results & discussion

3.1. Chemical composition of P. tricornutum and T. chui

The proximate composition of the whole and broken biomass, as well as the fishmeal, is provided in Table 3a. Protein content was higher in *T. chui* than *P. tricornutum*. Breaking the cell walls facilitated the release of more protein (to a level similar to that in *T. chui*) from *P. tricornutum*. We did not notice any major differences in protein content in broken cells of the *T. chui* compared to the whole alga. The lipid content was in the same range for the two whole microalgae but breaking the cell walls helped in increasing the availability of crude lipid from *P. tricornutum*. Such a lipid-releasing effect was not noted for *T. chui*. Review articles have discussed the effectiveness of different pretreatment techniques in facilitating the increased extractability of intracellular products (Alhattab et al., 2019). Microalgae from different taxonomic classes differ in size, cell shape, cell wall structure, and chemical constituents. Different disruption methods based on mechanical or non-mechanical techniques may therefore have different effects (Lee et al., 2017). Increased release of protein and lipid from bead-milled *P. tricornutum* suggests that the extraction method is efficient in successfully obtaining lipid and nitrogen from *P. tricornutum*. Bead-milling-caused improvement was noted for *T. chui*. Results from one of our previous studies indicated the efficacy of double extrusion to release more lipids from *T. chui* (Sørensen et al., 2021). These results could be indicating that the efficacy of the pretreatment is dependent on the microalga type and the target compound.

Table 3b
Amino acid composition (g/100 g) of the whole biomass from *Phaeodactylum tricornutum* and *Tetraselmis chui*.

	P. tricornutum	*T. chui*
Essential amino acids		
Arginine	2.1	2.5
Histidine	0.6	0.8
Isoleucine	1.8	1.7
Leucine	3.0	3.5
Lysine	2.3	2.6
Methionine	0.9	1.0
Phenylalanine	2.0	2.2
Threonine	1.8	2.1
Valine	2.2	2.5
Non-Essential amino acids		
Alanine	2.7	3.5
Aspartic acid	3.6	4.6
Glutamic acid	5.6	4.9
Glycine	2.0	2.6
Hydroxyproline	0.1	<0.1
Proline	2.4	2.3
Serine	1.6	1.8
Tyrosine	1.2	1.3

Table 3c
Water soluble peptide size (Da) distribution in whole and cell wall disrupted microalgae.

	PI	PB	TI	TB
Molecular weight				
> 20,000	4.8	0.2	0.3	0.5
20,000–15,000	0.2	0.3	<0.1	0.1
15,000–10,000	0.3	0.5	0.1	<0.1
10,000–8000	0.4	0.2	0.2	0.1
8000–6000	1.5	0.4	0.8	0.6
6000–4000	3.1	0.9	1.6	1.3
4000–2000	4.6	3.0	1.9	2.5
2000–1000	3.6	5.5	1.9	3.1
1000–500	4.8	7.3	2.5	3.2
500–200	8.4	8.7	4.0	4.6
200-	68.2	72.9	86.7	84.1

PI – *P. tricornutum* whole; PB – *P. tricornutum* broken; TI – *T. chui* whole; TB - *T. chui* broken; RD – Reference diet.

Amino acid composition of the intact microalgae was analyzed, and the information is given in Table 3b. Both microalgae contained all the essential amino acids, but there were some small variations in the content. In both the microalgae, small molecular weight peptides <200 MW dominated the proteins in them (Table 3c). Some larger peptides had a higher abundance in the intact *P. tricornutum* biomass compared to the broken type. The high content of low molecular weight peptides suggests more antioxidant activity because a previous study has reported the strong antioxidant properties of such small peptides (Sheih et al., 2009).

The fatty acid composition of the whole and broken microalgae biomass is presented in Table 3d. The fatty acid composition in the two strains of microalgae varied widely. We are unable to point out any effect of the bead milling process. The microalga, *P. tricornutum* had more Σ MUFA and EPA + DHA while *T. chui* had a higher content of ΣPUFA n-3. The lipid class information in Table 3e indicates that triacylglycerol content was higher in *P. tricornutum* than in *T. chui*. Free fatty acids were also higher in *P. tricornutum* than *T. chui*, but bead milling increased the free fatty acids in *T. chui*. The differences in free fatty acids were not reflected in the changes in monoacylglycerol or diacylglycerol which were at the same level regardless of microalgae or processing. The

Table 3d
Fatty acid composition (% of Bligh & Dyer extract) in whole and broken *P. tricornutum* and *T. chui*.

	PI	PB	TI	TB
C14:0	2.8	2.9	0.2	0.3
C16:0	6.4	8.2	9	9.7
C18:0	0.2	0.2	0.1	0.1
C16:1 n-7	16.7	19.5	0.3	0.6
C18:1 (n-9) + (n-7) + (n-5)	1.6	1.7	4.7	5.3
C20:1 (n-9) + (n-7)	<0.1	<0.1	0.5	0.5
C16:2 n-4	3.4	4.4	0.4	0.5
C16:3 n-4	3.8	3.1	<0.1	0.1
C18:2 n-6	1.6	1.6	1.7	1.9
C18:3 n-6	0.3	0.3	1.4	1.4
C20:4 n-6	2.4	3.1	0.2	0.3
C16:4 n-3	0.1	0.1	8.5	8.5
C18:3 n-3	0.4	0.4	7.4	7.7
C18:4 n-3	0.3	0.3	5.1	5.1
C20:4 n-3	0.1	0.1	0.2	0.2
C20:5 n-3 (EPA)	14.3	14.1	3.3	3.6
C22:6 n-3 (DHA)	0.5	0.4	<0.1	<0.1
SUM SFA	9.4	11.3	9.3	10.1
SUM MUFA	18.4	21.2	5.5	6.4
Sum PUFA n-6	4.3	5.1	3.3	3.6
Sum PUFA n-3	15.7	15.4	24.6	25.2
Sum PUFA total	27.2	28	28.3	29.6
n-6/n-3	0.3	0.3	0.1	0.1
Sum EPA + DHA	14.8	14.5	3.3	3.6
Sum identified	55	60.5	43.1	45.9
Sum unidentified	10.2	9.6	14.4	14.6

The following fatty acids were detected at levels lower than <0.1: C20:0, C22:0, C22:1 (n−11) + (n-9) + (n-7), C24:1 n-9, C20:2 n-6. C20:3 n-6, C22:4 n-6, C20:3 n-3, C21:5 n-3, C22:5 n-3. PI – *P. tricornutum* whole; PB – *P. tricornutum* broken; TI – *T. chui* whole; TB – *T. chui* broken; RD – Reference diet.

Table 3e
Lipid classes (% of Bligh & Dyer extract) in whole and broken *P. tricornutum* and T. *chui*.

	PI	PB	TI	TB
Triacylglycerol	5.5	6.5	3	2.7
Diacylglycerol	<0.5	<0.5	<0.5	<0.5
Monoacylglycerol	<1	<1	<1	<1
Free fatty acids	22	23	5.4	10
Cholesterol	<0.5	<0.5	1.4	1.9
Cholesterol ester	<0.5	1.5	0.9	1.1
Phosphatidylethanolamine	8.9	1.9	6.4	14
Phosphatidylinositol	<1	<1	<1	<1
Phosphatidylserine	<1	<1	<1	<1
Phosphatidylcholine	<1	<1	<1	<1
Lyso-Phosphatidylcholine	1.2	0.5	1	2.7
Total polar lipids	10.1	2.3	7.4	16.9
Total neutral lipids	28.4	31.1	10.9	16.1

PI – *P. tricornutum* whole; PB – *P. tricornutum* broken; TI – *T. chui* whole; TB - *T. chui* broken; RD – Reference diet.

Table 4
Phenolic compounds* (mg/g DW) in the differentially disrupted *P. tricornutum* extracts.

Disruption (%)	Cinnamic acid	Benzoic acid
0	0.6319 ± 0.0246	1.1799 ± 0.0758
48	1.8948 ± 0.1325	–
55	1.8862 ± 0.0245	–
61	2.3021 ± 0.2519	–
65	2.9371 ± 0.0484	1.1010 ± 0.0741
73	2.7847 ± 0.0983	1.0214 ± 0.1002

* Values (three replicates per differentially disrupted biomass analyzed on three parallels each) are means ± sd.

dominant lipid class in *P. tricornutum* was neutral lipids. Total neutral lipids were lower in *T. chui* compared to *P. tricornutum*. However, both total polar lipids and total neutral lipids increased in *T. chui* after bead milling.

3.2. Kinetics of bioactive compounds-release from P. tricornutum and T. chui

The effect of cell wall disruption on bioactive compound extraction was studied by analyzing the TPC, total carotenoids, TAC, chlorophyll A and B; extracts from *P. tricornutum* was obtained after 0–73% cell wall disruption and the corresponding range for *T. chui* was 0–99% (Tables 4 and 5). The degree of bead milling affected the release of TPC from *P. tricornutum* ($p < 0.05$, Fig. 2a); TPC increased from 6.97 in control (0% disruption) and peaked to 17.19 mg GAE/g DW when the disruption degree was 48%. Note that after 48% disruption, TPC content started to plateau. Chlorophyll A and total carotenoids also reached their maximum values at 48% disruption degree, while the highest release of chlorophyll B was observed in samples that were subjected to 60% or higher disruption degree. A previous study has reported TPC values ranging from 9.98 to 42.16 mg GAE/g extract of *P. tricornutum* by extraction with water and ethanol at different temperatures (Gilbert-López et al., 2017). In the same study, the authors reported higher levels of total carotenoids (0.71–36.82 mg/g extract). In contrast, Safafar et al. (2015) obtained a lower concentration of TPC in *P. tricornutum* (3.16 mg GAE/g), although the total carotenoids extracted were similar (4.60 mg/g).

The TAC of *P. tricornutum* extracts in our study remained constant irrespective of biomass disruption degree, although numerically highest values were observed in the samples with cell disruption levels of 55% and 65% (2.54 mM TE/g DW and 2.55 mM TE/g DW, respectively). This behavior may be explained by the antioxidant properties of all the above-mentioned compounds. Although the bioactive compound-release kinetics varied with the cell wall disruption degrees, we observed stable antioxidant capacity. The TAC values obtained in the present study are higher than the results reported in the published literature. Goiris et al. (2012) obtained antioxidant capacity values of 19.1 μmol TE/g DW for *P. tricornutum*, which is a thousand times lower than our observations. Safafar et al. (2015) obtained 6.79 μM TE/g for methanolic extract from 1 mg algae biomass DW/mL of methanol. Gilbert-López et al. (2017) reported 0.61 mmol TE/g in *P. tricornutum* extract, using pure ethanol for the extraction of bioactive components and at a temperature of 50 °C. A positive correlation was found between the degree of disruption and TPC ($r = 0.9454$, $p < 0.0001$), and between the degree of disruption and chlorophyll B ($r = 0.6159$, $p = 0.0065$); based on the values from the differentially processed *P. tricornutum* samples.

The observations about the release of bioactive compounds from *T. chui*, at different cell wall disruption levels (Fig. 2b), were different from those obtained for *P. tricornutum*. Overall, the concentrations of bioactive compounds were lower in *P. tricornutum* than in *T. chui*, which is indicated by using different Y-axis scales in Fig. 2a and b. Only the TPC was in the same range for the two algae. The TPC in *T. chui* plateaued beyond 60% cell disruption degree. The results are similar to the findings reported for *Tetraselmis* sp. by other authors. Maadane et al. (2015) obtained values of 25.5 mg GAE/g for ethanolic extracts of *T. chui* (1 g in 100 mL), while Widowati et al. (2017) extracted 16.87 mg GAE/g from methanolic extract of *T. chui*, prepared at a concentration of 50 ppm. Goiris et al. (2012) obtained lower values for extracts of *Tetraselmis* sp. (3.74 mg GAE/g DW) and *T. suecica* (1.71 mg GAE/g DW). It should be noted that none of the aforementioned studies performed by other scientists broke the microalgae cell walls.

A significant increase ($p < 0.05$) in TAC was observed after bead milling of *T. chui* biomass, up to 60% disruption degree. The maximum extraction of total carotenoids (76.47 mg/g DW) and chlorophyll A (186.89 mg/g DW) was achieved at 67% disruption degree, and the

Table 5
Phenolic compounds* (mg/g DW) in the differentially disrupted *T. chui* extracts.

Disruption (%)	Capsaicin	Cinnamic acid	4-vinylphenol	Dihydro-p-coumaric acid	Sinapic acid	Benzoic acid
0	5.687 ± 2.967	0.311 ± 0.020	0.683 ± 0.245	0.583 ± 0.175	–	0.148 ± 0.005
61	10.921 ± 0.219	0.987 ± 0.052	–	3.180 ± 0.112	0.219 ± 0.004	0.945 ± 0.071
67	8.061 ± 0.142	0.976 ± 0.067	1.716 ± 0.074	3.233 ± 0.364	0.182 ± 0.016	1.426 ± 0.144
77	12.810 ± 0.937	–	–	1.263 ± 0.149	0.103 ± 0.011	1.599 ± 0.135
83	8.490 ± 0.713	0.598 ± 0.029	0.992 ± 0.113	1.841 ± 0.349	–	0.747 ± 0.086
90	7.492 ± 2.293	0.442 ± 0.079	0.797 ± 0.074	1.608 ± 0.287	–	0.691 ± 0.050
95.5	5.999 ± 0.192	0.501 ± 0.066	–	1.734 ± 0.363	–	0.682 ± 0.042
99	16.441 ± 0.904	0.699 ± 0.063	–	2.337 ± 0.090	0.285 ± 0.032	0.786 ± 0.084

* Values (three replicates per differentially disrupted biomass analyzed on three parallels each) are means ± sd.

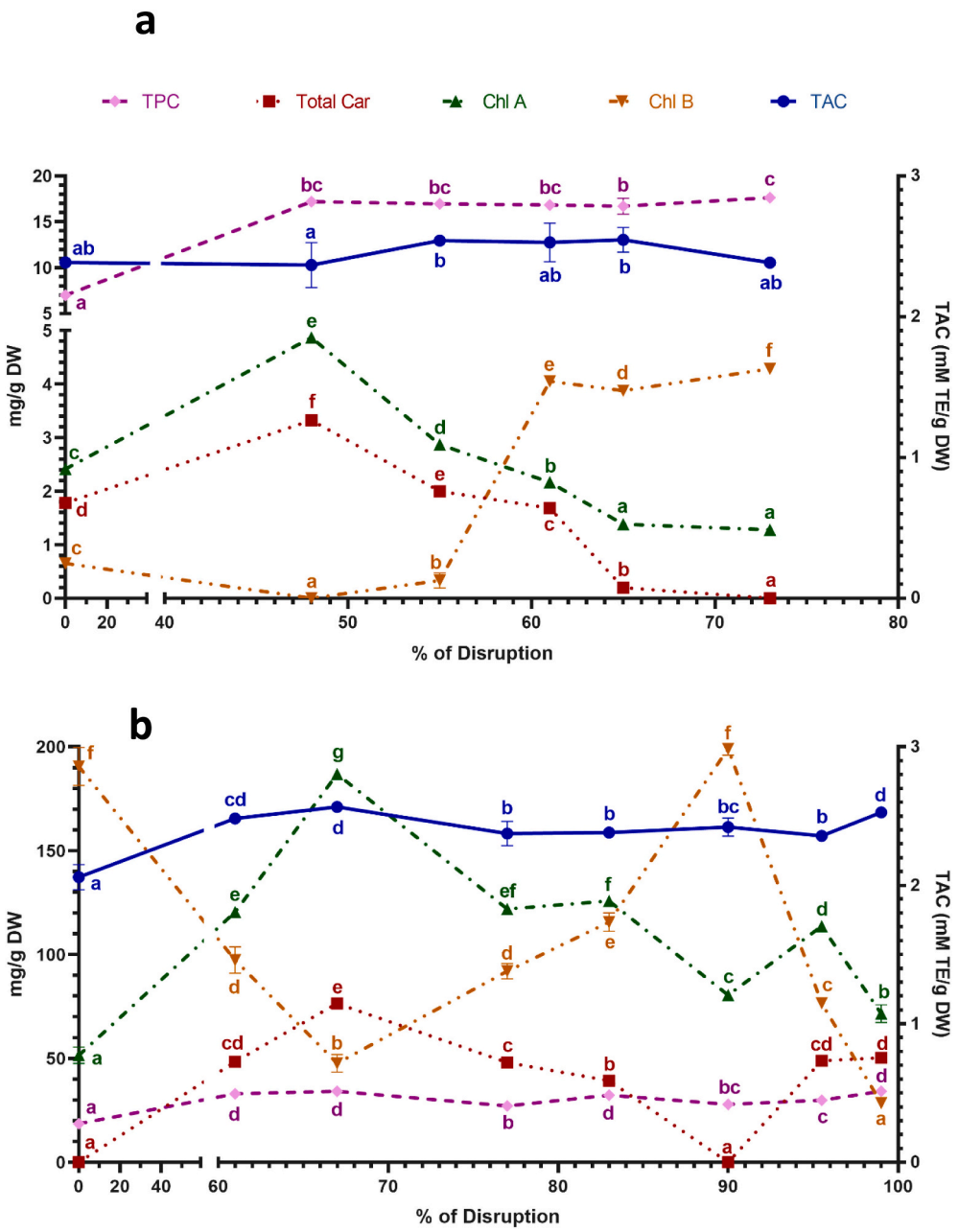

Fig. 2. Concentration of the polyphenols in the extracts from (a) *P. tricornutum* and (b) *T. chui*, after different bead milling treatments. Total phenolic compounds (TPC), total carotenoids (Total Car), chlorophyll A (Chl A), chlorophyll B (Chl B) and total antioxidant capacity (TAC). Different letters above each line indicate significant statistical differences ($p < 0.05$).

Table 6
Fatty acid profile (% of total fatty acids) in the experimental feeds that were fed to Atlantic salmon.

	PI	PB	TI	TB	RD
C14:0	5.03 ± 0.01	5.12 ± 0.03	5.21 ± 0	4.71 ± 0.02	5.05 ± 0.01
C16:0	16.5 ± 0.04	17.36 ± 0.06	19.68 ± 0	17.48 ± 0.04	16.37 ± 0.1
C18:0	2.55 ± 0.03	2.54 ± 0.02	2.93 ± 0.01	2.49 ± 0.01	2.57 ± 0.05
Σ SFA	24.08 ± 0.05	25.02 ± 0.07	27.82 ± 0.00	24.68 ± 0.05	23.99 ± 0.11
C16:1	8.97 ± 0.03	9.6 ± 0.04	5.52 ± 0.00	5.17 ± 0.02	8.89 ± 0.08
C18:1	11.17 ± 0.05	11.34 ± 0.05	13.92 ± 0.02	12.64 ± 0.03	11.31 ± 0.09
C20:1n-9	5.42 ± 0.04	5.37 ± 0.01	5.87 ± 0.15	5.97 ± 0.01	5.50 ± 0.03
C22:1n-11	8.45 ± 0.07	8.48 ± 0.04	10.12 ± 0.04	9.30 ± 0.00	8.58 ± 0.03
C22:1n-9	0.73 ± 0.02	0.75 ± 0.01	0.97 ± 0.02	0.82 ± 0.01	0.93 ± 0.13
C24:1n-9	0.93 ± 0.00	1.00 ± 0.01	1.03 ± 0.05	1.06 ± 0.04	0.86 ± 0.04
Σ MUFA	35.68 ± 0.09	36.54 ± 0.07	37.43 ± 0.03	34.96 ± 0.02	36.07 ± 0.10
C18:2n-6	2.90 ± 0.03	2.67 ± 0.02	3.09 ± 0.00	2.90 ± 0.01	2.88 ± 0.07
C18:3n-3	2.65 ± 0.02	2.42 ± 0.01	3.46 ± 0.01	3.57 ± 0.02	2.61 ± 0.07
C20:5n-3	10.60 ± 0.18	9.97 ± 0.04	8.04 ± 0.02	8.5 ± 0.02	11.3 ± 0.08
C22:5n-3	0.79 ± 0.04	0.73 ± 0.01	0.77 ± 0.07	0.80 ± 0.01	0.78 ± 0.04
C22:6n-3	9.70 ± 0.06	8.81 ± 0.04	8.59 ± 0.02	9.54 ± 0.00	9.76 ± 0.06
Σ PUFA	26.64 ± 0.17	24.59 ± 0.06	23.94 ± 0.00	25.31 ± 0.02	27.32 ± 0.14
Σ n-3	23.74 ± 0.17	21.92 ± 0.06	20.86 ± 0.00	22.41 ± 0.02	24.44 ± 0.12
Σ n-6	2.90 ± 0.03	2.67 ± 0.02	3.09 ± 0.00	2.90 ± 0.01	2.88 ± 0.07
Σ n-6/Σn-3	0.12 ± 0.16	0.12 ± 0.38	0.15 ± 1.73	0.13 ± 0.79	0.12 ± 0.01
EPA + DHA	20.3 ± 0.18	18.77 ± 0.06	16.63 ± 0.02	18.04 ± 0.01	21.05 ± 0.10

Values are mean ± sem and two feed samples were analyzed to obtain the data.

highest chlorophyll B level (199 mg/g DW) was obtained at 90% disruption degree. Other studies have reported 17 and 11 times higher values for total carotenoids in *T. suecica* and *Tetraselmis* sp. respectively (Maadane et al., 2015). The differences in results from the two microalgae could be explained by differences in their cultivation conditions, which is an important factor that determines the production of bioactive compounds. For *T. chui*, the degree of disruption had a positive correlation with TPC ($r = 0.7107$, $p = 0.0001$) and total carotenoids ($r = 0.4100$, $p = 0.0466$). Moreover, TAC showed a strong positive correlation with TPC ($r = 0.8996$, p = 0.0001), and with total carotenoids ($r = 0.6718$, $p = 0.0003$) and chlorophyll A ($r = 0.5726$, $p = 0.0035$). These results fully agree with those previously described in the literature; phenolic compounds, carotenoids, and chlorophylls contribute to antioxidant capacity (Goiris et al., 2012; Morowvat and Ghasemi, 2016; Pangestuti and Kim, 2011; Rodrigues et al., 2015; Safafar et al., 2015; Sansone and Brunet, 2019).

3.3. Phenolic profile of *P. tricornutum* and *T. chui*

The phenolic profiles of the two microalgae species studied were very different (Tables 4 and 5). Two polyphenols, cinnamic acid and benzoic acid, were extracted from *P. tricornutum* (Table 4). Both compounds belong to the phenolic acid subfamily. The extraction of cinnamic acid was enhanced by cell wall disruption. As for the *T. chui* biomass, six polyphenols were identified, namely capsaicin, cinnamic acid, 4-vinylphenol, dihydro-p-coumaric acid, sinapic acid, and benzoic acid (Table 5). The main compound obtained was capsaicin with maximum values at 99% disruption degree (16.44 mg/g DW). Dihydro-p-coumaric acid was the second most abundant polyphenol, and the values were 5 times lower than capsaicin, achieved by 61% (3.18 mg/g DW) and 67% (3.23 mg/g DW) disruption degrees (Table 5). Cinnamic acid and benzoic acid were also extracted at most of the disruption degrees, although at a concentration lower than in *P. tricornutum*. The phenolic compound, 4-vinylphenol was extracted only at disruption degrees of 67%, 83%, 90%, and in the control. The hydroxycinnamic acid, sinapic acid was detected only at disruption degrees of 61%, 67%, 77%, and 99%, and that too at very low concentrations.

To our knowledge, other studies have not reported the phenolic profile of *P. tricornutum* or *T. chui*. Adb El-Baky et al. (2009) reported the presence of cinnamic, gallic, 4-hydroxybenzoic and chlorogenic acids in *Spirulina* extracts. Bulut et al. (2019) reported the extraction of benzoic acid derivatives (gallic acid, 4-hydroxy benzoic acid and vanillic acid), cinnamic acid derivatives (caffeic acid, chlorogenic acid) and flavonols (quercetin), in ethanol/water and ethyl acetate extracts from *Scenedesmus* sp.

Capsaicin (*trans*-8-methyl-*N*-vanillyl-6-nonenamide) is a phenolic alkaloid of the capsaicinoid group, and the compound is specific to *Capsicum* spp. It is this active ingredient in chili peppers that causes the burning sensation. Although capsaicin is exclusively produced by plants

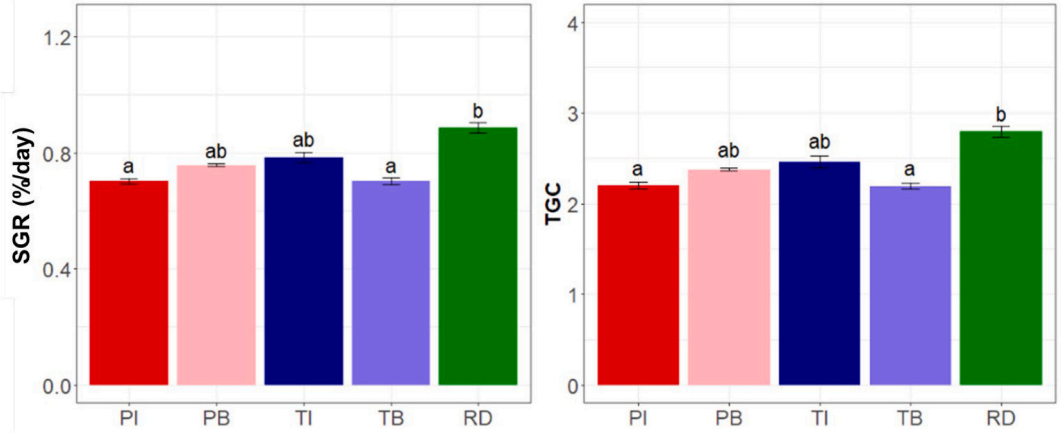

Fig. 3. Specific growth rate (SGR) and thermal growth coefficient (TGC) of Atlantic salmon fed the different experimental diets. RD- reference diet, PI- *Phaeodactylum* whole, PB- *Phaeodactylum* broken, TI- *Tetraselmis* whole, TB- *Tetraselmis* broken. Bar graphs were drawn using mean and sem of the data ($n = 3$ tanks) and different letters above the bars indicate significant differences ($p < 0.05$) between groups.

Table 7
Digestibility (ADC%) of ingredients in the diets fed to Atlantic salmon.

	PI	PB	TI	TB	Anova *P*-value
Dry matter	$42.06 \pm 1.95^{y/B}$	$29.89 \pm 4.15^{x/A}$	$11.50 \pm 4.25^{x/A}$	$41.75 \pm 1.98^{y/B}$	Alga: 0.03; Process: 0.03, Alga:Process: 0.001
Protein	$71.79 \pm 0.99^{y/B}$	$62.38 \pm 0.88^{x/A}$	$53.52 \pm 1.34^{x/A}$	$69.08 \pm 1.69^{y/B}$	Alga: 0.001; Process: 0.04, Alga:Process: 9.41×10^{-6}
Lipid	$58.23 \pm 0.29^{y/B}$	$71.40 \pm 2.17^{x/A}$	$8.56 \pm 3.94^{x/A}$	$51.93 \pm 1.23^{y/B}$	Alga: 0.001; Process: 0.001, Alga:Process: 0.001

Values obtained from 3 replicate tanks (feces from all fish pooled; 6 fish/tank) of each group are presented as mean ± sem. Different superscripts (A, B, C) indicate the significant differences between the groups based on the factor alga (PI vs TI and PB vs TB), and different superscripts (x, y) indicate the significant differences between the groups based on the factor process (PI vs PB and TI vs TB). PI - *P. tricornutum* whole; PB - *P. tricornutum* broken; TI - *T. chui* whole; TB - *T. chui* broken.

Table 8
Digestibility (ADC%) of dry matter and nutrients in the whole diets of Atlantic salmon.

	PI	PB	TI	TB	RD	Anova P-value
Dry matter	57.12 ± 0.60^{ac}	53.45 ± 1.30^{ab}	47.76 ± 1.31^{b}	57.12 ± 0.60^{ac}	63.89 ± 1.39^{c}	Alga: 0.01; Process: 0.01; Alga:Process: 0.001
Protein	79.32 ± 0.28^{ac}	76.49 ± 0.26^{ab}	73.67 ± 0.40^{b}	78.41 ± 0.50^{c}	82.31 ± 1.50^{c}	Alga: 0.001; Process: 0.03; Alga:Process: 0.001
Lipid	85.37 ± 0.05^{ab}	86.67 ± 0.54^{a}	74.84 ± 0.80^{d}	84.17 ± 0.23^{b}	91.76 ± 0.43^{c}	Alga: 0.001; Process: 0.001; Alga:Process: 0.001

Values obtained from 3 replicate tanks (feces from all fish pooled/tank) of each group are presented as mean ± sem. Statistically different values are indicated using different superscripts. PI - *P. tricornutum* whole; PB - *P. tricornutum* broken; TI - *T. chui* whole; TB - *T. chui* broken; RD – reference diet.

(chili peppers), previous studies have not reported the extraction of the compound from microalgae. We have now gathered evidence to indicate that microalgae can synthesize it; this compound was the primary polyphenol extracted from *T. chui* biomass. This significant finding could partly explain the differences in digestibility of nutrients in Atlantic salmon fed *T. chui*.

3.4. Fatty acids in the experimental feeds

Fatty acid composition in the feeds is given in Table 6. Total SFA (saturated fatty acids) and ΣMUFA were higher in the intact *T. chui* diet followed by the broken *P. tricornutum* diet (Table 6). Total PUFA and n-3 values were higher in the reference diet. The highest value of total n-6 fatty acid was noted for the intact *T. chui*. EPA + DHA was higher in the reference diet followed by the intact *P. tricornutum* diet. Ratio of total n-6/n-3 fatty acids was higher in intact *T. chui* diets.

3.5. Digestibility study

3.5.1. Digestibility of nutrients in the whole diet and microalgae

In 35 days, we observed a 40% increase in the weight of the fish in all groups, from an average start weight of 315 g to a final weight of 441 g. For the algae-fed groups, the average SGR and TGC values were 0.77% and 2.40, respectively. Fish fed the *P. tricornutum* whole (PI) and *T. chui* broken (TB) diets had significantly lower weight gain, SGR, and TGC (Fig. 3) compared to the reference diet fed fish. A longer feeding period would be necessary to further assess the impact of these dietary treatments on growth performance. Bead milling-caused increase in digestibility of diets and ingredient was noted for fish fed *T. chui* (Tables 7, 8). Fish fed *T. chui* (TI) had significantly lower digestibility of DM, protein and lipid in whole diets (Table 8). The ADC of protein in the intact *P. tricornutum* was higher than that in the intact *T. chui*. However, breaking the cell walls reduced the ADC of protein in *P. tricornutum* while it increased that of *T. chui*. The ADC of lipid in the intact *T. chui* was lower compared to the intact *P. tricornutum*. Only the digestibility of lipid in whole diets containing *P. tricornutum* was increased (not statistically significant, $p = 0.3823$) by bead milling, as noted for the digestibility of the ingredient. Overall, the ADC values of DM and protein were lower in both microalgae in the present experiment compared to *Nannochloropsis* sp. and *Desmodesmus* sp. reported in Gong et al. (2018). Bead milling improved the digestibility of lipid in both microalgae although the pretreatment released more lipids only from *P. tricornutum* (Table 3a). The ADC of DM, protein, and lipid of intact *P. tricornutum* was higher than intact *T. chui*. Nevertheless, bead milling improved the ADC values in *T. chui*. Bead milling is reported as an efficient method to

Table 9
Fatty acid profile (% of total fatty acids) in the fillets of Atlantic salmon fed five different diets.

	PI	PB	TI	TB	RD
C14:0	2.99 ± 0.05^{ab}	3.02 ± 0.05^{a}	2.77 ± 0.06^{b}	3.00 ± 0.01^{ab}	3.09 ± 0.02^{a}
C16:0	13.29 ± 0.08^{a}	13.52 ± 0.23^{ab}	14.02 ± 0.19^{b}	13.95 ± 0.14^{b}	13.40 ± 0.07^{ab}
C18:0	2.96 ± 0.02^{a}	2.97 ± 0.07^{ab}	3.22 ± 0.12^{b}	3.17 ± 0.00^{b}	3.00 ± 0.02^{ab}
C22:0	4.28 ± 0.06^{a}	4.14 ± 0.06^{ab}	4.10 ± 0.06^{a}	4.04 ± 0.10^{a}	4.85 ± 0.14^{b}
Σ SFA	23.52 ± 0.08^{a}	23.66 ± 0.23^{a}	24.11 ± 0.24^{ab}	24.15 ± 0.06^{ab}	24.34 ± 0.11^{b}
C16:1n-7	5.08 ± 0.03^{ab}	5.49 ± 0.09^{a}	3.89 ± 0.03^{c}	3.95 ± 0.04^{bc}	4.08 ± 0.06^{abc}
C18:1n-9	26.89 ± 0.21^{ab}	26.96 ± 0.18^{ab}	25.82 ± 0.46^{a}	28.16 ± 0.40^{b}	25.63 ± 0.14^{a}
C18:1n-7	3.23 ± 0.01^{abc}	3.24 ± 0.01^{ab}	3.13 ± 0.03^{bc}	3.26 ± 0.01^{a}	3.03 ± 0.01^{c}
C20:1n-9	4.41 ± 0.01^{bc}	4.02 ± 0.13^{a}	4.20 ± 0.11^{ac}	4.18 ± 0.06^{ac}	4.60 ± 0.08^{b}
Σ MUFA	39.60 ± 0.21^{a}	39.70 ± 0.31^{a}	37.04 ± 0.47^{b}	39.56 ± 0.51^{ab}	37.34 ± 0.28^{b}
C18:2n-6	8.33 ± 0.08^{ab}	8.32 ± 0.09^{abc}	7.60 ± 0.14^{bc}	8.58 ± 0.10^{a}	7.45 ± 0.06^{c}
C18:3n-3	3.07 ± 0.06^{ab}	3.06 ± 0.03^{ab}	3.58 ± 0.03^{bc}	3.70 ± 0.04^{c}	2.93 ± 0.01^{a}
C18:4n-3	1.07 ± 0.02^{a}	1.10 ± 0.03^{a}	1.48 ± 0.05^{b}	1.43 ± 0.01^{b}	1.22 ± 0.01^{ab}
C20:4n-3	1.12 ± 0.00^{a}	1.14 ± 0.01^{ab}	1.35 ± 0.03^{c}	1.26 ± 0.01^{bc}	1.23 ± 0.01^{abc}
C20:5n-3	4.81 ± 0.04^{ab}	5.17 ± 0.12^{a}	5.10 ± 0.15^{a}	4.41 ± 0.03^{b}	5.09 ± 0.05^{a}
C22:5n-3	1.88 ± 0.02^{a}	1.88 ± 0.02^{a}	1.82 ± 0.01^{ab}	1.72 ± 0.03^{b}	1.86 ± 0.02^{a}
C22:6n-3	13.03 ± 0.26^{a}	12.91 ± 0.81^{a}	14.75 ± 0.61^{b}	13.42 ± 0.84^{ab}	14.39 ± 0.24^{ab}
n-6	8.33 ± 0.08^{ab}	8.32 ± 0.09^{abc}	7.60 ± 0.14^{bc}	8.58 ± 0.10^{a}	7.45 ± 0.06^{c}
n-3	24.99 ± 0.28^{a}	25.27 ± 0.92^{a}	28.08 ± 0.50^{b}	25.95 ± 0.83^{ab}	26.71 ± 0.24^{ab}
EPA + DHA	17.85 ± 0.30	18.08 ± 0.92	19.85 ± 0.59	17.84 ± 0.85	19.48 ± 0.25
Σ PUFAs	33.32 ± 0.21^{a}	33.59 ± 0.90^{a}	35.68 ± 0.41^{b}	34.53 ± 0.73^{ab}	34.16 ± 0.31^{ab}
n-6/n-3	0.34 ± 0.01^{a}	0.33 ± 0.01^{a}	0.28 ± 0.01^{b}	0.33 ± 0.01^{a}	0.28 ± 0.00^{ab}

Values from 3 replicate tanks (6fish/tank) are presented as mean ± sem. Statistically different values are indicated using different superscripts. PI - *P. tricornutum* whole; PB - *P. tricornutum* broken; TI - *T. chui* whole; TB - *T. chui* broken; RD - Reference diet.

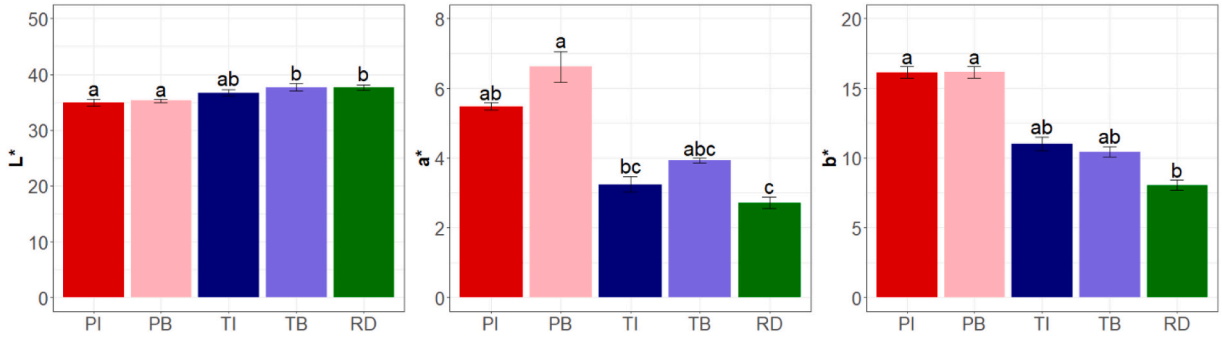

Fig. 4. Minolta L*, a* and b* values for dorsal muscle of Atlantic salmon. L*, lightness, a* and b*, color directions. +a* is the red direction, −a* is the green direction, +b* is the yellow direction, and -b* is the blue direction. RD- reference diet, PI- *Phaeodactylum* whole, PB- *Phaeodactylum* broken, TI- *Tetraselmis* whole, TB- *Tetraselmis* broken. Bar graphs were drawn using mean and sem of the data (n = 3 tanks) and different letters above the bars indicate significant differences ($p < 0.05$) between groups. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

increase the extractability of nutrients from *N. gaditana*, which in turn enhanced the ADC of the alga-derived protein and lipid in Nile tilapia (*Oreochromis niloticus*) (Teuling et al., 2019). However, microalgae respond differently to various methods that are employed to break cell walls. A recent study with European seabass reported lower ADC values of *T. chui* compared to *N. oceanica* (Batista et al., 2020). The ADC of protein and energy in feeds with *N. oceanica* was efficiently improved after pre-treating the algae biomass with an enzyme cocktail while mechanical treatment of *T. chui* was most efficient for increasing ADCs of nutrients and energy.

3.6. Fillet fatty acids

ΣSFA (saturated fatty acids) in the fillet of fish fed the reference diet was the highest compared to the value in the *P. tricornutum*-fed fish (Table 9). The fillet of the fish fed *P. tricornutum* had the highest ΣMUFA (monounsaturated fatty acids) compared to those fed intact *T. chui* and the reference diet. Values of n-6 fatty acid in the fillet of fish fed both intact *P. tricornutum* and broken *T. chui* were higher compared to the fish fed the reference diet. Fillet of fish fed *P. tricornutum* had lower n-3 fatty acids compared to fish fed intact *T. chui*. Total PUFA (polyunsaturated fatty acid) value was the highest in fish fed intact *T. chui* compared to *P. tricornutum*-fed fish. The Σn-6/Σn-3 was the highest in intact *P. tricornutum* fed fish, which had values similar to those fed the broken *P. tricornutum* and *T chui* fed fish.

Bead milling-caused decrease in EPA was evident in the fillet of *T. chui* fed fish, but not in the fillet of the *P. tricornutum* group (Table 9). In addition, the process was found to influence linoleic acid, sum n-6, and n-6/n-3, though at a very minute level. The processing effect on fatty acids deserves attention because the alteration of the above-mentioned fatty acids led to lipid accumulation in the liver of Atlantic salmon (Sørensen et al., 2021). A 100% soybean oil diet caused a selective accumulation of linoleic and oleic acid in the liver of Atlantic salmon (Ruyter et al., 2006); the levels of linoleic acid (% of total fatty acids) in the liver were 15.8/17.5 compared to 2.1/2.3 (in the control group). Furthermore, excess linoleic acid can elevate endocannabinoids in the liver of salmon, and in mice, the fatty acid counteracted the anti-inflammatory properties of EPA and DHA (Alvheim et al., 2013). EPA and DHA are known to have differential effects in a mouse model; while EPA can reduce hepatic triacylglycerol better than DHA, DHA can suppress hepatic inflammation and reactive oxygen species generation (Suzuki-Kemuriyama et al, 2016). In our study, we noted an increase in EPA in *P. tricornutum*, but the difference was not significant. Hence, future studies should find a way to increase the EPA and DHA content to ward off any adverse effects caused by other fatty acids that are released by mechanical or physical processing of microalgae.

Table 10
Pigments (ng/g) in the fillets of Atlantic salmon fed five different diets.

Pigments	PI	PB	TI	TB	RD
Fucoxanthinol	104.1 ± 6.03^{a}	140.3 ± 6.23^{a}	2.1 ± 0.53^{b}	1.4 ± 1.06^{b}	1.5 ± 1.45^{b}
Fucoxanthin	204.2 ± 15.41^{b}	283.9 ± 16.76^{a}	2.8 ± 0.48^{c}	2.4 ± 1.63^{c}	1.8 ± 1.66^{c}
Idoxanthin	4.8 ± 0.67	9.3 ± 1.71	4.6 ± 2.00	3.9 ± 1.58	3.2 ± 1.39
Astaxanthin	59.4 ± 3.73	64.5 ± 6.38	51.7 ± 2.77	61.9 ± 4.31	49.8 ± 1.11
Diatoxanthin	15.7 ± 1.10^{ab}	25.2 ± 2.39^{a}	0.1 ± 0.10^{c}	0.2 ± 0.21^{bc}	0.8 ± 0.29^{abc}
Lutein+astaxanthin cis mixture	16.5 ± 1.35^{a}	14.1 ± 0.83^{a}	62.7 ± 2.71^{b}	65.8 ± 2.49^{b}	10.7 ± 0.54^{a}
Lutein cis	4.4 ± 0.07^{ac}	5.6 ± 0.52^{ab}	8.6 ± 0.52^{b}	7.8 ± 1.38^{b}	1.1 ± 0.13^{c}

Values from 3 replicate tanks of each group (6 fish/tank) are presented as mean ± sem. Statistically different values are indicated using different superscripts. PI - *P. tricornutum* whole; PB - *P. tricornutum* broken; TI - *T. chui* whole; TB - *T. chui* broken; RD - Reference diet.

3.7. Fillet and skin pigmentation

The Minolta L*, a* and b* values of the flesh (dorsal part) of fish fed the five different diets were significantly different. L* was significantly higher in the broken *T. chui* and reference diet fed fish (Fig. 4). On the other hand, a*-value was significantly higher in the PB compared to RD (Fig. 4). For b*-value, both PI and PB had significantly higher values compared to the RD. No differences were noted for L*, a* or b*-values of the *T. chui* fed groups and the RD (Fig. 4). As described above, both microalgae biomasses used in this study contained high levels of antioxidant compounds and pigments and most of them were released at a higher degree of cell wall disruption. The higher a* and b* values of fish fed *P. tricornutum* incorporated feeds were not expected because previous feeding studies with this microalga have not reported similar changes in pigmentation. Microalgae have the potential to produce a wide range of carotenoids (Pignolet et al., 2013), but only a few known strains such as *Haematococcus pluvialis* are known to accumulate high concentrations (up to 50 mg/g) of astaxanthin (Minhas et al., 2016). It has been reported that *P. tricornutum* accumulates fucoxanthin, the orange-coloured xanthophyll (Derwenskus et al., 2019; Kim et al., 2012). None of the experimental diets were supplemented with synthetic astaxanthin or other carotenoids, and hence, the skin and flesh pigmentation is likely to be solely from the natural pigments. A small amount of astaxanthin may be derived from marine ingredients such as fishmeal and fish oil. The flesh from microalgae-fed fish contained higher (numerical values) levels of astaxanthin than those fed RD, and

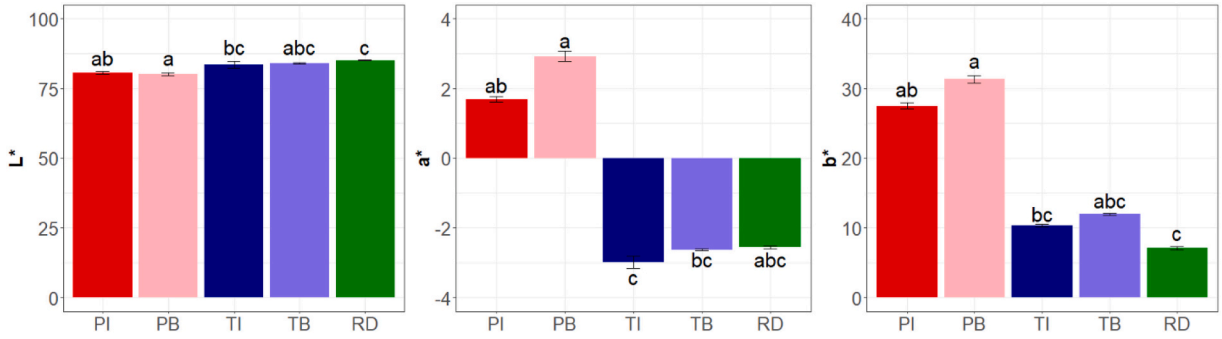

Fig. 5. Minolta L*, a* and b* values for dorsal skin of Atlantic salmon. L*, lightness, a* and b*, color directions. +a* is the red direction, −a* is the green direction, +b* is the yellow direction, and -b* is the blue direction. RD- reference diet, PI- *Phaeodactylum* whole, PB- *Phaeodactylum* broken, TI- *Tetraselmis* whole, TB- *Tetraselmis* broken. Bar graphs were drawn using mean and sem of the data (n = 3 tanks) and different letters above the bars indicate significant differences ($p < 0.05$) between groups. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

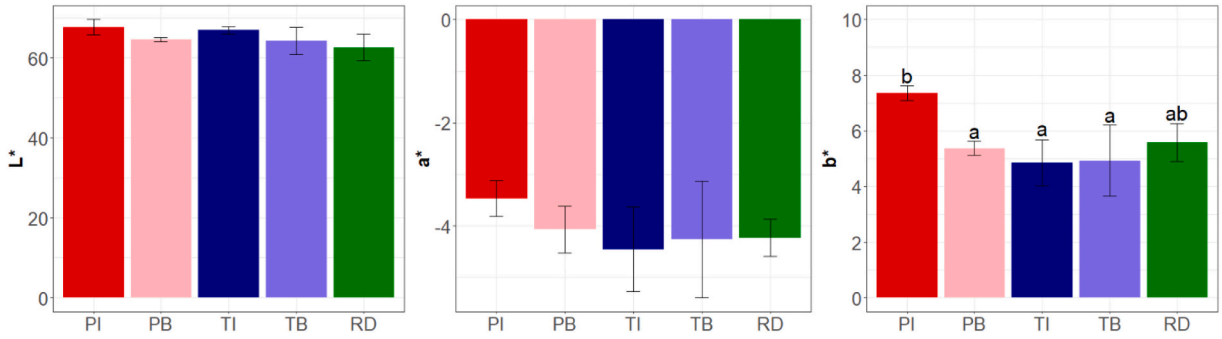

Fig. 6. Minolta L*, a* and b* values for belly skin of Atlantic salmon. L*, lightness, a* and b*, color directions. +a* is the red direction, −a* is the green direction, +b* is the yellow direction, and -b* is the blue direction. RD- reference diet, PI- *Phaeodactylum* whole, PB- *Phaeodactylum* broken, TI- *Tetraselmis* whole, TB- *Tetraselmis* broken. Bar graphs were drawn using mean and sem of the data (n = 3 tanks) and different letters above the bars indicate significant differences ($p < 0.05$) between groups. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 7. Skin and flesh color of salmon from RD (left) and PB (right) group. RD- reference diet, PB- *Phaeodactylum* broken.

the highest values were noted for fish fed PB. In the case of PB and TB-fed fish, the antioxidant protection of other microalgae-derived bioactive compounds appears to have spared dietary astaxanthin for effective deposition in salmon muscle tissue. Table 10 indicates that fucoxanthin, fucoxanthinol and diatoxanthin were higher in fish fed *P. tricornutum* compared to fish fed *T. chui.* Fish fed broken *P. tricornutum* had significantly more fucoxanthin in the fillets compared to fish fed the intact alga. Fish fed the reference diet had very low levels of the assessed carotenoids compared to the *P. tricornutum* fed fish. These results indicate the improved utilization of pigments from *P. tricornutum.* Fucoxanthin was reported to improve lipid and cholesterol metabolism in rats (Ha and Kim, 2013). High levels of fucoxanthin and fucoxanthinol in the *P. tricornutum* fed fish indicate that the former is hydrolyzed to the latter by intestinal enzymes, as reported by Sugawara et al. (2002). Diatoxanthin protects *P. tricornutum* from intense light (Pereira et al., 2021), and hence, the high content of the xanthophyll could reduce photooxidation of fatty acids in salmon fillets. It is known that compared to astaxanthin, only very less amounts of lutein are absorbed by Atlantic salmon, and the yellow pigment does not affect astaxanthin pigmentation (Olsen and Baker, 2006). Hence, the high lutein content in the fillet of fish fed *T. chui* is not important to devise strategies for high pigmentation in salmon.

The dorsal skin Minolta values were also significantly affected by the diets. Fish fed the PB diet had significantly lower L* compared to the RD group. The a*-values of the dorsal skin of the PI and PB fed groups were positive, but the values were negative for the fish fed TI, TB and RD. Significant differences were noted for the PB vs TI comparison (Fig. 5). The Minolta L* and a*-values for the ventral skin of the fish fed different diets were not significantly different (Fig. 6). Visual observation indicated that the ventral side of the fish fed *P. tricornutum* containing feeds was yellowish (Fig. 7). Minolta b*-value of the PI group was significantly

higher compared to the other alga-fed groups. Based on the Minolta values, visual observation, and the carotenoid content of the fillets of Atlantic salmon it can be stated that dietary incorporation of *P. tricornutum* at high levels, as tested in the present study, can contribute to enhanced salmon skin and fillet pigmentation.

4. Conclusion

Bead milling has the potential to be an effective pre-treatment procedure to release more phenolic compounds and pigments from *P. tricornutum* and *T. chui*. The industry can employ appropriate disruption degrees to obtain the polyphenols or pigments of interest. Based on the results from this study it can be stated that a 60% disruption of both *P. tricornutum* and *T. chui* can release enough polyphenols and other pigments of commercial value. Feeding Atlantic salmon with bead-milled *P. tricornutum* and *T. chui* biomass can be used as a strategy to improve the digestibility of lipids from both microalgae, but their effect on the different organs should be assessed carefully. Based on the digestibility and pigmentation results, *P. tricornutum* or intact *T. chui* could be good ingredients in Atlantic salmon feeds.

CRediT authorship contribution statement

Mette Sørensen: Conceptualization, Funding acquisition, Supervision, Methodology, Writing – original draft, Writing – review & editing, Project administration. **Katerina Kousoulaki:** Conceptualization, Funding acquisition, Supervision, Methodology, Writing – review & editing, Project administration, Writing – original draft. **Renate Hammerø:** Formal analysis, Investigation, Writing – original draft. **Marialena Kokkali:** Formal analysis, Investigation, Writing – original draft. **Dorinde Kleinegris:** Methodology. **Francisco J. Marti-Quijal:** Investigation, Writing – review & editing. **Francisco J. Barba:** Investigation, Writing – review & editing. **Anjana Mahesh Palihawadana:** Formal analysis. **Einar Skarstad Egeland:** Formal analysis, Writing – original draft. **Chris Andre Johnsen:** Formal analysis. **Odd Helge Romarheim:** Methodology. **Saraswathy Bisa:** Writing – review & editing. **Viswanath Kiron:** Conceptualization, Funding acquisition, Supervision, Methodology, Writing – review & editing, Project administration.

Declaration of Competing Interest

The authors of the article declare that they have no competing interests.

Data availability

We have included all the data in the manuscript.

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