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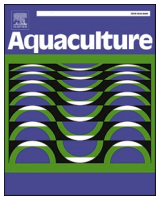

Utilization of *Nannochloropsis oceanica* in plant-based feeds by Atlantic salmon (*Salmo salar*)

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ABSTRACT

The phototropic microalga *Nannochloropsis oceanica* is a promising feed ingredient with the potential to provide nutrients including polyunsaturated fatty acids in aquafeeds. Complex and rigid cell walls limit the nutrient utilization of microalgae by Atlantic salmon. Here, we report results from two studies-a laboratory study on post-smolts and a farm trial on consumer-ready fish-that were conducted to understand the efficacy of both treated and untreated whole biomass.

In the laboratory study, we investigated if extrusion can be used as a feasible pre-treatment method to enhance digestibility, growth, feed utilization and health of the fish. Here, we employed post smolt Atlantic salmon with an initial average weight of 141.8 ± 28.2 g and they were fed one of the below mentioned experimental feeds in five replicate tanks for 84 days. Four low-fishmeal feeds were formulated; a plant-based control feed without the microalga (CTRL), two feeds containing 7.5 (NE7.5) and 15% (NE15) of the pre-extruded microalga and one feed containing 15% of the un-extruded microalga (NN15). In the farm study, fish of average weight 1.83 ± 0.01 kg were reared in pens for 197 days. In this trial, two experimental feeds were used, a control low-fishmeal feed (CT) and a feed containing 7.5% un-extruded microalga (NW).

In the laboratory study, all alga-supplemented feeds lowered weight gain, SGR and TGC compared to the control feed. The FCR and FI did not differ between the CTRL and NE7.5 groups but the parameters were significantly poor in fish fed feeds with the highest incorporation of the alga. Likewise, in the farm study feed conversion was not significantly affected by the inclusion of the microalga. The retention of lipid in the post-smolts showed a linear decrease with the incorporation of the microalga in the feed, and the protein retention was significantly reduced only at the highest incorporation level. The content of sum PUFA and EPA + DHA in fish fed microalgae were numerically and significantly higher in the farm and laboratory studies, respectively. The apparent digestibility coefficient (ADC) of protein and lipid in the post-smolts showed an inverse relation to the incorporation level of the microalga. The digestible lipid and energy retention efficiencies improved ($p < 0.05$) in fish fed pre-extruded microalgae. Expression of antioxidant and immune genes suggested that the microalga did not impart any negative effects on the post-smolts. The farm study indicated the ability of the alga to plausibly shape the intestinal micromorphology and affect the nutrient absorption as well as peristalsis.

Based on the laboratory study, we can state that the differences between the pre-treated and untreated microalga diets were only minor suggesting that inclusion level of the alga in plant-based diets had a more significant effect than the pre-treatment, at least at 15% incorporation level. The main conclusion from both the

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studies is that *Nannochloropsis oceanica* in the feeds of Atlantic salmon can facilitate a slight increase in deposition of n-3 fatty acids, EPA and DHA.

1. Introduction

Production of food from oceans can be increased by six-fold through appropriate innovation and sustainable management (Costello et al., 2020). Aquaculture is the fastest growing food production system, and intensive farming using formulated feeds is increasing at a greater rate compared to extensive systems (FAO, 2020). Intensified aquaculture production calls for the introduction of feed ingredients other than those derived from terrestrial crops, which are the dominant ingredients in modern feeds (Aas et al., 2019; Ytrestøyl et al., 2015). Increased use of plant ingredients in aquafeeds contributes to arable land conversion, more use of fresh water and increased greenhouse gas emissions. Feed ingredients with a lower environmental footprint include insect meal and microbial biomass that can be grown in waste streams from industry or other food production systems (Hua et al., 2019).

Microalgae are a diverse group of photosynthetic microorganisms that use carbon dioxide, light energy and inorganic nutrients to produce organic biomass and oxygen. Some can also be grown heterotrophically in the absence of light, using organic carbon sources. Microalgae are the primary producers of marine food webs and increased use of low trophic species in fish feeds will improve the environmental footprint of the food production sector. Phototrophic microalgae can be the future path deviators of the aquafeed sector, which aims to embrace sustainability. Microalgae can provide macronutrients such as protein and lipid, though the biochemical composition varies among algae species, strains and also their growth conditions (Shah et al., 2018; Tibbetts, 2018). The crude protein content of most species of microalgae is lower compared to fish meal and plant protein concentrates, which are used in commercial feeds for carnivorous fish, and microalgae may also contain higher concentrations of non-protein nitrogen (Laurens et al., 2012; Lourenço et al., 2004). All essential amino acids are present in microalgae (Tibbetts, 2018), at varying levels. Certain species of microalgae have the capacity to accumulate high lipid content (Shah et al., 2018). The most valuable species for fish nutrition may be those with the capacity to produce the long chain polyunsaturated fatty acids, especially EPA and DHA. Microalgae also contain vitamins, minerals, sterols, carotenoids, phenolic compounds, peptides, glucans and a wide range of functional biomolecules that may add value to their biomass (Buono et al., 2014; Yaakob et al., 2014). On the other hand, the indigestible carbohydrates, which are mostly associated with the rigid cell walls of microalgae, should be kept as low as possible in fish feeds, especially for carnivorous fishes because they limit the digestibility of nutrients and utilization of energy (Gong et al., 2018; Gong et al., 2019; Sørensen et al., 2016; Sørensen et al., 2017). The cell walls of microalgae differ both in terms of chemical composition and architecture (Bernaerts et al., 2018). An overview of cell wall composition in different microalgae and the techniques to break the cell walls have been described by Alhattab et al. (2019). The cell wall of *Nannochloropsis* spp. is made up of an inner cellulose-based layer and an outer algaenan-based layer (Scholz et al., 2014). The latter layer is very resistant to enzymes and chemicals, and hence *Nannochloropsis* cells cannot be easily ruptured (Alhattab et al., 2019). Use of mechanical methods such as bead milling, are shown to be efficient in increasing the digestibility of *N. gaditana* in Nile tilapia feeds (Teuling et al., 2019). This may also suggest that other methods such as thermomechanical treatment can be used to increase nutrient digestibility and accessibility of nutrients from *Nannochloropsis* spp. Extrusion processing is one such method and is commonly used in commercial fish feed production, where high temperature (120–130 °C), high pressure (20–30 bar) and shear forces are employed to transform the ingredients into a dough. Studies with Atlantic salmon (*Salmo salar*) have already shown that extrusion can increase the digestibility of feed ingredients (Gong et al., 2018). If extrusion can be demonstrated as an efficient method to rupture microalgal cell walls and increase digestibility of nutrients, the use of microalgae will start to gain traction among the aquafeed companies. Microalgae can be incorporated only at low levels (up to 10%) in the feeds for carnivores such as Atlantic salmon (Gong et al., 2020; Sørensen et al., 2016) unless the biomass is pretreated (Kiron et al., 2012; Kiron et al., 2016b).

The overall aim of this study was to investigate the effect of *Nannochloropsis oceanica* on growth, intestinal health and product quality of the fish.

2. Material and methods

Two feeding studies were performed to understand the effect of *N. oceanica* on Atlantic salmon: 1) a laboratory study, using Atlantic salmon (*Salmo salar*) post smolts reared in tanks and 2) a farm study employing on-growth phase in net pens.

2.1. Experimental design and feeds

The feeding trials were approved by the National Animal Research Authority (FDU: Forsøksdyrutvalget ID-5887) in Norway.

For the laboratory study, four nearly isoproteic (49% of dry matter) and isolipidic (21–22% of dry matter) feeds were formulated. All four diets contained low levels (<10%) of fish meal: the control feed contained no *N. oceanica* (CTRL), two test feeds contained 7.5% (NE7.5) and 15% (NE15) extruded *N. oceanica* and the fourth diet feed was similar to the NE15, but with un-extruded *N. oceanica* (NN15). The microalgae mainly replaced fish meal in the experimental feeds.

Similarly, for the on-growth phase study, two isoproteic (49%) and isolipidic (23%) feeds were fed to the experimental fish. The two feeds were: a control feed with no alga (CT) and a test feed with 7.5% unextruded *N. oceanica*, (NW). We employed 7.5% in the farm trial because the laboratory trial showed reduced utilization at the highest inclusion level.

Ingredients, chemical composition and fatty acid profile of the feeds are presented in Tables 1 and 2, respectively.

The test microalga *N. oceanica* used in the feeds was cultured in closed photobioreactors at Allmicroalgae, Lisbon, Portugal. After harvesting and dewatering by centrifugation, the biomass was spray dried at Algafarm (Pataias, Portugal) and marketed by Allmicroalgae – Natural Products® (Lisbon, Portugal).

SPAROS LDA (Olhão, Portugal) performed the extrusion treatment (hereafter mentioned as pre-treatment or extruded alga) of the microalga and manufactured the experimental feeds. The product from Allmicroalgae was passed through a twin-screw extruder after mixing with wheat meal, and the details about the pre-treatment of the microalga as well as feed manufacturing process of the experimental diets is reported in Gong et al. (2020). The dried extruded pellets were ground and mixed with the other feed ingredients before extruding again through the same twin-screw extruder that is mentioned above.

2.2. Fish and feeding

2.2.1. Laboratory study

The laboratory study was performed using Atlantic salmon that were obtained from Salten Smolt AS (Breivik, Bodø, Norway) on 7th May 2019. The fish were maintained at the Nord University Research Station until 11th July. The fish were fed EWOS micro 40 (Cargill, Halsa, Norway) during the holding period. On 11th July 2019, the fish was weighed and distributed to the experimental units; 540 fish with initial

Table 1
Ingredients (%) used in the experimental feeds.

Ingredients	CTRL	NE7.5	NE15	NN15	CT*	NW*
Fishmeal 70 LT[1]	10.00	6.60	5.00	5.00	15.00	10.00
Nannochloropsis extruded[2]		7.50	15.00			
Nannochloropsis un-extruded[2]				15.00		7.50
Potato protein concentrate[3]	12.00	12.00	11.50	11.50		
Soy protein concentrate[4]	12.00	12.00	11.50	11.50	15.00	15.00
Pea protein concentrate[5]	12.00	12.00	11.50	11.50	10.00	10.00
Corn gluten[6]	8.50	8.50	8.37	8.37	5.00	5.00
Wheat gluten[7]	7.00	7.00	7.00	7.00	12.00	13.60
Wheat meal[8]	14.00	10.67	7.27	7.27	15.30	11.65
Fish oil[9]	9.65	9.25	8.80	8.80	7.00	7.00
Rapeseed oil[10]	9.65	9.25	8.80	8.80	16.00	15.30
Soy Lecithin[11]	0.50	0.50	0.50	0.50		
Vitamin & Mineral Premix[12]	1.00	1.00	1.00	1.00	1.00	1.00
Monocalcium phosphate[13]	2.08	2.08	2.08	2.08	1.50	1.70
L-histidine[14]	0.10	0.10	0.10	0.10	0.10	0.10
L-threonine[15]	0.20	0.30	0.30	0.30	0.25	0.25
L-tryptophan[16]	0.10	0.13	0.16	0.16	0.10	0.10
L-lysine					0.50	0.55
DL-methionine[17]	0.40	0.40	0.40	0.40	0.10	0.10
Yttrium oxide[18]	0.02	0.02	0.02	0.02		
ZEOFeed[19]	1.00	1.00	1.00	1.00	1.00	1.00
Antioxidant					0.11	0.11
Carophyll Pink 10%					0.04	0.04
Proximate composition (Analyzed)						
Dry matter (g/kg dry matter)	941	895	949	939	943	948
Crude protein	489	490	491	489	492	487
Crude lipid	229	202	215	219	233	231
Ash	68	86	102	104	98	93
Carbohydrate	214	222	192	188	177	189
Gross energy (MJ/kg)	24.2	25.2	22.6	23.6	23.3	23.6

CTRL: Plant-based control feed; NE7.5: Extruded *N. oceanica* 7.5% feed; NE15: Extruded *N. oceanica* 15%; NN15: Un-extruded *N. oceanica* 15%.

* Feeds used in the farm trial- CT: Plant-based control feed; NW: whole *N. oceanica* 7.5%.

[1] Norvik 70: 70.3% crude protein (CP) 5.8% crude fat (CF); Sopropeche, France.

[2] Allmicroalgae: *Nannochloropsis*: 2.8% moisture, 36.6% CP, 14.3% CF, 9.4% fiber, 22.8% ash, 17.5 KJ g-1 energy, 2.1% lysine and 0.9% methionine, Lisbon, Portugal.

[3] Prostar: CP: 81%, CF: 3.1%; Avebe, The Netherlands.

[4] Soycomil P: 63% CP, 0.8% CF; ADM, The Netherlands.

[5] NUTRALYS F85F: 78% CP, 1% CF; Roquette Frères, France.

[6] Corn gluten meal: 61% CP, 6% CF; COPAM, Portugal.

[7] VITEN®: 80% CP, 7.5% CF; Roquette Frères, France.

[8] Wheat meal: 11.7% CP, 1.6% CF; Casa Lanchinha, Portugal.

[9] SAVINOR UTS, Portugal.

[10] Henry Lamotte Oils GmbH, Germany.

[11] Lecico P700IPM; LECICO GmbH, Germany.

[12] PREMIX, Premix especialidades agrícolas e pecuárias, Lda, Portugal. Vitamins (IU or mg/kg feed): DL-alpha tocopherol acetate, 100 mg; Sodium menadione bisulphate, 25 mg; Retinyl acetate, 20,000 IU; DL-cholecalciferol, 2000 IU; Thiamin, 30 mg; Riboflavin, 30 mg; Pyridoxine, 20 mg; Cyanocobalamin, 0.1 mg; Nicotinic acid, 200 mg; Folic acid, 15 mg; Ascorbic acid, 1000 mg; Inositol, 500 mg; Biotin, 3 mg; Calcium panthotenate, 100 mg; Choline chloride, 1000 mg; Betaine, 500 mg. Minerals (g or mg/kg feed): Cobalt carbonate, 0.65 mg; Copper sulphate, 9 mg; Ferric sulphate, 6 mg; Potassium iodide, 0.5 mg; Manganese oxide, 9.6 mg; Sodium selenite, 0.01 mg; Zinc sulphate,7.5 mg; Sodium chloride, 400 mg; Calcium carbonate, 1.86 g; excipient Wheat middling.

[13] MCP: 21.8% Phosphorus, 18.4% Calcium; Fosfitalia, Italy.

[14] L-Histidine: 98%; Ajinomoto Eurolysine SAS, France.

[15] THREAMINO®: 98% L-Threonine; Evonik Nutrition & Care GmbH, Germany.

[16] TRYPAMINO®: 98% Tryptophan; Evonik Nutrition & Care GmbH, Germany.

[17] DL-Methionine for Aquaculture™: 99% Methionine; Evonik Nutrition & Care GmbH, Germany.

[18] Sigma Aldrich, USA.

[19] ZeoFeed®, ZEOCEM, Slovak Republic.

Table 2
Fatty acid profile (% of total fatty acids) of the experimental feeds.

Fatty acid %	CTRL	NE7.5	NE15	NN15	CT	NW
C14:0	3.55	3.74	3.45	3.38	3.54	3.47
C16:0	13.83	14.69	13.51	13.41	13.30	14.00
C18:0	3.31	3.41	2.98	2.92	3.02	3.15
C18:2n-6	15.66	15.19	15.19	15.04	11.91	12.35
C18:3n-3	4.85	4.59	4.54	4.49	3.93	3.90
C18:4n-3	1.11	1.07	1.07	1.06	1.29	1.24
C20:5n-3	6.69	6.58	7.92	7.94	7.67	7.19
C22:6n-3	4.16	3.79	3.82	3.87	5.28	4.87
EPA + DHA	10.85	10.38	11.74	11.80	12.95	12.06
ΣSFAs	20.70	21.84	19.94	19.70	19.87	20.57
ΣMUFAs	42.90	42.72	42.70	42.10	46.97	46.98
Σn-3 PUFAs	16.81	16.04	17.34	17.35	19.48	18.46
ΣPUFAs	32.47	31.23	32.53	32.39	31.39	30.81
Σn-3/ Σn-6	1.07	1.06	1.14	1.15	2.82	2.63

Values are expressed as mean value of 2 replicate samples per diet.
SFAs, Saturated fatty acids; MUFAs, Monounsaturated fatty acids (C16:1n-9, C18:1n-9, C18:1n-7 and C20:1n-11in the case of the lab study, C16:1n-7, C18:1n-9, C18:1n-7, C22: 1n-9 in the case of cage study); n-3 PUFAs, Omega-3 polyunsaturated fatty acids; PUFAs, Polyunsaturated fatty acids.

weight 141.8 ± 28.2 g were randomly allocated to the experimental units (n = 27 fish per tank; fish with average weight in the range 300–362 and standard deviation in the range 36–71). The fish were starved for 2 days after transferring to the allocated tanks (5 replicate tanks/treatment) and then switched directly to the experimental feeds, the ingredient composition and the fatty acid profile of which are given in Tables 1 and 2.

The feeding experiment was carried out in a flow-through system. In total, 20 circular fiberglass tanks (800 L) were used for the study. Each tank was supplied with sea water pumped from Saltenfjorden, from a depth of 250 m. During the experiment, water flow rate was maintained at 1000 L per h, and the average temperature and salinity of the rearing water were 8.0 °C and 35 ‰, respectively. Oxygen saturation was always above 95.8% saturation, measured at the outlet water. A 24-h photoperiod was maintained throughout the feeding period. The fish were fed ad libitum using automatic feeders (Arvo Tech, Finland); administered at two time points every day, from 08:00–09:00 and 14:00–15:00 during the 84-day trial. After each feeding, the uneaten feeds were collected from the water drains in a 17 l tank-mounted solid waste collector (Aquatic Eco-Trap, Pentair Aquatic Eco-Systems1, Fl, USA), before they were strained out from the water in a steel wire mesh. Left-over feeds of each experimental tank were collected to determine the feed intake. A recovery test was performed without fish in the tanks to obtain the correction factor of dry matter loss while determining the feed intake, as described in our previous publication (Gong et al., 2019).

2.2.2. Farm study

The farm study was carried out at GIFAS (Inndyr, Nordland) site Langholmen, in small-scale net pens (5x5x5 m), where environmental conditions are comparable to those in commercial-scale salmon farms. For the 197-day feeding study, 6 net pens were used, i.e., 3 net pens per diet. Each net pen was stocked with 80 salmon of average weight 1.83 kg. Feed was delivered by hand and to satiation. Uneaten pellets, collected from the underwater funnels, were counted to determine the feed consumption. The net pens were inspected daily to collect and register the weight of the dead fish. The trial ended when the final weight of the fish was double that of the initial weight.

2.3. Fish sampling and data collection

At the beginning and end of the post smolt experiment, all fish (540) were individually weighed, and their lengths were recorded. Before handling, fish were anesthetized using tricainemethanesulfonate (MS 222, 140 mg/L). At the termination of the experiment, six fish per tank were pooled to assess the final chemical composition. These fish were packed in plastic bags, immediately frozen and kept at −40 °C until analyses. Feces were collected from the remaining fish in the tanks. Fecal matter, collected from individual fish by stripping, were pooled to obtain enough material for chemical analysis.

As for the farm trial samples, 6 fish per pen were employed for determining the proximate chemical composition, energy and fatty acid profile. The Norwegian Quality Cut parts from 8 fish per cage were collected for analysing the product quality (astaxanthin and lipid content) using the PhotoFish (AkvaGroup AS, Bryne, Norway). Visual evaluation of fillet color was performed using the Roche SalmoColour FanTM (DSM Nutritional Products Ltd., Basel, Switzerland). Texture (force and area associated with fillets) was analyzed by TA-XT2 texture analyser (Stable Micro System, Surrey, UK) using a 60° steel blade that traversed through 90% of the fish at a speed of 1 mm s^{-1}. Texture was analyzed on duplicate blocks of NQC part (32 × 32 × 15 mm, length×width×height), prior to onset of rigor, according to the description of Johnsen et al. (2011).

2.3.1. Gene expression analysis

Distal intestine was dissected out and transferred to cryotubes. These cryotubes were frozen in liquid nitrogen, and later stored at −80 °C. Standard protocols for RNA extraction, quality and quantity check and quantification of mRNA levels were followed, as reported previously (Sørensen et al., 2021). The normalization factor was calculated based on the relative quantities of the two most stable genes, *ef1ab* and *rnap2* (Vasanth et al., 2015). The mRNA levels of antioxidant-related (*superoxide dismutase1, sod1*; *nuclear factor erythroid 2-related factor 2, nrf2*), gut mucosa-related (*immunoglobulin, igt*), inflammation-related (*interleukin 17d, il17d*; *transforming growth factor beta—tgfb*, *annexin 1* and *2*, *anxa1*, *anxa2*), antimicrobial (*cathelicidin 1* and *2, cath1*, *cath2*; *galectin* 3, *gal3*), transporter protein (neutral amino acid transporter, *slc6a19*), mucin-related (*muc5b*) and β-glucan receptor (*sclra* and *cr3*), genes were assessed in this study.

2.3.2. Histomorphometric analysis of the distal intestine

After understanding the differences in expression of the above-mentioned genes, we wanted to know if the alga can develop intestinal inflammation in market size fish. Sections of the anterior segment (as rings) of the distal intestine (around 3 cm in length) were dissected from 5 fish per tank (n = 15 per diet). The contents of the distal intestine were flushed out by rinsing with 10% neutrally buffered formalin (NBF). The sections were then fixed with 10% NBF for 24 h at room temperature at the GIFAS research station. The subsequent histological steps were undertaken at the histology laboratory of the Research station located at Mørkvedbukta, Nord University, Bodø, Norway. Fixed tissues were cut (approximately 0.5 cm in thickness) longitudinally and processed with increasing concentration of ethanol, followed by xylene bath. The tissues were then embedded in paraffin (Sørensen et al., 2017). For each sample collected from a fish, a tissue section of 4 μm in thickness was prepared using Leica microtome. The prepared slides were air dried for at least 24 h before they were stained with Alcian blue - periodic acid–Schiff (AB-PAS) at pH 2.5. Photomicrographs at 40× magnification were generated by a camera (Leica MC170HD, Heersbrugg, Switzerland) fitted on a light microscope (Leica DM1000, Wetzlar, Germany) and by using a software, Leica Application Suite (LAS V4.12.INK, Heerbrugg, Switzerland). An open source image analysis software, ImageJ 1.53e (Schneider et al., 2012) was used to generate quantitative and semi-quantitative data from all the photomicrographs.

For the histomorphometric analysis, along the longitudinal axis of the distal intestine, 5 different locations, within the same intestinal zone, were selected to get 5 well-oriented villi. The ImageJ tools ('straight' and 'segmented lines') were employed to quantify the following histomorphometric indices: thickness of serosa layer (SA); thickness of tunica muscularis layer (ML); thickness of sub mucosa (SM); height of intestinal villi (FH); width of intestinal villi (WV); height of enterocytes (HE); and width of lamina propria (LP). Based on the description in previous publications (Baeverfjord and Krogdahl, 1996; Bakke-McKellep et al., 2007; Knudsen et al., 2008; Silva et al., 2015; Urán et al., 2008a; Urán et al., 2008b; Urán et al., 2008c; Urán et al., 2009), a semi-quantitative ordinal scoring strategy (from 1 to 4) for the following indices was adopted to study the morphological changes induced by the diets: NC, number of mucous cells per distinct villi area; IL, number of intra-epithelial lymphocytes per distinct villi area; SV, score for the presence of supra nuclear vacuoles in the enterocytes of intestinal villi (Supplementary Table 1).

2.3.3. Chemical analyses

The fish samples from each tank/pen were homogenized using an industrial food processor (Foss Tecator, 2096 homogenizer, Hilleroed, Denmark). The whole body samples as well as fecal samples were freeze-dried (VirTis benchtop, Warminster, PA, USA) for 72 h prior to the chemical analysis.

The fish, experimental feeds and freeze-dried feces were finely ground by mortar and pestle and homogenized prior to analyses of dry matter (105 °C for 20 h; ISO 6496:1999), crude protein (Kjeldahl Auto System, Tecator Systems, Höganäs, Sweden; ISO 5983:1987), ash (incineration in a muffle furnace at 540 °C for 16 h; ISO 5984:2002) and energy (IKA C200 bomb calorimeter, Staufen, Germany; ISO 9831:1998). Yttrium in both feces and feeds from the post smolts experiment was analyzed by employing inductive coupled plasma mass spectroscopy (ICP-MS) by Eurofins (Moss, Norway; NS-EN ISO 11885). All the samples were analyzed in duplicate. Total lipid content of the fish was determined by ethyl-acetate extraction method. Total lipid content of the feed and feces was analyzed employing the Soxhlet method with acid hydrolysis (Soxtec HT 6209, Tecator, Höganäs, Sweden; modified AOAC method 954.020), by Eurofins® (Moss, Norway). Fatty acid profile of fish and feed was measured by gas chromatography (GC) of methyl-ester derivatives of the fatty acids of the lipids extracted from the samples. For this, the homogenized samples were lyophilized for 72 h before the lipids were extracted and analyzed in duplicate. Total lipid from the fish for fatty acid analysis was extracted according to the method of Bligh and Dyer (1959). The fatty acid methyl esters (FAMEs) were prepared according to the AOCS Official Method Ce 1b-89. FAMEs were separated and quantitated using a Scion 436 GC equipped with a flame ionization detector, a splitless injector and a DB-23 column (Agilent Technologies, Santa Clara, USA).

Standard mixtures of FAMEs were used for identification and quantitation of common fatty acids in samples (FAME MIX 2/ GLC-473, Nu-Chek Prep, Elysian, MN, USA). The quantification was performed using the relative percentage area of the total FA using Compass CDS, Bruker Co-operation software.

2.4. Formulae for deriving feed and fish growth performance indices

Fish growth performance was assessed based on different indices, derived employing the following equations:

$$\text{Condition factor } \left(\text{g/cm}^3\right)(\text{CF}) = \frac{\text{W}_\text{f}\ (\text{g})}{\text{FL}^3} \times 100$$

$$\text{Weight gain } (\%)(\text{WG}) = \left(\frac{\text{W}_\text{f} - \text{W}_\text{i}}{\text{W}_\text{i}}\right) \times 100$$

$$\text{Specific growth rate } \left(\%\text{day}^{-1}\right)\ (\text{SGR}) = \left(\frac{\text{Ln}\ (\text{W}_\text{f}) - \text{Ln}\ (\text{W}_\text{i})}{\text{d}}\right) \times 100$$

$$\text{Thermal growth coefficient (TGC)} = \frac{(W_f)^{1/3} - (W_i)^{1/3}}{(T \times d)} \times 1000$$

$$\text{Feed conversion ratio (FCR)} = \frac{\text{Total feed intake in dry basis (g)}}{\text{Weight gain (g)}}$$

$$\text{Protein efficiency ratio (PER)} = \frac{\text{Weight gain (g)}}{\text{Total protein ingested (g)}}$$

where, W_f = final body weight of fish (g/fish), W_i = initial body weight of fish (g/fish), T = rearing temperature in °C and d = feeding days, FL = fork length of fish (cm).

Apparent Digestibility Coefficient (ADC) of nutrients and dry matter were calculated according to following equations:

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

$$ADC_{dry\ matter} = \left[1 - \left(\frac{Marker_{feed}}{Marker_{faeces}}\right)\right] \times 100$$

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

$$\text{Nutrient (or Energy) retention}(\%) = \frac{(W_f \times N_f(\text{or } E_f) - W_i \times N_i(\text{or } E_i)}{\text{NI (or EI)}} \times 100$$

where N_f = final nutrient content of the body; N_i = initial nutrient content of the body, E_i = initial energy content of the body, E_f = final energy content of the body, NI = nutrient intake or EI = energy intake. Retention of a digested nutrient was calculated based on values for each tank.

$$\text{Nutrient (or Energy) retention efficiency}_{\text{digested}}(\%) = \frac{\text{Nutrient (or Energy) retention}(\%)}{\text{ADC}(\%)} \times 100$$

2.5. Statistical analysis

Statistical analyses were performed using SPSS 19.0 software and R-4.0.2. The data were tested for normality (Shapiro–Wilk normality test) and equality of variance (Levene's test. All data from the tank experiment were analyzed using one-way analysis of variance (ANOVA). Thereafter, Duncan's multiple comparison test was used to identify the significant differences among the means of the experimental groups. In the case of non-parametric data, first we transformed the data and checked the assumptions. If the transformed data did not follow both the assumptions, Kruskal-Wallis and Dunn's test were employed to analyse the data. When the homogeneity of variance assumption alone was violated, Welch one-way test followed by Games-Howell post-hoc test was employed to understand the differences between the groups.

The effect of diet in the farm study was analyzed by either parametric unpaired two-samples *t*-test or non-parametric Wilcoxon test. For all the data, except the histomorphometric analysis and general fillet quality characteristics data, from the farm trial, pen was used as the experimental unit. A dimension reduction method Uniform manifold approximation and projection, UMAP, was also employed to understand the differences (based on Canberra distance (Faisal et al., 2020)) between the histomorphometric parameters of the fish groups in the farm study.

In both experiments, a significance level of $P < 0.05$ was chosen to indicate the differences.

3. Results

3.1. Growth and feed performance

The post smolts had good growth during the laboratory trial and the final weights increased by 123%–157% compared to the start weights (Table 3a). There were significant differences in the final weights, SGR and TGC of the diet groups. Weight gain ($P = 0.001$), SGR ($P = 0.002$) and TGC ($P = 0.001$) were significantly higher in fish fed the CTRL compared to fish fed the alga incorporated feeds. There were no differences between the alga-fed groups. The FCR ($P = 0.001$) of the fish fed the CTRL and NE7.5 did not differ significantly, and the feed utilization was improved in these two groups compared to the NE15 and NN15 groups. There were no differences in feed utilization between the groups fed the high incorporation level. There were no differences between the PER of the fish fed the CTRL and the NE7.5 diets, but these values were higher ($P < 0.001$) compared to the groups fed the NE15 and NN15 diets. There were no differences between the PER of the high level of the alga fed groups. The CF of the CTRL group was significantly higher compared to the NN15 group, while the CF of the two groups fed NE7.5 and NE15 ranked in between.

The results from the farm study indicated that the growth performance of the fish fed the NW diet was similar to that of the fish fed the control diet (Table 3b). However, the statistical analyses detected significant differences and decreasing trends; in the case of feed conversion ratio and the protein efficiency ratio, respectively.

3.2. Chemical composition

The proximate composition and energy content of whole body of fish from the tank study is presented in Table 4a. The moisture content ($P < 0.05$) and protein content ($P < 0.05$) were higher in fish fed the NN15 diet compared to the other groups, which were not significantly different between them (Table 4a). Lipid content was lower for fish fed the NE7.5 diet compared to the other groups. We did not find any differences in the ash and energy content of the diet groups (Table 4a). Although the numerical values of the proximate composition of fish fed the NW diet, from the farm study, were lower compared to the control group, we did not observe any significant differences between the values (Table 4b).

The fatty acid profile of the flesh from the post-smolts reared in tanks

Table 3a
Growth performance and feed utilization of Atlantic salmon employed in the laboratory study.

	CTRL	NE7.5	NE15	NN15	ANOVA P-value
IBW (g)	141.84 ± 0.08	142.01 ± 0.34	141.69 ± 0.43	141.68 ± 0.16	0.832
FBW (g)	356.51 ± 5.53^{b}	328.82 ± 6.88^{a}	316.29 ± 4.75^{a}	315.62 ± 6.98^{a}	0.001
WG (%)	151.34 ± 3.89^{b}	131.57 ± 5.09^{a}	123.26 ± 3.8^{a}	122.79 ± 5.13^{a}	0.001
SGR (% day $^{-1}$)	1.10 ± 0.02^{b}	1.00 ± 0.03^{a}	0.96 ± 0.02^{a}	0.95 ± 0.03^{a}	0.002
TGC	2.79 ± 0.05^{b}	2.50 ± 0.07^{a}	2.38 ± 0.06^{a}	2.37 ± 0.07^{a}	0.001
FCR	0.73 ± 0.02^{a}	0.75 ± 0.01^{a}	0.85 ± 0.02^{b}	0.85 ± 0.03^{b}	0.001
PER	2.97 ± 0.07^{b}	3.03 ± 0.05^{b}	2.54 ± 0.05^{a}	2.56 ± 0.08^{a}	< 0.001
CF	1.48 ± 0.01^{b}	1.44 ± 0.01^{ab}	1.42 ± 0.02^{ab}	1.41 ± 0.02^{a}	0.044

IBW: Initial body weight; FBW: Final body weight; WG: Weight gain; SGR: Specific growth rate; TGC: Thermal growth coefficient; FCR: Feed conversion ratio; PER: Protein efficiency ratio; CF: Condition factor.
Values are expressed as mean ± SEM (n = 5 replicate tanks). Values in the same row with different superscript letters indicate significant differences ($P < 0.05$).

Table 3b
Growth performance and feed utilization of Atlantic salmon, farm study.

	CT	NW	P-value
IBW (kg)	1.83 ± 0.01	1.83 ± 0.01	0.836
FBW (kg)	4.23 ± 0.11	4.07 ± 0.05	0.268
SGR (% day $^{-1}$)	0.42 ± 0.01	0.40 ± 0.00	0.254
TGC	3.75 ± 0.12	3.56 ± 0.03	0.217
FCR	1.13 ± 0.01^{a}	1.17 ± 0.01^{b}	0.046
PER	1.89 ± 0.02°	1.78 ± 0.04°	0.077
CF	1.32 ± 0.01	1.30 ± 0.01	0.427

IBW: Initial body weight; FBW: Final body weight; WG: Weight gain; SGR: Specific growth rate; TGC: Thermal growth coefficient; FCR: Feed conversion ratio; PER: Protein efficiency ratio; CF: Condition factor.
Values are expressed as mean ± SEM (n = 3 replicate pens). $^{\circ}$ indicates a trend in significant differences (0.05 < P < 0.1).

Table 4a
Proximate composition and energy content in the whole body of Atlantic salmon reared in tanks (laboratory study).

	CTRL	NE7.5	NE15	NN15	ANOVA P-value
Moisture (%)	68.46 ± 0.37^{a}	68.89 ± 0.12^{a}	68.49 ± 0.4^{a}	69.89 ± 0.28^{b}	0.017
% of dry matter					
Protein	55.93 ± 0.92^{a}	55.75 ± 0.47^{a}	56.62 ± 0.8^{a}	58.7 ± 0.46^{b}	0.031
Lipid	37.85 ± 1.24^{b}	34.53 ± 0.53^{a}	37.47 ± 0.81^{b}	38.21 ± 0.74^{b}	0.032
Ash	6.37 ± 0.18	6.88 ± 0.16	6.73 ± 0.21	6.76 ± 0.13	0.237
Energy (kJ/g)	26.04 ± 0.19	25.93 ± 0.13	25.93 ± 0.10	26.19 ± 0.27	0.730

Values are expressed as mean ± SEM (n = 5 replicate tanks).
Values in the same row with different superscript letters indicate significant differences (P < 0.05).

Table 4b
Proximate composition and energy content in the whole body of Atlantic salmon reared in the farm pens (farm study).

	CT	NW	P-value
Moisture (%)	65.64 ± 0.27	65.56 ± 0.49	0.891
% of dry matter			
Protein	51.16 ± 0.52	50.77 ± 0.53	0.636
Lipid	47.98 ± 0.80	46.60 ± 0.57	0.229
Ash	5.55 ± 0.10	5.94 ± 0.19	0.141
Energy (kJ/g)	26.84 ± 0.59	26.71 ± 1.41	0.938

Values are expressed as mean ± SEM (n = 3 replicate pens).

is given in Table 5a. Sum saturated fatty acids, dominated by the C16:0, was significantly higher in fish fed the CTRL diet, while no differences were observed in the values of the fish fed the microalga-incorporated diets. Significant differences were also noted in the MUFA (dominated by C18:1n-9, oleic acid) as well as in the n-6 PUFA (dominated by C18:2n-6, linoleic acid), showing slightly higher relative levels in fish fed the CTRL and NE7.5 than those fed NE15 and NN15. On the other hand, the n-3 fatty acids, sum PUFAs as well as EPA + DHA were lower in fish fed the CTRL and we detected significantly higher values in fish fed the microalga. No significant differences were noted in these values of the flesh of fish fed the microalga-incorporated diets.

Regarding the values from the farm study, we observed a significant difference only in the palmitoleic acid (C16:1n-9); this fatty acid had lower levels in the fish fed the NW diet. Similarly, there was a decreasing trend in the case of gadoleic acid (C20:1n-11). The higher numerical values of EPA and DHA in the fish fed the NW diet are noteworthy, based on the values of the fatty acids/% of total fatty acids and g/100 g fillet

Table 5a
Fatty acid profile (% of total fatty acids) in the fillets of Atlantic salmon fed the experimental feeds.

Fatty acid %	CTRL	NE7.5	NE15	NN15	ANOVA P-value
SFAs					
C14:0	2.81 ± 0.02	2.78 ± 0.02	2.77 ± 0.02	2.81 ± 0.02	0.288
C16:0	13.10 ± 0.1^{b}	12.15 ± 0.12^{a}	12.25 ± 0.07^{a}	12.33 ± 0.11^{a}	< 0.001
C:18	3.33 ± 0.06^{b}	2.94 ± 0.06^{a}	2.85 ± 0.02^{a}	2.86 ± 0.04^{a}	< 0.001
C22:0	1.54 ± 0.03^{c}	1.86 ± 0.09^{a}	1.73 ± 0.03^{ab}	1.66 ± 0.06^{bc}	< 0.001
ΣSFAs	20.78 ± 0.14^{b}	19.73 ± 0.12^{a}	19.61 ± 0.07^{a}	19.66 ± 0.15^{a}	< 0.001
MUFAs					
C16:1n-9	3.55 ± 0.02^{a}	3.74 ± 0.02^{b}	3.88 ± 0.01^{c}	4.06 ± 0.02^{d}	< 0.001
C18:1n-9	34.82 ± 0.18^{c}	34.35 ± 0.15^{b}	33.75 ± 0.11^{a}	33.34 ± 0.18^{a}	< 0.001
C18:1n-7	3.21 ± 0.01	3.20 ± 0.01	3.20 ± 0.01	3.20 ± 0.01	0.789
C20:1n-11	2.64 ± 0.10	2.72 ± 0.13	2.57 ± 0.09	2.84 ± 0.08	0.301
ΣMUFAs	44.23 ± 0.14^{b}	44.03 ± 0.14^{b}	43.41 ± 0.14^{a}	43.44 ± 0.2^{a}	0.001
n-6 PUFAs					
C18:2n-6	12.91 ± 0.09^{b}	12.90 ± 0.07^{b}	12.67 ± 0.05^{a}	12.57 ± 0.05^{a}	< 0.001
C20:2n-6	0.91 ± 0.01^{b}	0.80 ± 0.01^{a}	0.83 ± 0.01^{a}	0.83 ± 0.01^{a}	< 0.001
Σn-6 PUFAs	13.82 ± 0.08^{b}	13.78 ± 0.08^{b}	13.51 ± 0.04^{a}	13.40 ± 0.05^{a}	< 0.001
n-3 PUFAs					
C18:3 n-3	3.79 ± 0.03^{b}	3.74 ± 0.02^{b}	3.65 ± 0.02^{a}	3.61 ± 0.04^{a}	< 0.001
C18:4 n-3	0.92 ± 0.01^{a}	1.13 ± 0.03^{c}	1.09 ± 0.01^{c}	1.04 ± 0.02^{b}	< 0.001
C20:4n-3	0.78 ± 0.01	0.77 ± 0.01	0.77 ± 0.01	0.77 ± 0.00	0.867
C20:5n-3	4.04 ± 0.06^{a}	4.26 ± 0.06^{a}	4.55 ± 0.05^{b}	4.66 ± 0.13^{b}	< 0.001
C22:5n-3	1.46 ± 0.01^{a}	1.41 ± 0.03^{a}	1.52 ± 0.00^{b}	1.57 ± 0.01^{b}	< 0.001
C22:6n-3	8.57 ± 0.14^{a}	9.93 ± 0.15^{b}	9.76 ± 0.14^{b}	9.78 ± 0.26^{b}	< 0.001
Σn-3 PUFAs	19.54 ± 0.2^{a}	21.23 ± 0.15^{b}	21.35 ± 0.19^{b}	21.42 ± 0.4^{b}	< 0.001
ΣPUFAs	33.36 ± 0.28^{a}	35.01 ± 0.12^{b}	34.86 ± 0.23^{b}	34.82 ± 0.39^{b}	0.002
EPA + DHA	12.60 ± 0.17^{a}	14.19 ± 0.16^{b}	14.31 ± 0.18^{b}	14.44 ± 0.38^{b}	< 0.001
Σn-3/Σn-6	1.41 ± 0.01^{a}	1.54 ± 0.01^{b}	1.58 ± 0.01^{b}	1.60 ± 0.02^{b}	< 0.001

SFAs, Saturated fatty acids; MUFAs, Monounsaturated fatty acids; n-6 PUFAs, Omega-6 polyunsaturated fatty acids; n-3 PUFAs, Omega-3 polyunsaturated fatty acids; PUFAs, Polyunsaturated fatty acids.
Values are expressed as mean ± SEM (n = 5 replicate tanks). Values in the same row with different superscript letters indicate significant differences (P < 0.05).

(Table 5b).

3.3. Digestibility

The ADCs of dry matter, protein, lipid, ash and energy in the four feeds are presented in Table 6. The ADCs of DM in the CTRL and NE7.5 diet were significantly higher compared to the NE15 and NN15 groups. The lowest digestibility was observed in the NN15 group. Protein digestibility differed significantly among all four feed groups; the highest

Table 5b
Fatty acid profile (% of total fatty acids) and fatty acid content in the fillets of Atlantic salmon fed the experimental feeds in the farm trial.

	CT	NW	P-value	CT*	NW*	P-value
SFAs						
C14:0	2.52 ± 0.01	2.29 ± 0.03	0.634	0.35 ± 0.00	0.31 ± 0.00	0.100
C16:0	11.80 ± 0.03	11.9 ± 0.16	0.854	1.65 ± 0.00	1.63 ± 0.02	0.100
C18:0	3.04 ± 0.02	3.03 ± 0.05	0.414	0.42 ± 0.00	0.42 ± 0.01	0.365
C22:0	0.88 ± 0.02	0.84 ± 0.01	0.400	0.12 ± 0.00	0.11 ± 0.00	0.100
ΣSFAs	18.18 ± 0.08	18.07 ± 0.24	0.680	2.54 ± 0.01	2.48 ± 0.03	0.1627
MUFAs						
C16:1n-9	3.07 ± 0.02[a]	2.90 ± 0.03[b]	0.014	0.43 ± 0.00[a]	0.40 ± 0.00[b]	0.005
C18:1n-9	37.27 ± 0.4	36.9 ± 0.05	0.549	5.21 ± 0.06	5.06 ± 0.01	0.1586
C18:1n-7	3.44 ± 0.03	3.40 ± 0.04	0.469	0.48 ± 0.00	0.47 ± 0.01	0.108
C20:1n-11	2.77 ± 0.04°	2.67 ± 0.02°	0.108	0.39 ± 0.01[a]	0.37 ± 0.00[b]	0.036
ΣMUFAs	44.46 ± 1.98	35.6 ± 7.43	0.356	6.22 ± 0.28	4.88 ± 1.02	0.318
n-6 PUFAs						
C18:2n-6	11.89 ± 0.12	11.84 ± 0.05	0.772	1.66 ± 0.02	1.62 ± 0.01	0.100
C20:2n-6	0.99 ± 0.01	0.98 ± 0.01	0.405	0.14 ± 0.00	0.13 ± 0.00	0.118
Σn-6 PUFAs	12.88 ± 0.11	12.81 ± 0.05	0.638	1.80 ± 0.02°	1.76 ± 0.01°	0.090
n-3 PUFAs						
C18:3n-3	4.62 ± 0.05	4.45 ± 0.04	0.298	0.65 ± 0.01	0.61 ± 0.00	0.100
C18:4n-3	0.90 ± 0.12	0.71 ± 0.09	0.285	0.13 ± 0.02	0.10 ± 0.01	0.253
C20:4n-3	0.86 ± 0.01	0.81 ± 0.02	0.145	0.12 ± 0.00°	0.11 ± 0.00°	0.079
C20:5n-3	4.52 ± 0.05	4.66 ± 0.13	0.403	0.63 ± 0.01	0.64 ± 0.02	0.776
C22:5n-3	1.85 ± 0.03	1.83 ± 0.04	0.680	0.26 ± 0.00	0.25 ± 0.01	0.272
C22:6n-3	7.37 ± 0.06°	7.95 ± 0.20°	0.087	1.03 ± 0.01	1.09 ± 0.03	0.151
Σn-3 PUFAs	19.99 ± 0.18	20.42 ± 0.45	0.449	2.80 ± 0.03	2.80 ± 0.06	0.980
PUFAs	32.87 ± 0.09	33.23 ± 0.41	0.470	4.60 ± 0.01	4.55 ± 0.06	0.518
EPA + DHA	11.89 ± 0.05	12.61 ± 0.32	0.148	1.66 ± 0.01	1.73 ± 0.04	0.272
Σn-3/Σn-6	12.88 ± 0.11	12.81 ± 0.05	0.638	1.80 ± 0.02°	1.76 ± 0.01°	0.090

Values are expressed as mean ± SEM (n = 3 replicate pens, 18 fish/group). ° indicates a trend in significant difference (0.05 < P < 0.1). Values in the same row with different superscript letters indicate significant differences (P < 0.05). *Fatty acid in g/100 g fillet was calculated using the formula given in the guidelines of FAO (FAO, 2012). The equation is (% of a particular fatty acid in total fatty acids/100) x (lipid in g/100 g fillet x fatty acid conversion factor). Fatty acid conversion factor = 0.933 - (0.143/lipid in g/100 g fillet).

digestibility was noted for the CTRL diet, and the digestibility gradually decreased with the alga incorporation, with the lowest value for the NN15 diet. In addition, extrusion of *Nannochloropsis* impacted protein digestibility; higher values were noted for the NE15 group compared to the NN15 group. The ADC of lipid was significantly affected by the alga incorporation. The digestibility was the highest for the fish fed the CTRL diet (89%) and the value gradually decreased with the incorporation of the pre-treated microalga, reaching 79% in the fish that received the NE15 diet (P < 0.001). The lipid digestibility of the fish fed the NN15 was not different than those fed the NE7.5 diet. Furthermore, extrusion reduced the lipid digestibility, as evidenced by the values of the NE15 and NN15. The changes in the ADC of energy had the same trend as lipid digestibility, with the highest ADC for fish fed the CTRL diet and we observed a reduction in energy digestibility with the incorporation of pre-treated microalga (P < 0.001). There were no differences between the energy digestibility of the NE15 and NN15 groups. The ADC of ash was the lowest for the fish fed the CTRL diet (87.43%, P < 0.001). There were no differences in ash digestibility among the alga fed groups.

Table 6
Apparent digestibility coefficients (%) of dry matter, protein, lipid and energy in different experimental feeds.

	CTRL	NE7.5	NE15	NN15	ANOVA P-value
Dry matter	66.37 ± 0.3[c]	65.78 ± 0.42[c]	61.8 ± 0.47[b]	59.57 ± 0.48[a]	<0.001
Protein	85.12 ± 0.21[d]	83.71 ± 0.35[c]	81.3 ± 0.41[b]	80.21 ± 0.08[a]	<0.001
Lipid	88.87 ± 0.33[c]	82.13 ± 0.84[b]	78.94 ± 0.3[a]	81.18 ± 0.45[b]	<0.001
Ash	87.43 ± 0.16[a]	91.19 ± 0.20[b]	90.79 ± 0.16[b]	90.76 ± 0.09[b]	< 0.001
Energy	80.86 ± 0.16[c]	79.68 ± 0.24[b]	72.97 ± 0.51[a]	73.89 ± 0.29[a]	<0.001

Values are expressed as mean ± SEM (n = 5 replicate tanks).
Values in the same row with different superscript letters indicate significant differences (P < 0.05).

3.4. Protein, lipid and energy retention

The retention of protein, lipid and energy in the fish differed among the experimental diets (Table 7). Retention of protein as well as protein retention efficiency were similar in the fish fed CTRL and NE7.5 diets, and the values were significantly higher than those of the fish fed the NE15 and NN15 diets. No differences were noted in the protein retention in the high level-alga fed groups. The retention of lipid (gross) showed a linear decrease; from the CTRL to NN15 (P = 0.001) groups. The highest retention was noted for the CTRL diet, followed by the NE7.5 diet. The lipid retention of NN15 diet was significantly lower than that of the CTRL and NE7.5 diets, but not significantly different from that of the NE15 diet. The lipid retention efficiency of NN15 was significantly lower

Table 7
Retention and retention efficiency (%) of lipid, protein and energy in Atlantic salmon fed the experimental feeds.

Parameter	CTRL	NE7.5	NE15	NN15	ANOVA P-value
Gross (retention)					
Protein	52.50 ± 1.15[b]	52.15 ± 0.88[b]	45.98 ± 1.00[a]	45.41 ± 1.38[a]	< 0.001
Lipid	77.02 ± 1.69[c]	74.04 ± 1.38[bc]	69.27 ± 1.46[ab]	65.21 ± 2.06[a]	0.001
Energy	53.52 ± 1.10[c]	51.63 ± 0.68[bc]	49.63 ± 0.93[b]	45.05 ± 1.29[a]	< 0.001
Digested (retention efficiency)					
Protein	62.97 ± 0.41[b]	62.37 ± 1.37[b]	56.62 ± 1.32[a]	56.51 ± 2.22[a]	0.011
Lipid	88.49 ± 0.86[b]	90.28 ± 2.85[b]	87.90 ± 2.19[b]	80.19 ± 2.90[a]	0.045
Energy	67.49 ± 0.44[b]	64.83 ± 1.07[ab]	68.14 ± 1.37[b]	60.83 ± 2.03[a]	0.010

Values are expressed as mean ± SEM (n = 5 replicate tanks).
Values in the same row with different superscript letters indicate significant differences (P < 0.05).

than the values in the other groups. The differences in retention of energy followed the same pattern as gross lipid. The energy retention efficiency was significantly lower (60) for the fish fed NN15 than CTRL (67) and NE15 (68), while the value of the fish fed NE7.5 ranged in between (64).

3.5. Expression of selected genes in the intestine of Atlantic salmon post-smolts

Although the expression of *sod* decreased with the inclusion of the alga, only the value of the NN15 group was significantly lower compared to the CTRL group (Fig. 1). There were no significant differences in the expression of *nrf2* and *gal3*. In the case of *cath1*, the value of the NN15 group was significantly lower compared to both the CTRL and NE7.5 groups. However, we did not observe any significant difference for *cath2*. The expression of the gene, *il17d*, was significantly lower in the extruded-alga fed groups compared to the CTRL group, but significant difference was noted only for the NE7.5 vs CTRL comparison. The gene *slc6a19* was not significantly affected, but there was an apparently lower expression for the NE15 group. The genes *anxa1* and *anxa2* had lower expression in the alga-fed groups, but significant differences were not detected for the expression of *anxa2*, between the NN15 and NE7.5 groups. Furthermore, while the gene *igt* was not significantly altered, *muc5b* was reduced in the groups fed diets with the alga, and significant differences were noted for the high alga inclusion level fed groups compared to the CTRL group. The expression pattern of *sclra* and *cr3* in the different feed groups were similar; *sclra* was significantly lower in the NE7.5 group compared to the CTRL group and *cr3* was significantly lower in the NE7.5 group compared to both the CTRL and NE15 groups.

3.6. Histomorphometric analysis of the distal intestine of fish from the farm study

The histomorphometric indices in the distal intestine of salmon after being fed the experimental diets for 197 days are presented in Table 8. We found a differential clustering of the two groups, based on dimensionality reduction by considering the Canberra distance (Supplementary Fig. 1). The results showed that the muscle layer thickness, submucosa thickness, villi height, villi width, enterocyte height, supranuclear vacuoles were significantly higher in fish fed the NW diet. A decreasing trend was noted for serosa thickness and lamina propria width in the fish fed the NW diet. The intraepithelial lymphocyte counts were not different between the two groups.

3.7. Product quality of slaughtered fish from the farm study

The visual flesh pigmentation measured with SalmoFan ranged between 25 and 26 and the values associated with the fish fed the two diets were not significantly different (Fig. 2a). The astaxanthin content in the muscle was significantly lower in the fish fed the NW diet (Fig. 2b). No differences were observed for the lipid content in the flesh (Fig. 2c). The fillet textural characteristics (force and area) of the two feeding groups did not differ significantly (Fig. 3).

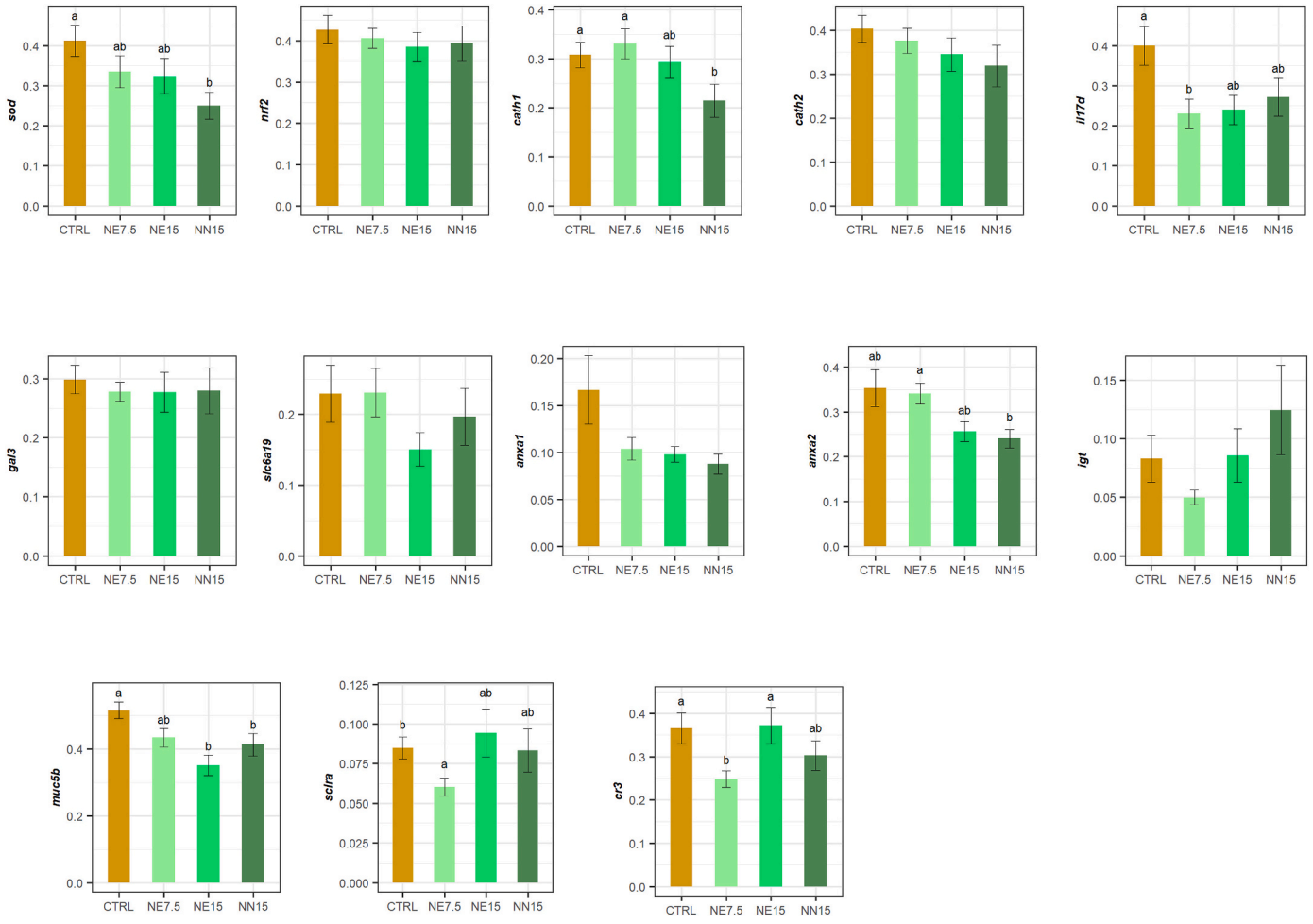

Fig. 1. Relative mRNA levels of selected genes in the intestine of salmon from the tank study. Each bar graph shows mean ± SEM of mRNA levels (n = 20 fish/group) of the gene (y axis title) expressed in the intestine. Different letters above the bars indicate significant differences ($P < 0.05$).

Table 8
Histomorphometric indices measured in the distal intestine of Atlantic salmon.

Indices	CT	NW	P-value
SA (μm)	$16.18 \pm 1.32^{\circ}$	$13.17 \pm 1.15^{\circ}$	0.075
ML (μm)	195.46 ± 14.44^{a}	305.68 ± 15.13^{b}	1.33×10^{-5}
SM (μm)	65.13 ± 5.92^{a}	98.50 ± 7.23^{b}	1.314×10^{-4}
FH (μm)	1460.94 ± 81.04^{a}	2178.51 ± 94.15^{b}	3.345×10^{-6}
WV (μm)	84.40 ± 3.56^{a}	115.83 ± 3.25^{b}	5.802×10^{-7}
HE (μm)	32.88 ± 1.41^{a}	56.92 ± 1.67^{b}	1.126×10^{-11}
LP (μm)	$11.23 \pm 0.75^{\circ}$	$9.51 \pm 0.39^{\circ}$	0.079
NC (score)	2 (1)	3 (1)	0.272
IL (score)	$3\ (0)^{\circ}$	$3\ (1)^{\circ}$	0.089
SV (score)	$3\ (0)^{a}$	$4\ (0)^{b}$	4.0×10^{-4}

CT control diet; NW, diet with the microalga *Nannochloropsis oceania*; SA, thickness of serosa layer; ML, thickness of muscle layer; SM, thickness of sub mucosa; FH, height of villi; WV, width of villi; HE, height of enterocytes; LP, width of lamina propria; NC, number of mucous cells per distinct villi area; IL, number of intraepithelial lymphocytes per distinct villi area; SV, score for supra nuclear vacuoles. Values are presented as mean ± SEM, n = 15 fish per diet group (5 fish/tank). Significant differences ($p < 0.05$) between diet groups are indicated by different superscripts (a or b) on each row after conducting a parametric unpaired two-samples *t*-test or a non-parametric unpaired two-samples Wilcoxon test. $^{\circ}$ indicates a trend in significant difference ($0.05 < P < 0.1$). Median, interquartile range (IQR) is reported for score data.

4. Discussion

Finding replacements to fish meal in the feeds of carnivorous fishes like Atlantic salmon, is the right move towards sustainable management of salmon farming. Hence, researchers are finding evidence to reveal the benefits of new ingredients that can be used in aquafeeds. Microalgae that have high-quality protein with essential amino acids and which are the backbone of the aquatic food web, are good alternative ingredients to fish meal. Hence, we evaluate different microalgae for their suitability in salmon feeds, and in the present study, we examined if pre-treatment of *N. oceanica* could improve their utilization by Atlantic salmon, by conducting laboratory and farm studies on post-smolts and market size fish, respectively.

4.1. Fish performance and feeding efficiency

The performance of the post-smolts, during the experimental period, was in general very good and was slightly better than the results reported by Sørensen et al. (2017), wherein the examined incorporation levels of *N. oceanica* were 10 and 20% in fish meal based feeds for Atlantic salmon. The SGR values were in the same range as those reported in Gong et al. (2019). In the latter report, Gong et al. tested the microalga *Scenedesmus* sp. at 10% and 20% in plant-based diets with a fish meal content in the range of 10% - 2.5% for the control and high microalgae feed, respectively.

The present study showed reduced growth for all alga-fed groups. This result is in line with other studies with Atlantic salmon that tested different incorporation levels of microalgae such as *Phaeodactylum tricornutum*, *Desmodesmus* sp., *N. oceanica* and *Scenedesmus* sp. (Gong et al., 2019; Kiron et al., 2016b; Sørensen et al., 2016; Sørensen et al., 2017). These results suggest that higher incorporation levels such as 15% of specific microalgae in the feeds for salmonids are likely to affect growth and nutrient utilization.

Feed intake and FCR values were higher in fish fed the highest incorporation levels of the microalga, irrespective of pre-treatment; this suggests that the fish compensated the lower energy utilization of the feeds by increased feed intake. Sørensen et al. (2017) have also indicated that there were no palatability issues with the incorporation of this algal ingredient in the feeds for Atlantic salmon. Higher feed intake and lower weight gain explains the higher FCR values for the high incorporation levels.

4.2. Nutrient digestibility and retention and fatty acid profile

The ADC values for protein, lipid and energy were reduced when the microalga was incorporated into the feeds of the post smolts; the macronutrient and energy digestibility values were reduced with increasing inclusion of the microalga. The reduction in protein digestibility of feeds, with more extruded microalga, suggests that the thermomechanical treatment was not good enough to improve the digestibility to the same level as that observed for the CTRL group. But the lower digestibility of the NN15 diet compared to the NE15 diet points to some improvement in protein digestibility by the extrusion processing.

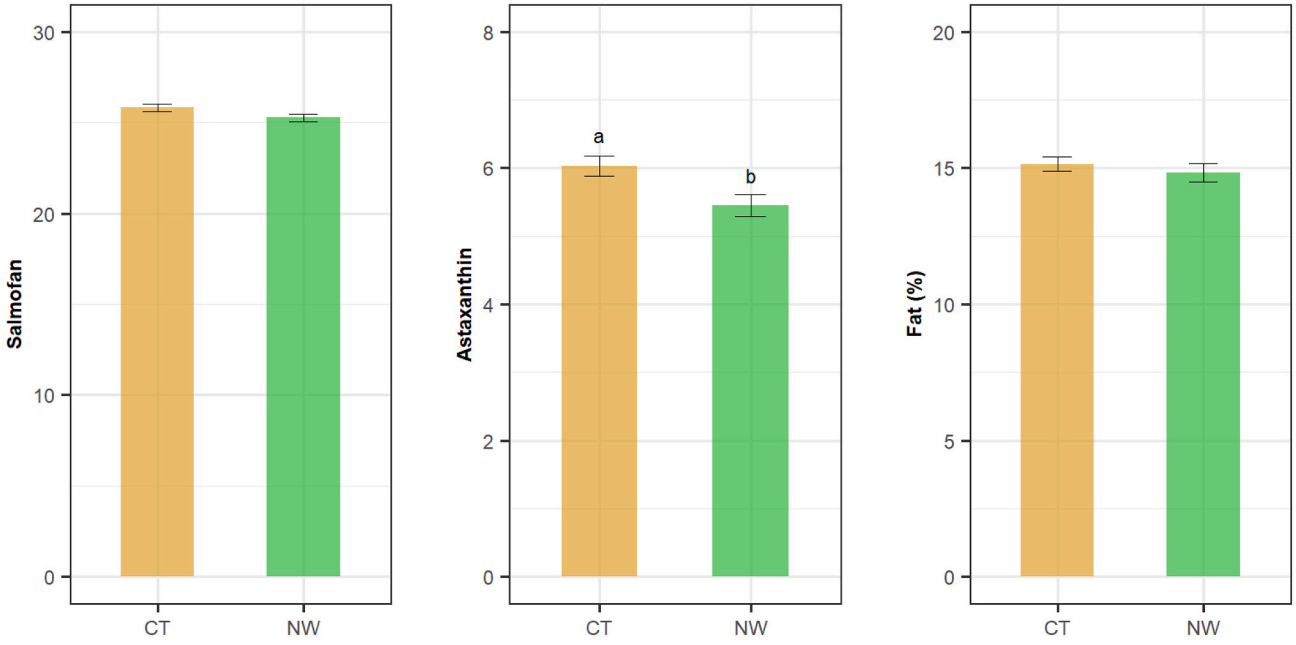

Fig. 2. General quality characteristics of fillet from Norwegian Quality Cut obtained from the experimental fish. (a) visual color (a), (b) astaxanthin concentration and (c) fat % (as is). Different letters above the bars (mean ± SEM, n = 24 fish/diet group) indicate significant differences ($P < 0.05$).

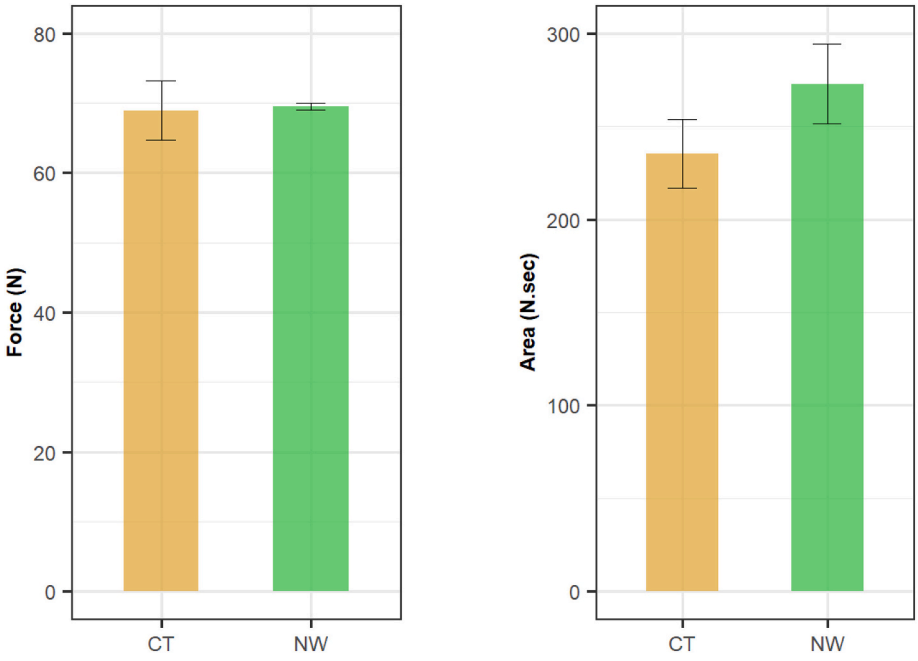

Fig. 3. Texture parameters that indicate the quality of fillet. (a) force, (b) area. Each bar graph shows mean ± SEM (n = 3 tanks/group).

Furthermore, no negative effects were noted for protein digestibility when 10% extruded *N. oceanica* was included into the feeds of Atlantic salmon (Gong et al., 2020). Improved lipid digestibility of the NN15 diet compared to the NE15 diet suggests that lipids were more easily digested in the un-extruded microalga at higher incorporation level. In contrast to this observation, extrusion was recently proven as an efficient pre-treatment technique to extract lipid from *N. oceanica* (Li et al., 2020), and a previous study has reported the values of the PUFAs such as C18:2n-6, C18:3n-3, C20:4n-6 and C20:5n-3 (0.97, 0.50, 0.37 and 2.34% dry weight, respectively) in the alga (Zanella and Vianello, 2020). Increased extractability is usually associated with improved accessibility of nutrients, which is positively correlated with digestibility of nutrients, especially lipids (Teuling et al., 2017; Teuling et al., 2019). The reduced lipid digestibility in the fish fed the NE15 diet in the present experiment is likely due to the mixing of wheat with the microalga prior to the pre-treatment. It is well known that starch and lipid form complexes during heat processing, which reduces the digestibility of starch (Wang et al., 2020). However, not much is known about the effect of such complex formation on lipid digestibility.

The protein retention values of the alga-fed groups were higher than the values reported by Gong et al. (2019). On the other hand, retention of lipid had a similar trend, as described in the aforementioned report, that is, a reduction with increasing incorporation of the microalga. The lower digested lipid and energy retention noted for the fish fed the NN15 diet compared to the fish fed the NE15 diet points to the effect of the pre-treatment of the microalgal biomass.

The fatty acid profile in the experimental feeds was slightly affected by the incorporation of the microalga. The fatty acid that changed the most with the incorporation of microalga was C16:1n-9; approximately 31% higher in NN15 and NE15 compared to the control group. Although we noted a higher level of C16:1n-9 in NE7.5 (post-smolts), the results from the farm study indicated a reduction in this fatty acid when the fish were fed 7.5% whole alga. The next abundant fatty acid in the fish fillet from the laboratory study was C20:5n-3, which was almost 19% higher in the feeds with the highest alga incorporation compared to the control group. The dietary microalga had a positive effect on the fatty acid profile of the flesh. The increased content of n-3 PUFAs, especially the valuable EPA + DHA level (1.73 g/100 g fillet) in fish fed the microalga-incorporated diets indicate that *Nannochloropsis* spp. is a promising salmon feed ingredient. It was reported previously that EPA + DHA in Atlantic salmon fed 100% capelin oil and 100% sardine oil was 1.6 and 2.9 g/100 g portion, respectively (Bell and Tocher, 2022). Although not significantly different, the microalga fed fish from the farm study also had higher values for n-3 PUFAs and EPA + DHA. The beneficial effects of microalgae as a PUFA source in fish feed have also been documented in other reports (Gong et al., 2019; Gong et al., 2020; Katerina et al., 2020; Kousoulaki et al., 2016; Sørensen et al., 2016).

4.3. Alteration in the antioxidant and immune genes

Inclusion of alga was found to lower the antioxidant genes in the intestine of the post-smolts, but the lowering was particularly prominent in the non-treated alga fed NN15. On the contrary, a higher expression of *sod1* was recently reported in the distal intestine of Atlantic salmon fed 30% of pre-treated *N. oceanica* (Sørensen et al., 2021). In a previous study, with high inclusion of fish meal feeds, an apparent lowering of *sod* in salmon fed 20% lipid extracted *N. oceanica* was also noticed compared to fish that did not receive the alga (Sørensen et al., 2017). Furthermore, serum SOD activity also exhibited a similar response pattern in the same study. Thus, the inclusion of 15% *N. oceanica* seems to be associated with reduced expression of *sod*, based on our two studies. On the other hand, inclusion of lipid extracted *Desmodesmus* sp. was not found to decrease ($p > 0.05$, not significantly) the serum SOD activity (Kiron et al., 2016b).

Cathelicidins are activated in response to microbial breaches (Ho et al., 2013), and in the current study, we observed only a lowering of the expression of both *cath1* and *cath2*. On the other hand, *Desmodesmus* sp. was found to apparently increase ($p > 0.05$) the mRNA levels of *cath1* and *cath2* (Kiron et al., 2016b).

The expression of the proinflammatory gene, *il17d*, was lower in the *N. oceanica* fed groups. On the other hand, in our previous studies we observed apparently higher levels of this gene in the fish fed lipid-extracted *N. oceanica* and not in the fish that received lipid extracted *Desmodesmus* sp. (Kiron et al., 2016b; Sørensen et al., 2017). Genes linked to inflammatory responses, such as *gal3*, *anxa1* and *anxa2*, behaved differently; *gal3* was not altered by the alga feeding and the latter two were lowered with the alga feeding. The gene *anxa1* is involved in intracellular and anti-inflammatory processes, while loss of *anxa2* is linked to enhanced cell-matrix adhesion (Buckingham et al.,

2006; Rankin et al., 2013; Vago et al., 2012). However, we did not find any difference in *gal3* expression, which has a role in intestinal epithelial intercellular adhesion (Jiang et al., 2014). In addition, the immunoglobulin-related gene *igt* was not affected, but the expression of the gel-forming mucin-related gene, *muc5b*, was reduced after the alga feeding although it is reported, that certain immunoglobulins in humans are associated with mucins, especially Muc5B (Fahrbach et al., 2013). Furthermore, *Nannochloropsis* spp. is known to encode one 1,3-β-glucan synthase gene (Wang et al., 2014). However, the expression of two salmon beta glucan receptors, namely *sclra* and *cr3* (Kiron et al., 2016a), as well as that of *il17d* was lower in the NE7.5 group compared to the control group, suggesting a downregulation of the inflammatory response triggered by the activation of glucan receptors. We have reported a similar insignificant expression of *cr3* when the fish were fed 30% pre-treated *N. oceanica* (Sørensen et al., 2021), as observed in the case of NE15.

The histomorphometric indices pointed to improved gut health in the fish fed the NW diet during the farm study. The higher villi height and width, thickness of mucosa and submucosa layers, along with increased height of enterocytes and presence of supra nuclear vacuoles indicate the ability of the microalga to improve the nutrient absorptive area in the intestine (Yamauchi et al., 2010). We observed an increase in only ash digestibility of the post-smolts of the NE7.5 group. The increased height of enterocytes, thickness of mucosa and slight increase in number of mucus cells per villi area may be an adaptation response to increased amounts of indigestible carbohydrates in the feed. Indigestible carbohydrates in the feed can increase fold height, enterocyte vacuolization, and weight of the distal intestine segment in Atlantic salmon (Sørensen et al., 2011). The higher distal intestine somatic index reported in Atlantic salmon fed inulin was presumed to be due to hypertrophy in the muscular layers caused by increased peristalsis as a result of more lumen filling (Bakke-McKellep et al., 2007).

The evaluation of product quality parameters such as visual color, astaxanthin content, texture and lipid content was performed on fish of edible size, i.e., with an average gutted weight > 3.5 kg. The color of salmon fillet is important for the aquaculture industry and 40 ppm astaxanthin was added to the feed to ensure pigmentation of the marketed salmon. Utilization of astaxanthin in the feed depends on numerous factors, such as inclusion level of carotenoids in the feeds, their bioavailability and size of the fish. Digestibility of astaxanthin varies between 40 and 60%, but it is reduced by low water temperatures (Ytrestøyl et al., 2005). The present experiment used feeds rich in plant derived protein and lipid, to resemble commercial fish feeds (Aas et al., 2019). The slightly reduced astaxanthin content in the NW- fed fish, compared to the control fish could be because of the lower fishmeal content in the NW diet. Marine ingredients are important for the uptake and utilization of astaxanthin, due to their content of long-chain polyunsaturated fatty acids, phospholipids and cholesterol (Bjerkeng et al., 1999; Olsen et al., 2005; Ytrestøyl et al., 2019), and the bioaccessibility of carotenoids is affected more at low water temperature (Ytrestøyl et al., 2019). High correlation between visual observation and analyzed values of astaxanthin (Christiansen et al., 1995) is the basis of the Photofish technology that predicts astaxanthin concentration based on captured images. The Photofish detected small, but significant, differences in astaxanthin concentration between the two groups. However, the relatively high concentration of flesh astaxanthin was assessed visually, using Salmofan, which was not powerful to detect significant differences between the color in the two groups fed the two experimental diets.

The fillet texture and lipid content did not vary between the dietary treatments. The lipid content in the NQC was slightly higher than values reported by other researchers (Einen et al., 1999), but lower than values reported for rainbow trout (Mørkøre et al., 2001).

5. Conclusion

Growth and feed performance of Atlantic salmon was not impacted distinctly when they were offered thermomechanically pre-treated *Nannochloropsis oceanica*. The observed differences in the nutrient and energy digestibility could be the effect of microalga inclusion rather than the effect of pre-treatment technique applied on the microalgal biomass. Pre-extrusion of the alga improved only the digestible lipid and energy retention efficiencies in Atlantic salmon. The alterations in the antioxidant and immune genes do not imply any adverse effects of the alga. Although the alga had adverse effects on the growth (both 7.5 and 15%) and FCR (at 15%) of the fish, the inclusion of alga enhanced the value of flesh, through increased deposition of EPA and DHA. These observations on the treated alga may suggest that methods other than the pre-treatment employed in this study should be tested to rupture the cell walls of *Nannochloropsis oceanica*.

Declaration of Competing Interest

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

Data availability

All data are included in the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2022.738651.

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