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Approaches to improve utilization of *Nannochloropsis oceanica* in plant-based feeds for Atlantic salmon

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ABSTRACT

Rigid cell walls of microalgae lower the digestibility and nutrient bioavailability in carnivorous fish. Extrusion is a thermo-mechanical process and a scalable technology that may break cell walls and improves nutrient utilization. It can be hypothesized that certain feed additives may further improve microalgae nutrient digestibility and feed utilization by fish. The aim of the current study was to investigate i) the effect of incorporation of 10% pre-extruded *Nannochloropsis oceanica* on nutrient digestibility, growth and feed utilization of Atlantic salmon post smolts, and ii) the ability of feed additives in improving the feed utilization. Four low fish meal feeds were formulated; a control without the microalga *N. oceanica* (CO), a feed containing 10% of the pre-extruded microalga (NC), and two feeds containing 10% of the pre-extruded microalga and supplemented with either 0.06% Digestarom® (ND) or 1% ZEOFeed (NZ). Fish (initial average weight of 227.3 ± 3.4 g) in 5 replicate tanks of each of the study groups were fed one of the experimental feeds for 68 days. The apparent digestibility of dry matter in the NC and NZ groups were significantly higher compared to the control group (CO). The digestibility of lipid was significantly lower, and digestibility of ash was higher in the alga-fed groups (NC, ND and NZ) compared to the control group (CO). The incorporation of 10% pre-extruded *N. oceanica* in plant-based commercial-like feeds did not affect the growth, feed utilization and whole body proximate composition of salmon. No effects of the feed additives were observed on growth, feed utilization and histomorphology of distal intestine of salmon, but cell proliferation (PCNA) was higher for fish fed the alga alone as well as the alga-ZEOfeed combination. There were no differences in polyunsaturated fatty acids in whole body of fish fed the different feeds. It is noteworthy that whole-body EPA + DHA levels of fish fed the algae feeds were maintained at the same levels as fish fed the control feed that contained 50% more fish meal and 10% more fish oil.

1. Introduction

Aquaculture production in Norway has increased from around 150, 000 t in the 1990s to more than 1.35 million tonnes in 2018, and is dominated by Atlantic salmon, accounting for around 95% of the total volume (SSB, 2018). Future growth of salmon farming depends on high-quality sustainable ingredients that promote good growth and feed utilization, maintain fish health and preserve the nutritional quality of the end product.

Chemical composition of some microalgae signifies their potential as feed ingredients for Atlantic salmon (Becker, 2007; Shields and Lupatsch, 2012; Tibbetts, 2018). These microorganisms are good sources of amino acids, n-3 polyunsaturated fatty acids (n-3 PUFAs) and astaxanthin (Shah et al., 2018). However, only a few of them are successfully commercialized and used in salmon feeds. The heterotrophic microorganism *Schizochytrium* sp. is a good source of the n-3 PUFA, docosahexaenoic acid (DHA), and hence may be a good replacer of fish oil (Kousoulaki et al., 2015; Sprague et al., 2017; Sprague et al., 2015). On the other hand, the photoautotrophic microalga *Haematococcus* sp. accumulate astaxanthin, and therefore, can be a good alternative to

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synthetic astaxanthin in salmon feeds (Griffiths et al., 2016).

Replacement of both fish meal and currently used plant-derived ingredients in salmon feeds with microalgae remains a challenge. Thorough testing is essential to understand the effects of microalgae on feed quality, growth, feed utilization, nutrient digestibility, and health of the animal, and end product quality (Glencross et al., 2007; Ringø et al., 2009). Our previous studies have shown that microalgae such as *Nannochloropsis oceanica (N. oceanica)* can be used at modest inclusion levels; up to 10%, without negatively affecting the performance and health of salmon (Sørensen et al., 2017). However, we found that nutrient digestibility (e.g. lipid) of the microalga-incorporated salmon feeds was lower compared to the fish-meal-based reference feeds (Gong et al., 2018; Sørensen et al., 2017). By proving that cost-effective processing technologies can disrupt cell walls or special feed additives can improve nutrient availability of microalgae, the salmon feed industry can be encouraged to rely on microalgae (Teuling et al., 2017; Tibbetts et al., 2017). Recent research suggested that extrusion can effectively disrupt the cells of *Nannochloropsis* and make the intracellular biocompounds available for further use (Gong et al., 2018; Wang et al., 2018).

Feed additives such as Digestarom® (a phytogenic ingredient) and zeolites (microporous aluminosilicate) are known to improve the performance and health of farmed animals (Jeney et al., 2015; Papaioannou et al., 2005). Studies with rainbow trout (*Oncorhynchus mykiss*) reported that supplementation of feeds with 0.1% Digestarom® PEP 1000 (containing 1.2% carvacrol) or 0.1% Digestarom® PEP MGE 1000 (containing 0.6% thymol) improved feed efficiency compared to control feed, although the body weight of the fish was unaltered (Giannenas et al., 2012). Furthermore, Digestarom® P.E.P. MGE was found to lower the fillet fat and slightly increase protein content in channel catfish (*Ictalurus punctatus*) (Peterson et al., 2014).

Clinoptilolite, a natural zeolite, in feeds for farmed animals had positive effect on nutrient digestibility, growth and feed utilization (Ghasemi et al., 2016; Kanyılmaz et al., 2015). Not many studies have reported the effects of zeolite on fish, but a previous study on gilthead sea bream suggested that inclusion of clinoptilolite into the feed can promote growth rate and feed efficiency (Kanyılmaz et al., 2015). Furthermore, zeolite (bentonite and mordenite) improved the growth and feed utilization in rainbow trout (Eya et al., 2008). The improved growth and nutrient utilization in the fish fed zeolites were attributed to the detoxifying effects of the compound (Ghasemi et al., 2016).

In our previous studies, we evaluated the potential of microalgae in high fish meal and fish oil feeds of Atlantic salmon (Kiron et al., 2012; Kiron et al., 2016; Sørensen et al., 2017). In the present study, we aimed to understand the nutritional value of microalgae in commercial-like feeds; i.e. feeds high in plant and low in marine ingredients. In addition, we tried to understand the effect of two feed additives on Atlantic salmon. The aims of the present study were to investigate the potential of: i) thermo-mechanical processed (extruded) *N. oceanica* as an ingredient in high plant-low marine ingredient salmon feed and ii) two different feed additives to improve the nutrient digestibility and utilization of the *Nannochloropsis*-incorporated feeds.

2. Material and methods

2.1. Experimental design and feeds

This feeding trial was approved by the National Animal Research Authority (FDU: Forsøksdyrutvalget ID-5887) in Norway.

Four nearly isoproteic (42–44% of dry matter) and isolipidic (28–30% of dry matter) feeds were formulated. The ingredient composition is provided in Table 1, chemical and amino acid composition is given in Table 2 and the information of the fatty acids is presented in Table 3. Four low fish meal feeds were employed in the current study; the control feed containing 15% fish meal and no *N. oceanica* (CO), a basal test feed containing 7.5% fish meal and 10% of the microalgae (NC), and two other test feeds similar to the feed NC, but supplemented with either 0.06% Digestarom® PEP MGE150 (Biomin GmbH, Getzersdorf, Austria; ND), or 1% ZEOFeed (ZEOCEM AS, Bystré, Slovakia; NZ). Digestarom® PEP MGE150 contains a blend of essential oils from oregano, anise, and citrus peel and the main active compounds are carvacrol, thymol, anethol, and limonene (Peterson et al., 2014; Rodrigues et al., 2018). ZEOFeed is a clinoptilolite and a natural zeolite that comprise a microporous arrangement of silica and alumina tetrahedral

Table 1
Ingredient composition (%) of the four experimental feeds.

Ingredients	CO	NC	ND	NZ
Fishmeal 70 LT FF (NORVIK)[a]	15.00	7.50	7.50	7.50
Nannochloropsis extruded[b]	–	10.00	10.00	10.00
Soy protein concentrate[c]	16.00	16.00	16.00	16.00
Pea protein concentrate[d]	10.00	10.00	10.00	10.00
Wheat gluten[e]	11.30	13.00	13.00	13.24
Wheat meal[f]	9.44	7.04	6.98	5.80
Faba beans[g]	7.00	7.00	7.00	7.00
Fish oil (SAVINOR)[h]	10.00	9.05	9.05	9.05
Rapeseed oil[i]	15.00	15.00	15.00	15.00
Vitamin & Mineral Premix INVIVO[j]	1.00	1.00	1.00	1.00
Lutavit C35[k]	0.03	0.03	0.03	0.03
Lutavit E50[l]	0.05	0.05	0.05	0.05
Choline chloride[m]	0.20	0.20	0.20	0.20
Monocalcium phosphate[n]	2.00	2.90	2.90	2.90
Calcium carbonate[o]	2.22	0.00	0.00	0.00
L-lysine[p]	0.40	0.60	0.60	0.60
L-threonine[q]	0.20	0.30	0.30	0.30
L-tryptophan[r]	0.04	0.11	0.11	0.11
DL-methionine[s]	0.10	0.20	0.20	0.20
Yttrium oxide[t]	0.02	0.02	0.02	0.02
Digestarom®[u]			0.06	
ZEOFeed[v]				1.00

CO: Plant based control feed; NC: *N. oceanica* 10% feed; ND: *N. oceanica* 10% + Digestarom® PEP MGE150 0.06% feed; NZ: *N. oceanica* 10% + ZEOFeed 1% feed.

[a] NORVIK 70: 70.3% crude protein (CP) 5.8% crude fat (CF), Sopropeche, France.
[b] Allmicroalgae, Portugal.
[c] Soycomil P: 63% CP, 0.8% CF, ADM, The Netherlands.
[d] NUTRALYS F85F: 78% crude protein, 1% crude fat, ROQUETTE Frères, France.
[e] VITAL: 80% CP, 7.5% CF, Roquette Frères, France.
[f] Wheat meal: 11.7% CP, 1.6% CF, Casa Lanchinha, Portugal.
[g] Faba beans: 28.5% CP; 1.2% CF, Ribeiro & Sousa Cereais, Portugal.
[h] SAVINOR UTS, Portugal.
[i] Henry Lamotte Oils GmbH, Germany.
[j] PREMIX 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.
[k] ROVIMIX STAY-C35, DSM Nutritional Products, Switzerland.
[l] ROVIMIX E50, DSM Nutritional Products, Switzerland.
[m] ORFFA, The Netherlands.
[n] MCP: 21.8% phosphorus, 18.4% calcium, Fosfitalia, Italy.
[o] CaCO3: 40% Ca, Premix Lda., Portugal.
[p] Biolys: 54.6% Lysine, Evonik Nutrition & Care GmbH, Germany.
[q] ThreAMINO: 98% L-Threonine, Evonik Nutrition & Care GmbH, Germany.
[r] TrypAMINO: 98% Tryptophan, Evonik Nutrition & Care GmbH, Germany.
[s] DL-Methionine for Aquaculture: 99% Methionine, Evonik Nutrition & Care GmbH, Germany.
[t] Sigma Aldrich, USA.
[u] BIOMIN Holding GmbH, Austria.
[v] ZEOCEM, Slovak Republic.

Table 2
Chemical composition of the four experimental feeds.

	CO	NC	ND	NZ
Proximate composition				
Dry matter	94.98	94.06	94.79	95.35
% of dry matter				
Protein	44.43	43.06	42.30	42.89
Lipid	29.48	28.17	30.28	29.47
Ash	8.90	8.85	9.04	9.63
Carbohydrate[a]	17.2	19.9	18.4	18.0
Energy (KJ g^{-1})[b]	23.8	23.0	23.5	23.3
Amino acids (% of dry matter)				
Alanine	1.9	1.7	1.8	1.8
Arginine	2.8	2.6	2.6	2.5
Aspartic acid	4.1	3.6	3.9	3.8
Cysteine	0.5	0.5	0.6	0.6
Glutamic acid	9.5	9.0	9.4	9.2
Glycine	2.1	1.8	1.9	1.8
Histidine	1.0	0.9	1.0	0.9
Leucine	3.4	3.1	3.2	3.2
Lysine	3.0	2.7	2.8	2.8
Isoleucine	1.8	1.7	1.8	1.7
Methionine	0.8	0.8	0.8	0.7
Phenylalanine	2.2	2.1	2.1	2.1
Proline	3.1	3.0	2.9	2.9
Serine	2.3	2.1	2.1	2.1
Threonine	1.9	1.8	1.9	1.8
Tryptophan	0.5	0.6	0.6	0.6
Tyrosine	1.5	1.4	1.4	1.4
Valine	2.0	1.9	2.0	2.0

CO: Plant-based control feed; NC: *N. oceanica* 10% feed; ND: *N. oceanica* 10% + Digestarom® PEP MGE150 0.06% feed; NZ: *N. oceanica* 10% + ZEOFeed 1% feed.

[a] Carbohydrate (% of dry matter) was calculated as 100 - (Protein of dry matter + Lipid of dry matter + Ash of dry matter).

[b] The gross energy content of feeds was not analyzed but calculated based on 23.7, 39.5 and 17.2 KJ g^{-1} for protein, lipids and starch, respectively.

Table 3
Fatty acid composition (% of total fatty acids) of the experimental feeds.

Fatty acids	CO	NC	ND	NZ
C14:0	2.8	2.7	2.7	2.7
C15:0	0.3	0.2	0.2	0.2
C16:0	10.2	9.9	10	9.9
C16:1n-7	3.2	3.4	3.4	3.4
C17:0	0.3	0.2	0.2	0.2
C18:0	2.3	2.2	2.2	2.2
C18:1n-9	39.1	39.9	40.0	40.1
C18:2n-6	14.3	14.5	14.4	14.4
C18:3n-3	6.0	6.1	6.1	6.1
C18:3n-6	0.1	0.1	0.1	0.1
C18:4n-3	0.9	0.8	0.9	0.9
C20:0	0.5	0.5	0.5	0.5
C20:1n-9	1.6	1.5	1.5	1.5
C20:2n-6	0.1	0.2	0.2	0.2
C20:4n-6	0.4	0.4	0.4	0.4
C20:4n-3	0.3	0.2	0.2	0.2
C20:5n-3	5.5	5.7	5.6	5.6
C22:0	0.3	0.3	0.3	0.3
C22:1	1.6	1.4	1.4	1.4
C22:5n-6	0.1	0.1	0.1	0.1
C22:5n-3	0.7	0.7	0.7	0.7
C22:6n-3	4.5	4.0	4.0	4.0
C24:0	0.1	0.1	0.1	0.1
C24:1n-9	0.3	0.3	0.3	0.3
ΣSFAs	16.8	16.3	16.4	16.3
ΣMUFAs	45.9	46.6	46.7	46.8
ΣPUFAs	33.2	33.0	32.8	32.8
Σn-6 PUFAs	15.2	15.5	15.3	15.4
Σn-3 FUFAs	18.0	17.6	17.5	17.5
n-3/n-6	1.19	1.14	1.14	1.14
EPA + DHA	10.0	9.7	9.6	9.6

CO: Plant-based control feed; NC: *N. oceanica* 10% feed; ND: *N. oceanica* 10% + Digestarom® PEP MGE150 0.06% feed; NZ: *N. oceanica* 10% + ZEOFeed 1% feed.

(EFSA, 2013).

The test microalga *N. oceanica* (contained 2.8% moisture, 36.6% protein, 14.3% lipid, 9.4% fiber, 22.8% ash, 17.5 KJ g^{-1} of energy, 2.1% lysine and 0.9% methionine) used in the feeds was cultured in closed photobioreactors at Allma®, 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 of the microalgae and manufactured the experimental feeds. The microalgae were pre-processed, by passing them through an extruder, prior to mixing them with other ingredients to prepare the experimental feeds. The pre-extrusion of algae was carried out as follows: *N. oceanica* (98.5%) powder was blended with wheat meal (1.5%) in a double-helix mixer (model 500 l, TGC Extrusion, France). The mixture was then passed through a pilot-scale twin-screw extruder (model BC45, CLEXTRAL, France) with a screw diameter of 55.5 mm to produce pellets (2.0 mm diameter size). The extrusion conditions were as follows: feeder rate 65 kg/h; screw speed 243 rpm; steam addition at conditioner 3%; water addition at extrusion barrel 1295 mL/min; temperature in the barrel was 112–113 °C recorded in section 3; moisture level of the dough at die exiting was 26%. The extruded alga pellets were dried in vibrating fluid bed dryer (model DR100, TGC Extrusion, France). The chemical composition of pre-extruded *N. oceanica* + wheat meal was 3.3% moisture, 36.4% protein, 14.2% lipid, 9.3% fiber, 22.6% ash, 17.4 KJ g^{-1} of energy, 2.0% lysine and 0.9% methionine.

The experimental feeds were produced by mixing all the powder ingredients and pre-extruded alga pellets in a double-helix mixer (model 500 l, TGC Extrusion, France) and ground (below 400 μm) in a micropulverizer hammer mill (model SH1, Hosokawa-Alpine, Germany). Feeds (pellet size: 3.0 mm) were manufactured with a twin-screw extruder (model BC45, Clextral, France) with a screw diameter of 55.5 mm. Extrusion conditions for the experimental feeds were: feeder rate (80–89 kg/h), screw speed (235–244 rpm), water addition (approximately 230 mL/min), temperature recorded in barrel section 1 was 34–36 °C and highest temperature was observed in barrel 3, varying between 124–127 °C. Extruded pellets were dried in a vibrating fluid bed dryer (model DR100, TGC Extrusion, France). After cooling, oils were added by vacuum coating (700 mbar, for approximately 50 s) (model PG-10VCLAB, Dinnissen, The Netherlands). Immediately after coating, feeds were packed in sealed plastic buckets and shipped to Nord University Research Station, Bodø, Norway for the feeding trial.

2.2. Fish and feeding

Atlantic salmon (*Salmo salar*) post-smolts were obtained from Cermaq, Hopen, Bodø, Norway (Aquagen strain, Aquagen AS, Trondheim, Norway) and maintained at the Research Station, Nord University for approximately 5 months. The fish were fed Spirit Supreme 75 and Spirit Supreme 150 (Skretting, Stavanger, Norway) during the holding period. At the start of the experiment, a total number of 600 fish with initial weight 227.3 ± 4.0 g were randomly allocated to the experimental units (n = 30 fish per tank). The fish were starved for 2 days after the distribution to the experimental tanks and then switched directly to the experimental feeds.

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 hour, and the average temperature and salinity of the rearing water were 7.5 °C and 35‰, respectively. Oxygen saturation was always above 85% recorded for water at the outlet. 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 68-day trial. After each feeding, the uneaten feeds that settled in the steel wire mesh of each experimental tank were collected.

2.3. Fish sampling and data collection

At the beginning and end of the experiment, all the fish (600) were individually weighed and their lengths were recorded. Before handling, fish were anesthetized using tricainemethanesulfonate (MS 222, 140 mg/l). Fish that were sampled for histology, whole body composition and organosomatic indexes were humanely euthanized by a sharp blow to the head. At 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. Three fish from each tank were weighed, dissected and the visceral organs (without heart and kidney) and liver from each fish were removed and weighed for calculation of organosomatic indexes. The distal intestine of these fish was sampled for histomorphology evaluation. Faeces were collected from the remaining fish in the tanks. Fecal matter was obtained from individual fish by stripping and pooled to obtain enough material for chemical analysis.

2.4. Chemical analyses

The fish samples from each tank were homogenized using an industrial food processor (Foss Tecator, 2096 homogenizer, Hilleroed, Denmark) before analyzing the whole body proximate composition of fish fed the experimental feeds. Both fecal samples and whole body samples were freeze dried (VirTis benchtop, Warminster, PA, USA) for 72 h prior to the chemical analysis.

The fish, experimental feeds and freeze-dried faeces 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), crude lipid (Soxtec HT6, Tecator, Höganäs, Sweden; ISO 6492:1999), 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). The amino acid analyses were performed according to ISO 13903:2005. Yttrium in both faeces and feeds 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 faeces 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 composition 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 samples 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 (GLC-473, Nu-Chek Prep, Elysian, MN, USA).

2.5. Histological analysis

Approximately 1 cm of the anterior part of the distal intestine was sampled and luminal contents were rinsed off with 10% neutral buffered formalin (NBF), and the tissue was fixed in 10% NBF for 24 h. Formalin-fixed samples were dehydrated in an alcohol gradient, equilibrated in xylene and embedded into paraffin blocks. For each fish, approximately 5 μm thick longitudinal sections were cut using microtome, after which they were mounted onto a glass slide.

2.5.1. Immunohistochemistry

Samples of the distal intestine from six fish per feed group were used for the immunohistochemistry analysis of the proliferating cell nuclear antigen (PCNA). The sections were dewaxed and rehydrated. Antigen retrieval was done by autoclaving the sections for 10 min at 120 °C in citrate buffer (10 mM/l citric acid monohydrate, pH 6). For quenching of endogenous peroxidase, sections were incubated with 3% hydrogen peroxide in water for 30 min. To prevent nonspecific binding, the sections were blocked with normal horse serum containing 5% BSA in PBS for 20 min at room temperature. Sections were then incubated with the primary antibody anti-PCNA mouse monoclonal antibody to PCNA (M0879, Dako Cytomation, Bath, United Kingdom) at a dilution of 1:500 in 1% BSA/TBS overnight at 4 °C. The sections were then incubated with secondary antibody horse anti-mouse biotinylated against IgG at dilution 1:1000 for 30 min at room temperature. Subsequently the slides were incubated with ABC reagent (Vectastatin PK6102, Vector Laboratories, Burlingame, CA, USA) for 30 min at room temperature. Brown staining was obtained by dropping 3,3-Diaminobenzidine (DAB) substrate (D7679 Sigma-Aldrich Corp. St.Louis, MO, USA) on top of the slides to form a dark brown insoluble precipitate. Hematoxylin was used for counterstaining. The sections were washed with PBS (3 × 5 min) between each step in the protocol.

For the analysis of cell proliferation, 20 well-oriented and intact villi per fish were selected. This generated 120 microphotographs per feed group that were captured at × 40 magnification by a camera (Leica MC170HD, Heersbrugg, Switzerland) mounted on light microscope (Leica DM1000, Wetzlar, Germany) using a software, Leica Microsystems Framework (LAS V4.12.INK, Heerbrugg, Switzerland). All the images were analyzed with ImageJ 1.52a (Schneider et al., 2012).

The total area of a villus (TVA) was demarcated by 'Freehand selections' tool, and measured by 'Analyze' menu in ImageJ. The PCNA stained area of a villus (PSA) was estimated using 'Colour Threshold' in ImageJ. For that, 'Brightness' in the 'Colour Threshold' was decreased until only the PSA was covered, while 'Thresholding method' was set to 'Default', 'Threshold colour' to red and 'Colour space' to HSB (hue, saturation and brightness). The PSA could then be selected and measured (Fig. 1). This value was used to calculate the cell proliferation index (CPI), ratio between PSA and TVA. Mean ± SEM values of CPI are presented.

2.6. Calculations and statistical analysis

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

$$\text{Weight gain (\%)(WG)} = \left(\frac{W_f - W_i}{W_i}\right) \times 100$$

$$\text{Feed intake (\%BW day}^{-1}\text{) (FI)} = \left(\frac{\text{Daily feed intake in dry basis (g)}}{\sqrt{W_f \times W_i}}\right) \times 100$$

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

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

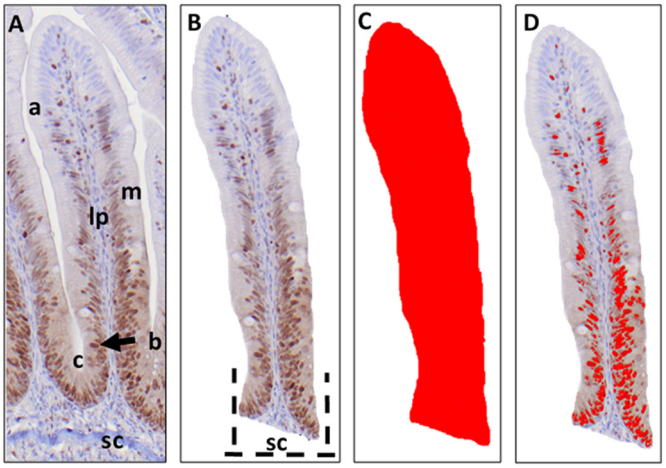

Fig. 1. Morphometric technique used to calculate the cell proliferation index (CPI) of the distal intestine of Atlantic salmon (*Salmo salar*). A. Simple intact villus at x10 magnification. a- absorptive vacuoles, b- PCNA-negative enterocyte, c- crypt, lp- lamina propria, m- mucous cell, sc- stratum compactum, arrow- PCNA-positive enterocyte. B. The selected boundaries of the villus included the epithelial part from tip of villus to its base and the crypt boundary was perpendicular to sc (which were not included) and parallel to lp. C. Total area of villus (TVA). D. The PCNA stained area of a villus (PSA).

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

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

$$\text{Hepato} - \text{somatic index (\%)(HSI)} = \frac{\text{Liver weight of fish (g)}}{W_f\,(g)} \times 100$$

$$\text{Viscero} - \text{somatic index (\%)(VSI)} = \frac{\text{Viscera weight of fish (g)}}{W_f\,(g)} \times 100$$

$$\text{Condition factor (g/cm}^3\text{)(CF)} = \frac{W_f\,(g)}{FL^3} \times 100$$

where, W_f = final body weight of fish (g/fish), W_i = initial body weight of fish (g/fish), T is the temperature in °C and d is 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 faeces, respectively, and $Nutrient_{feed}$ and $Nutrient_{faeces}$ represent the nutrient contents (% dry matter) in the feed and faeces.

All statistical analyses were performed using SPSS 22.0 software package for Windows. The data were tested for normality (Shapiro–Wilk normality test) and equality of variance (Levene's test). For parametric data, one way analysis of variance (ANOVA) was performed. Thereafter, Tukey's multiple comparison test was used to identify the significant differences among the means of the experimental groups. For non-parametric data, Kruskal-Wallis test, followed by Dunn's multiple comparison test, was performed to decipher the significant differences between the groups. A significance level of $p < .05$ was chosen to indicate the differences.

3. Results

3.1. Experimental feeds

All the experimental feeds were nearly isoproteic, isocaloric and balanced for EPA + DHA. The content of amino acids (AA's) in the feeds were balanced to meet the AA requirements of Atlantic salmon (NRC 2011); through the dietary supplementation of crystalline amino acids, lysine, methionine, threonine and tryptophan. The content of lysine and methionine was 2.7–3.0% and 0.7–0.8% of feed (dry basis), respectively (Table 2). The polyunsaturated fatty acids, namely EPA + DHA were similar in the feeds (2.7–2.9% of dry basis; based on information from Table 3).

3.2. Apparent digestibility coefficients of feeds

Digestibility of DM, protein, lipid and ash differed significantly among the four feeds ($p < .05$; Table 4). The DM digestibility was significantly lower in CO-fed fish compared to fish fed NC and NZ, while that in ND-fed fish were ranked in between the CO and the other two algae-fed groups. Protein digestibility was higher ($p < .05$) in fish fed NC than those fed ND while the values of the CO and NZ groups were similar and lie between those of NC and ND. Lipid digestibility was highest in fish fed CO, while no differences were observed among the alga-fed groups. Digestibility values of ash in alga-fed fish were positive while the values of the fish fed CO were negative but no significant differences ($p < .05$) were detected among treatments.

3.3. Growth and feed utilization

The growth and feed utilization are given in Table 5. The fish grew from an initial average weight of 227.3 g to a final mean body weight of 419.6 g during the experimental period of 68 days. There were no significant differences in final weight, weight gain, specific growth rate, thermal growth coefficient, feed conversion ratio, feed intake or protein efficiency ratio of the different groups. There were no significant differences in condition factor or viscero-somatic indices (VSI) of the feed groups. Hepato-somatic indices (HSI) ranged between 1.10 and 1.19; the highest value was for the ND group compared ($p < .05$) to the lowest value of the NC group.

3.4. Proximate composition of whole body

The proximate composition of fish fed the four experimental feeds,

Table 4
Apparent digestibility coefficients (ADC %) of dry matter, lipid, protein and ash in Atlantic salmon fed the experimental feeds.

	CO	NC	ND	NZ	*p* value
Dry matter	63.3 ± 0.52^{b}	67.5 ± 0.41^{a}	65.3 ± 0.34^{ab}	66.1 ± 0.89^{a}	0.008
Protein	87.8 ± 0.11^{ab}	88.5 ± 0.07^{a}	86.5 ± 0.54^{b}	87.9 ± 0.60^{ab}	0.032
Lipid	94.3 ± 0.28^{a}	91.3 ± 0.04^{b}	91.1 ± 0.32^{b}	91.9 ± 0.52^{b}	0.002
Ash	−24.0 ± 2.05^{b}	12.9 ± 2.66^{a}	13.9 ± 1.06^{a}	7.7 ± 0.18^{a}	< 0.001

CO: Plant-based control feed; NC: *N. oceanica* 10% feed; ND: *N. oceanica* 10% + Digestarom® PEP MGE150 0.06% feed; NZ: *N. oceanica* 10% + ZEOFeed 1% feed. Values are expressed as mean ± SD (n = 5 replicates). Values in the same row with different superscript letters indicate significant difference ($p < .05$).

Table 5
Growth performance, feed utilization and somatic indices of Atlantic salmon for experimental period.

	CO	NC	ND	NZ	p value
Growth parameter					
IBW(g)	227.94 ± 5.93	228.51 ± 1.82	225.27 ± 1.48	227.31 ± 4.24	0.628
FBW (g)	422.77 ± 22.16	415.05 ± 25.01	417.28 ± 21.08	423.26 ± 11.20	0.898
WG (%)	85.44 ± 7.80	81.61 ± 10.41	86.23 ± 4.74	85.21 ± 8.28	0.802
FI (% BW day^{-1})	0.83 ± 0.05	0.84 ± 0.05	0.82 ± 0.03	0.83 ± 0.02	0.836
SGR (% day^{-1})	0.91 ± 0.63	0.87 ± 0.08	0.90 ± 0.66	0.91 ± 0.38	0.774
FCR	0.90 ± 0.01	0.95 ± 0.05	0.89 ± 0.04	0.89 ± 0.02	0.109
PER	2.49 ± 0.05	2.39 ± 0.14	2.53 ± 0.12	2.52 ± 0.07	0.140
TGC	2.74 ± 0.21	2.64 ± 0.28	2.72 ± 0.22	2.76 ± 0.12	0.815
Somatic indices					
HSI	1.16 ± 0.03^{ab}	1.10 ± 0.59^{b}	1.19 ± 0.06^{a}	1.15 ± 0.02^{ab}	0.042
VSI	8.22 ± 2 0.27	8.30 ± 2.72	8.55 ± 0.50	8.38 ± 0.51	0.635
CF	1.41 ± 0.03	1.42 ± 0.03	1.44 ± 0.03	1.42 ± 0.03	0.332

CO: Plant-based control feed; NC: *N. oceanica* 10% feed; ND: *N. oceanica* 10% + Digestarom® PEP MGE150 0.06% feed; NZ: *N. oceanica* 10% + ZEOFeed 1% feed. IBW, Initial body weight; FBW, Final body weight; WG, Weight gain; FI, Feed intake; SGR, Specific growth rate; FCR, Feed conversion ratio; PER, Protein efficiency ratio; TGC, Thermal growth coefficient; HSI, Hepato-somatic index; VSI, Viscero-somatic Index; CF, Condition factor.
Values are expressed as mean ± SD (n = 5 replicates). Values in the same row with different superscript letters show significant differences ($p < .05$).

Table 6
Proximate composition and energy of the whole fish on a dry matter basis (%).

	CO	NC	ND	NZ	p value
Protein	50.26 ± 0.35	50.72 ± 1.06	50.67 ± 0.64	50.65 ± 0.79	0.762
Lipid	41.94 ± 1.08	42.22 ± 1.65	39.26 ± 3.38	39.14 ± 2.14	0.075
Ash	5.40 ± 0.14	5.75 ± 0.38	5.60 ± 0.42	5.53 ± 0.15	0.366
Energy (KJ g^{-1})	29.05 ± 0.17^{ab}	28.82 ± 0.14^{b}	28.99 ± 0.10^{ab}	29.14 ± 0.23^{a}	0.048

CO: Plant based control feed; NC: *N. oceanica* 10% feed; ND: *N. oceanica* 10% + Digestarom® PEP MGE150 0.06% feed; NZ: *N. oceanica* 10% + ZEOFeed 1% feed.
Values are expressed as mean ± SD (n = 5 replicates). Values in the same row with different superscript letters indicate significant difference ($p < .05$).

sampled at the termination of the experiment, is provided in Table 6. No significant differences were observed in protein, lipid or ash content of the experimental groups. The energy content was significantly higher in NZ and lowest in fish fed NC ($p < .05$).

3.5. Fatty acid composition of fish whole body

The fatty acid composition of the whole body is given in Table 7. Significant differences were observed for saturated fatty acids (SFAs) and polyunsaturated fatty acids (PUFAs). The SFAs was significantly higher in fish fed CO compared with fish fed NZ ($p < .05$). The monounsaturated fatty acids (MUFAs) and n-3 PUFAs of the four groups were not significantly different. The n-6 PUFAs were significantly lower in fish fed CO compared to other groups ($p < .05$). Overall, the PUFAs were significantly higher in fish fed NZ compared to other groups ($p < .05$). As for the individual fatty acids, linoleic acid (LA), C18:2 n-6 dominated the n-6 fatty acids and it was lower in fish fed the CO feeds than in those fed the algal feeds ($p < .05$). The eicosapentaenoic acid (EPA, C20:5n-3) was found to be at the same level in fish fed the feeds with microalga even with a 50% reduction in fish meal and a 10% reduction in the fish oil compared to the CO-fed fish.

3.6. Histology of distal intestine

The morphology of distal intestine is shown in Supplementary Fig. 1. Villi height and width of fish fed CO, NC, ND and NZ is presented in Table 8. No significant differences were noted among the feeds. Proliferating cell nuclear antigen-positive cells were predominantly observed at the base of the distal intestinal villi and more diluted along the rest of the villi area (Fig. 2). Morphometric analysis of proliferating cells indicated a slight increase of CPI for all the microalgae incorporated feeds compared to the control group, but only the NC and NZ were significantly higher (Fig. 3).

4. Discussion

4.1. Apparent digestibility coefficients of feeds

The digestibility of protein, lipid and ash of the control feed used in the present trial were similar or even higher compared to fishmeal-based feed reported in our previous studies (Kiron et al., 2016; Sørensen et al., 2017). The digestibility of protein and lipid in the microalga-incorporated feeds in the present study were higher than those reported for 10% and 20% incorporation of *N. oceanica* in Atlantic salmon (Sørensen et al., 2017). These findings suggest that pre-processing of *N. oceanica* by extrusion, rendered intracellular nutrients more accessible for digestion. Effect of extrusion on cell disruption was not investigated in the present study. Other extrusion studies with *N. oceanica* have reported changes in the cell morphology characterized by wrinkled and shrunken cells; some cells with broken walls and others with emptied content (Wang et al., 2018). Extrusion may not have completely ruptured the cells; an even stronger treatment, i.e. a combination of enzymatic hydrolysis and high pressure homogenization could only achieve 95% disruption degree with another microalga *Neochloris oleoabundans* (Wang et al., 2015). Bead milling is an efficient mechanical method that increased the ADC of protein and lipid in tilapia fed the processed *Nannochloropsis gaditana* (Teuling et al., 2019). The efficiency of high-pressure homogenization was demonstrated using *Chlorella vulgaris*; it was reported that the process increased the ADC of protein, lipid, energy, total carbohydrate, starch and most essential amino acids and fatty acids in Atlantic salmon (Tibbetts et al., 2017).

Incorporation of the microalga (NC) even improved digestibility of dry matter and ash compared to the control group in the present study. Increased digestibility of ash was also observed in Nile tilapia and African catfish when they were fed *Nannochloropsis gaditana* (Teuling et al., 2017). Negative ash digestibility values are explained by drinking of sea water (Thodesen et al., 2001). Element analyses were not performed in the present experiment. However, for salmonids reared in

Table 7
Fatty acid composition (% of total fatty acids) of the whole fish.

Fatty acids	CO	NC	ND	NZ	P value
C14:0	2.78 ± 0.08^{a}	2.82 ± 0.04^{a}	2.80 ± 0.12^{a}	2.62 ± 0.04^{b}	0.005
C15:0	0.24 ± 0.05	0.22 ± 0.04	0.22 ± 0.04	0.20 ± 0.00	0.532
C16:0	10.86 ± 0.11^{a}	10.78 ± 0.11^{a}	10.70 ± 0.22^{ab}	10.52 ± 0.04^{b}	0.009
C17:0	0.20 ± 0.00	0.20 ± 0.00	0.20 ± 0.00	0.20 ± 0.00	1.000
C18:0	2.70 ± 0.07^{a}	2.58 ± 0.04^{b}	2.62 ± 0.04^{ab}	2.60 ± 0.70^{ab}	0.028
C20:0	0.30 ± 0.00	0.30 ± 0.00	0.30 ± 0.00	0.30 ± 0.00	1.000
C22:0	0.14 ± 0.05	0.14 ± 0.05	0.16 ± 0.05	0.18 ± 0.04	0.585
ΣSFAs	17.34 ± 0.19^{a}	17.14 ± 0.15^{ab}	17.08 ± 0.37^{ab}	16.78 ± 0.08^{b}	0.010
C16:1n-7	3.20 ± 0.00^{b}	3.32 ± 0.04^{a}	3.30 ± 0.70^{a}	3.20 ± 0.00^{b}	< 0.001
C18:1n-9	37.30 ± 0.22	37.36 ± 0.32	37.40 ± 0.29	37.58 ± 0.30	0.472
C20:1n-9	3.42 ± 0.10	3.38 ± 0.04	3.38 ± 0.13	3.42 ± 0.10	0.862
C22:1n-9	3.04 ± 0.15	2.98 ± 0.15	2.96 ± 0.20	2.96 ± 0.13	0.846
C24:1n-9	0.50 ± 0.00^{a}	0.42 ± 0.04^{b}	0.44 ± 0.05^{ab}	0.50 ± 0.00^{a}	0.004
ΣMUFAs	47.52 ± 0.16	47.60 ± 0.14	47.60 ± 0.14	47.72 ± 0.10	0.203
C18:2n-6	11.82 ± 0.11^{b}	12.12 ± 0.08^{a}	12.10 ± 0.21^{a}	12.22 ± 0.13^{a}	0.003
C18:3n-6	0.22 ± 0.04	0.24 ± 0.05	0.24 ± 0.05	0.22 ± 0.04	0.848
C20:2n-6	0.90 ± 0.00	0.90 ± 0.07	0.88 ± 0.04	0.92 ± 0.04	0.629
C20:3n-6	0.30 ± 0.00	0.30 ± 0.00	0.32 ± 0.04	0.30 ± 0.00	0.418
C20:4n-6	0.30 ± 0.00^{b}	0.40 ± 0.00^{a}	0.36 ± 0.05^{a}	0.40 ± 0.00^{a}	< 0.001
C22:5n-6	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	1.000
Σn-6 PUFAs	13.86 ± 0.13^{b}	14.20 ± 0.07^{a}	14.16 ± 0.19^{a}	14.30 ± 0.21^{a}	0.002
C18:3n-3	4.18 ± 0.08	4.26 ± 0.11	4.26 ± 0.11	4.30 ± 0.07	0.299
C18:4n-3	1.02 ± 0.04	1.04 ± 0.11	1.00 ± 0.07	1.00 ± 0.00	0.778
C20:3n-3	0.30 ± 0.00	0.32 ± 0.04	0.30 ± 0.00	0.30 ± 0.00	0.418
C20:4n-3	0.80 ± 0.00	0.76 ± 0.05	0.78 ± 0.04	0.76 ± 0.05	0.455
C20:5n-3	2.86 ± 0.05	2.94 ± 0.05	2.98 ± 0.08	3.02 ± 0.13	0.056
C22:5n-3	1.20 ± 0.00	1.20 ± 0.00	1.20 ± 0.00	1.24 ± 0.05	0.083
C22:6n-3	6.82 ± 0.13	6.60 ± 0.20	6.58 ± 0.22	6.64 ± 0.20	0.233
Σn-3 FUFAs	17.20 ± 0.00	17.08 ± 0.13	17.12 ± 0.16	17.26 ± 0.08	0.097
ΣPUFAs	31.06 ± 0.08^{b}	31.30 ± 0.07^{b}	31.28 ± 0.21^{b}	31.60 ± 0.18^{a}	< 0.001
n-3/n-6	1.24 ± 0.00^{a}	1.21 ± 0.01^{b}	1.21 ± 0.01^{b}	1.21 ± 0.01^{b}	0.011
EPA + DHA	9.68 ± 0.08	9.54 ± 0.20	9.56 ± 0.19	9.66 ± 0.13	0.449

CO: Plant based control feed; NC: *N. oceanica* 10% feed; ND: *N. oceanica* 10% + Digestarom® PEP MGE150 0.06% feed; NZ: *N. oceanica* 10% + ZEOFeed 1% feed. Values are expressed as mean ± SD (n = 5 replicates). Values in the same row with different superscript letters indicate significant difference ($p < .05$).

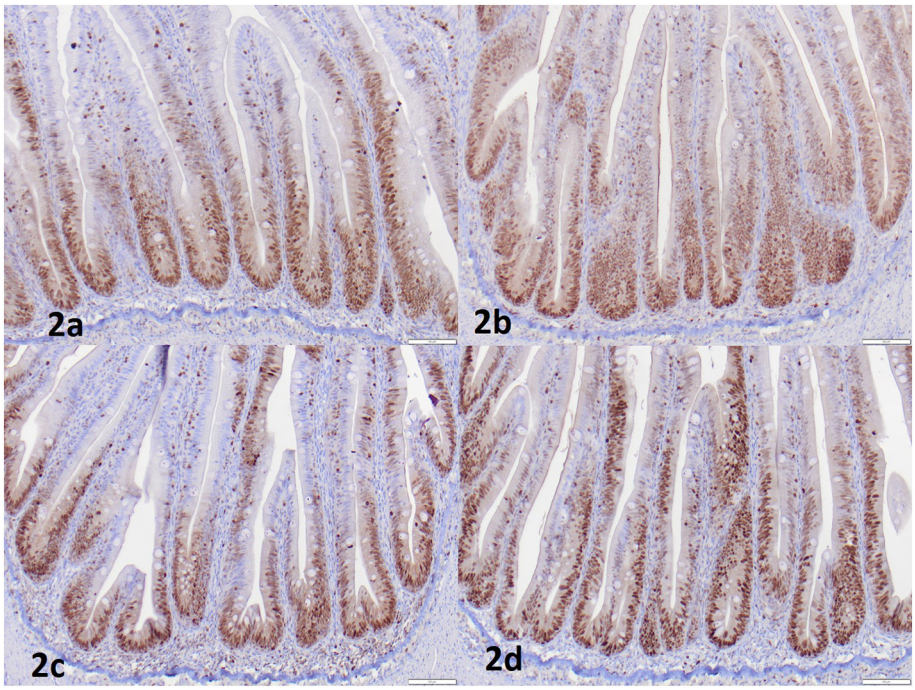

Fig. 2. Immunohistochemistry distal intestine of fish fed control feed (2a), or feeds with 10% extruded *N. oceanica* without additives (2b), or 10% extruded *N. oceanica* with Digestarom® (2c) or 10% extruded *N. oceanica* with ZEOFeed (2d). The bottom-right line is denoting 100 μm scale bar.

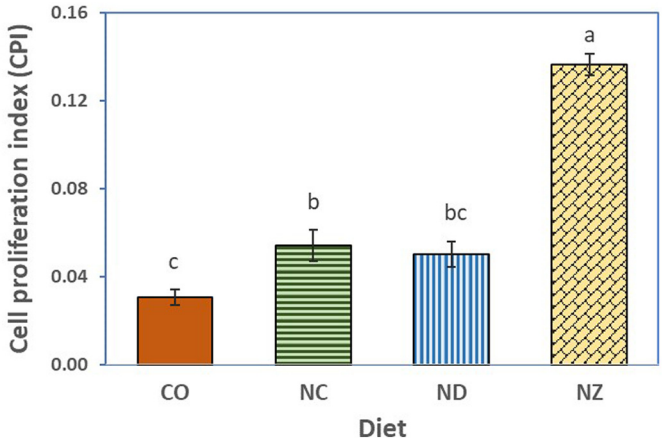

Fig. 3. Cell proliferating index in fish fed control feed, or feeds with extruded *N. oceanica* without (NC) or with Digestarom® (ND) or Zeofeed (NZ). Values are presented as means ± SEM, $n = 6$ fish per treatment group. Significant differences are denoted with different superscript ($p < .05$).

Table 8
Villi height and width (μm) in fish fed the different experimental feeds.

	CO	NC	ND	NZ
Villi height	610 ± 73.2	589 ± 50.4	552 ± 19.9	586 ± 56.2
Villi width	115 ± 4.7	101 ± 3.4	104 ± 4.5	103 ± 2.5

CO: Plant-based control feed; NC: *N. oceanica* 10% feed; ND: *N. oceanica* 10% + Digestarom® PEP MGE150 0.06% feed; NZ: *N. oceanica* 10% + ZEOFeed 1% feed.
Values are expressed as mean ± SD ($n = 6$ fish per diet). No significant differences were observed among the feeds ($p > .05$).

seawater there is a high correlation between ADC of ash and absorption of Ca and Mg, some of the key minerals in seawater (Thodesen et al., 2001). Negative ADC of ash is thus a strong indication of high drinking rate. Differences in pellet quality can also alter the ash digestibility (Aas et al., 2011; Gong et al., 2019). Pellet quality was not analyzed in the present experiment, but is affected by incorporation of microalgae in the feeds (Gong et al., 2019. In the study of Gong et al., (2019) there were no differences between the pellet qualities of 10% *Scenedesmus* incorporated feed and those without the alga. A 20% incorporation of the alga resulted in a doubling of the hardness and 87% more negative ash value compared to the control feed.

Earlier studies have reported reduced digestibility of lipids in feeds with more SFAs (Kousoulaki et al., 2016; Kousoulaki et al., 2015). Salmonids have limited capacity to digest SFAs at low temperature when the SFA levels are high (Menoyo et al., 2003; Menoyo et al., 2007; Ng et al., 2004). The SFA levels were similar among feeds (Table 3) and are therefore not a likely explanation for the reduced lipid digestibility noted for the microalga-incorporated feeds. Lipid digestibility is also dependent on the position of the fatty acids on the triacylglycerol (TAG) (Mu and Høy, 2004; Nielsen et al., 2005). The location of the SFAs in the tested microalgal TAG are unknown, and the effect of the position on lipid digestibility warrants further investigation. Reduction in lipid digestibility with incorporation of *N. oceanica* can also be explained by the carbohydrate composition as well as the chemical and mechanical properties of the cell walls (Glencross et al., 2012; Teuling et al., 2017; Tibbetts et al., 2017). Microalgae have complex carbohydrates such as cellulose, pectins and hemicelluloses (Baudelet et al., 2017; Scholz et al., 2014). Carnivorous fishes do not have the capacity to digest non-starch polysaccharides (NSPs) and they are only non-nutritive fillers in feeds (Irvin et al., 2016; Krogdahl et al., 2005). Earlier studies have shown that NSPs have negative effects on lipid and energy digestibilities of fish feed (Aslaksen et al., 2007; Espinal-Ruiz et al., 2014; Irvin et al., 2016; Leenhouwers et al., 2006; Refstie et al., 1999). Aslaksen et al. (2007) and Lekva et al. (2010) found a linear reduction in digestibility of lipid with increasing cellulose level (0–18%) in feeds for Atlantic salmon and Atlantic cod (*Gadus morhua* L.). Insoluble fiber, such as cellulose, interfere with digestion by increasing the gastric emptying rate, i.e. by reducing the time for digestion and absorption. Soluble fibers of the NSP fraction from cereals and legumes, disturb fat micelle formation and increase viscosity of gut contents, leading to a reduced gastric emptying rate, which may affect fat digestion in farmed fish (Espinal-Ruiz et al., 2014; Leenhouwers et al., 2006; Øverland et al., 2009; Refstie et al., 1999; Sinha et al., 2011).

4.2. Growth performance and feed utilization of the fish

Atlantic salmon readily accepted the experimental feeds and there were no mortalities during the experiment. The overall growth performance and feed utilization were similar to earlier studies on Atlantic salmon (Austreng et al., 1987; Hatlen et al., 2012), or even better compared to Atlantic salmon of comparable size fed fishmeal-based feeds (Kiron et al., 2016; Sørensen et al., 2017). Feeding Atlantic salmon with 10% pre-extruded *N. oceanica* had no negative effect on feed intake, final mean body weight, weight gain, specific growth rate, and thermal growth coefficient. The present findings suggest that if the feeds are carefully balanced for essential amino acids and other essential nutrients, fishmeal incorporation can be reduced to 7.5% or even lower without compromising the growth (Kousoulaki et al., 2018; Kousoulaki et al., 2013). In contrast to Sørensen et al. (2017), who reported higher feed intake when salmon were fed defatted *N. oceanica*, there were no differences in feed intake in the present experiment. These findings are in line with Kiron et al. (2012) and Sprague et al. (2015) who reported no effect on feed intake when Atlantic salmon were fed *Nanofrustulum* sp. or *Tetraselmis* sp. at 10% inclusion rate, or *Schizochytrium* sp. at 11% inclusion level. In contrast, Atlantic salmon fed feeds containing 12% dried whole cells of the microalga *Phaeodactylum tricornutum* had reduced feed intake (Sørensen et al., 2016).

The growth of the fish in the present experiment was not impacted as noted for Atlantic salmon fed *Nanofrustulum* sp. or *Tetraselmis* sp. at 10% inclusion rate (Kiron et al., 2012). Other studies have reported negative effects on growth and/or feed conversion ratio when Atlantic salmon were fed feeds with *Desmodesmus* sp. (10/20%), *Schyzochrytrium* sp. (11%), or *P. tricornutum* (12%) (Kiron et al., 2016; Sørensen et al., 2016; Sprague et al., 2015). Taken together, the contrasting results suggest that direct comparison of microalgae varieties across experiments are difficult. The responses in the fish depend on the species and size, feed formulation, nutrient contents of feeds and their availability.

Improved growth, feed utilization and health effects have been reported in fish fed plant essential oils- supplemented feeds (Sutili et al., 2018). Giannenas et al. (2012) investigated the effect of supplementing two phytogenic feed additives containing either 1.2% carvacrol or 0.6% thymol on the performance of rainbow trout and found a significantly higher feed efficiency compared to the control group fed a basal diet. Nutrient digestibility were also improved in farmed land animals, e.g., broiler chickens, when their feeds were supplemented with Digestarom® (Murugesan et al., 2015). In line with our results, studies with channel catfish (*Ictalurus punctatus*) and gilthead seabream (*Sparus aurata*) also reported no effects on digestibility of dry matter and protein, growth performance and FCR when feeds were supplemented with 0.02% Digestarom® PEP MGE150 (Peterson et al., 2014; Rodrigues et al., 2018). The second additive tested in the present study is a clinoptilolite and a natural zeolite. Zeolites can be natural or synthetic materials with unique structure and physicochemical properties (e.g. detoxifying effects; antioxidant effect, effects on microbiota) (Ghasemi et al., 2016; Pavelić et al., 2018). It is used as a mycotoxin-binder in the feeds of terrestrial animals and it also improves gut health by preventing diarrhea in calves and pigs (Ghasemi et al., 2016; Papaioannou et al., 2005). Although the ability of clinoptilolite as health and growth

promoters in fish have not been studied much, there are reports suggesting improved growth rate and feed utilization in fish species such as gilthead sea bream and rainbow trout (Eya et al., 2008; Kanyılmaz et al., 2015). ZEOfeed did not have any significant effect on nutrient digestibility, FCR or growth of salmon in the present experiment. The dissimilar effects of these two feed additives noted in several studies may be attributed to the fish species, inclusion levels of the additives and duration of feeding period. Long-term feeding trials with species-specific optimal doses should confirm the benefits of the feed additives.

Histomorphological changes are not likely to explain the differences in nutrient digestibility as no clear differences were noted on villi length, width and gut health among the fish fed the different feeds. The immunohistochemistry analysis was performed to get an in depth understanding of the tissue homeostasis and the technique has earlier been used to study toxic mechanisms (Sanden and Olsvik, 2009) and intestinal inflammation (Bjørgen et al., 2018; Romarheim et al., 2010). The PCNA has a regulatory role in DNA replication and control of cell cycle. Although increased PCNA staining cannot be used as an independent indicator of cell activity (Maga and Hübscher, 2003), the increased staining in the ZEOfeed group is suggestive of greater cell proliferation in the intestine of this group. There were no other signs of ill-health to indicate the negative effect of the increased cell proliferation. Further in depth studies should gather more information about the effect of the increased cell proliferation on intestinal health.

4.3. Proximate composition of the fish

The whole body proximate composition of Atlantic salmon was not affected by either the intake of the microalgae or the feed additives. Whole body protein of fish in the present study was lower and lipid content of fish was higher than values (protein 55–58% of DM, lipid 29–37% of DM) reported for Atlantic salmon fed microalgae feed (Kiron et al., 2016; Sørensen et al., 2017). The proximate composition can vary with life stages of the fish and is also influenced by endogenous factors such as genetics, size and sex, as well as exogenous factors such as feed composition, feeding frequency and environment (Shearer, 1994). The ash content of the fish in the present study was in line with the values reported for fish fed microalgae feed (Kiron et al., 2016; Sørensen et al., 2016; Sørensen et al., 2017). It should be noted that because of the unavailability of the initial fish samples the nutrient retention values that would have given more valuable information cannot be discussed here. Additional studies are required to document nutrient retention efficiencies of fish fed these diets.

4.4. Fatty acid composition of the fish

In salmonid fish, the whole body fatty acid compositions are closely related to the fatty acid profile of the feed (Sissener, 2018; Sprague et al., 2016; Teimouri et al., 2016). The fatty acid composition in the experimental feeds used in the present experiment showed only minor differences and was also reflected in the whole body composition of the fish fed the different experimental feeds. The major differences observed for the n-6 PUFAs in whole body of fish fed algae feeds could be attributed to LA and arachidonic acid (C20:4n-6, ARA). The higher content of PUFA in fish fed NZ also can be explained by an increased content of LA, ARA and a trend towards increased EPA. The most noteworthy finding in this study was that the whole-body EPA + DHA levels of fish fed the algae diets were maintained at the same levels as the CO diet, even with a 50% reduction in fish meal and a 10% reduction in fish oil.

5. Conclusion

The present study showed that incorporation of 10% pre-extruded *Nannochloropsis oceanica* in plant-based commercial-like feeds reduced the lipid digestibility but did not affect the growth, feed utilization or body proximate composition. A slightly increased cell proliferation was observed for fish fed the microalga and was further increased by supplementation of feeds with ZEOfeed. Otherwise, the feed additives Digestarom® and ZEOfeed did not demonstrate any distinct advantage at their respective inclusion levels in salmon feed. The content of EPA and DHA was unaffected when fishmeal/fish oil was reduced from 15%/10% to 7.5%/9%, respectively.

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CRediT authorship contribution statement

Yangyang Gong: Investigation, Formal analysis, Writing - review & editing. **Solveig L. Sørensen:** Investigation, Formal analysis, Writing - review & editing. **Dalia Dahle:** Investigation. **Nimalan Nadanasabesan:** Formal analysis. **Jorge Dias:** Investigation. **Luisa M.P. Valente:** Supervision, Funding acquisition. **Mette Sørensen:** Conceptualization, Methodology, Investigation. **Viswanath Kiron:** Conceptualization, Methodology, Investigation, Project administration, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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