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# Microalgae as functional feed for Atlantic salmon: effects on growth, health, immunity, muscle fatty acid and pigment deposition

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Microalgae are increasingly being investigated as functional feed additives in a variety of fish species, but our knowledge on how microalgae supplementation affects Atlantic salmon remains limited. We hypothesized that microalgae inclusion of 8% in the feed would improve performance, fatty acid and pigment deposition as well as health and immunity of Atlantic salmon reared in recirculating aquaculture systems (RAS). We fed Atlantic salmon smolts with five different microalgae enriched diets containing *Tetraselmis chuii* (TC), *Arthrospira platensis* (AP), *Schizochytrium limacinum* (SL) or *Chlorella vulgaris*, either intact (CVI) or as broken cell wall derivative (CVB) or a control diet (CD). After eight weeks of feeding in brackish water (13 psu), all groups were transferred to seawater (32 psu) for additional two weeks. Our results indicate that CVB improved feed conversion and protein retention, but reduced condition factor ( $p < 0.05$ ) compared to fish fed with a control diet. Voluntary feed intake decreased in seawater, but was similar among diet groups. The amount of docosahexaenoic acid was particularly high in SL-fed fish and alpha-linolenic acid was enriched in fish fed CVI, CVB and TC ( $p < 0.05$ ). Following seawater transfer, fat content and monounsaturated fatty acids decreased in the muscle, while polyunsaturated fatty acids increased. Lutein was present in all muscle samples, but highest concentrations were found in CVB-, CVI- and TC-fed fish. In the anterior intestine, microalgae supplementation induced differentially regulated trout protein 1 (*drtp1*) expression in CVI- and CVB-fed fish, but reduced the expression of interleukin 1 and 10 receptor (*il1r2* & *il10rb*) in CVI-fed fish. In the liver, feeding CVI and SL induced complement C1q like 2 (*c1ql2*) expression, while reducing serum amyloid A5 (*saa5*) expression. Superoxide-

dismutase protein concentration was induced in the liver of fish fed SL, while myeloperoxidase was reduced in most microalgae-fed groups. In conclusion, we show that commercially relevant microalgae can be used as functional feed additives for Atlantic salmon promoting different health aspects without negatively affecting their growth performance when cultivated in RAS.

#### KEYWORDS

microalgae, functional feed, bioactive compound, fatty acids, carotenoids, immunity, fish health, Atlantic salmon

## 1 Introduction

Feed in aquaculture is a finite resource, which provides nutrients but should also promote the growth and health of farmed fish. Consequently, interest is growing in developing functional feeds that guarantee good fish health, improve performance and mitigate farming related stressors. Although a variety of compounds have been investigated as functional ingredients in fish feed, microalgae have only recently been considered. These single cell algae contain different types of polysaccharides, sulfolipids, polyunsaturated fatty acids and pigments (Ricchio and Lauritano, 2020). Green algae such as *Chlorella* sp. and *Tetraselmis* sp. are rich in pigments such as chlorophylls and carotenoids, while heterotrophic *Schizochytrium* (herein considered as microalgae) contain high amounts of docosahexaenoic acid (DHA; Nakahara et al., 1996; Ren et al., 2010).

The diverse chemical composition of microalgae holds potential for a variety of biological activities, including antioxidant (Carballo et al., 2018; Teimouri et al., 2019), antimicrobial (Guzmán et al., 2019) as well as anti-inflammatory activities (Fujii, 2000; Guzmán et al., 2003). Feeding microalgae to different fish species was found to affect their health and immunity. Including *Chlorella vulgaris* into the feed, for instance, counteracted soy-bean meal-induced intestinal inflammation in Atlantic salmon *Salmo salar* (Grammes et al., 2013) and zebrafish *Danio rerio* (Bravo-Tello et al., 2017). Dietary administration of *Chlorella sorokiniana* further stimulated humoral innate immunity in rainbow trout *Oncorhynchus mykiss* (Chen et al., 2021), while *Tetraselmis chuii* and *Phaeadactylum tricornutum* increased phagocytotic and complement activity in gilthead seabream *Sparus aurata* (Cerezuela et al., 2012a).

Although health and immune promoting effects are described for different microalgae species, the majority of studies in fish has investigated microalgae as a source to replace fishmeal or fish oil in the feed (Carvalho et al., 2020; Kousoulaki et al., 2020; Sarker et al., 2020a). Studies evaluating the functional properties of different microalgae species under challenging environmental conditions are currently missing.

Such challenging environmental conditions can occur during the production of Atlantic salmon in recirculating aquaculture systems (RAS). Production of Atlantic salmon in RAS is globally expanding (Bergheim et al., 2009; Davidson et al., 2021). This is because these systems allow a controlled production environment

with a high level of biosecurity and a significantly reduced discharge of waste products into the aquatic environment (Dalsgaard et al., 2013; Ahmed and Turchini, 2021). However, the RAS environment is considered particularly challenging for fish health due to higher stocking densities (Calabrese et al., 2017), accumulation of waste products (Ruyet et al., 2008), as well as water disinfection treatment (Soleng et al., 2019; Stiller et al., 2020). Dietary mitigation under these conditions may be a promising strategy to improve overall health and performance of salmon cultivated in RAS. Currently the use of a brackish water phase in RAS before seawater transfer is investigated as an alternative to shorten the production time in the sea (Ytrestøyl et al., 2020; Ytrestøyl et al., 2023). While it is well established that transfer to seawater of Atlantic salmon is associated with a drastic stress-related reduction in appetite (Usher et al., 1991), little is known about whether microalgae, which contain different amino and fatty acids, could potentially increase feed intake in this critical time period.

A thorough assessment of the fish's health and immune status in functional feeding studies requires investigating both transfer of functional components from feed to fish and their subsequent effects *in vivo*. The former is usually performed using analytical chemistry to trace fatty acids, pigments and other functional compounds. The latter requires investigating different aspects of the fish's health and immune status. Functional feeds are expected to provoke a local response in the intestine (Bae et al., 2020; López Nadal et al., 2020), but also systemic effects may occur. These are reflected in physiological alterations of the blood plasma, the spleen and the liver, being constantly exposed to antigens from the bloodstream (Bayne et al., 2001; Wu et al., 2016).

In this study, we aimed to elucidate whether functional diets enriched with different commercially relevant microalgae species at 8% inclusion in the feed can improve performance, health and immune status of Atlantic salmon reared in RAS. Since seawater transfer of salmon smolts is a particular critical time period following land-based rearing in RAS (Usher et al., 1991; Karlsen et al., 2018), we evaluated diet dependent effects during this time-period in addition. Alongside with performance indicators, fatty acid and pigment profiles, a set of putative biomarkers on gene and protein level in plasma, liver, intestine and spleen were used to evaluate the health and immune status of the salmon.

## 2 Materials and methods

### 2.1 Feed formulation

Six isonitrogenous and isoenergetic (on dry matter basis; see Table 1) experimental diets were formulated based on the nutrient requirements of Atlantic salmon (National Research Council, 2011). The diets were designed to include one of the following microalgae: *Chlorella vulgaris*, *Tetraselmis chuii* (TC), *Arthrospira platensis* (AP) or *Schizochytrium limacinum* (SL) at an inclusion level of 8%. Two different *Chlorella vulgaris* were included in the experimental design, one had an intact (CVI) and the other a broken cell wall (CVB). The microalgae were obtained from a commercial supplier and were cultivated in both open and closed bioreactors under commercial settings (Supplementary Table 1). All microalgae were spray-dried after harvesting. Inclusion of the microalgae was done in exchange for wheat starch, wheat gluten and canola oil. In contrast to other studies basal feed components (e.g. fish meal and fish oil) were kept constant in the diet formulation and hence allowed to evaluate the direct effect of every microalgae ingredient. The experimental diets were pelletized (Type 14U175, Amandus Kahl, Hamburg, Germany) at temperatures below 60°C to pellets with 4 mm diameter and stored at 4°C in the dark before and during the trial.

### 2.2 Experimental setup

The experiment was conducted at the facilities of the Fraunhofer IMTE, Büsum, Germany. Atlantic salmon smolts were obtained from Jurassic Salmon, Poland and were acclimated for two months in a recirculating aquaculture system. During acclimation, the fish were fed

a commercial salmon diet (Aller Aqua, Denmark). Water treatment of the RAS (7.6 m<sup>3</sup>, turnover rate 4 times h<sup>-1</sup>) consisted of a moving bed biofilter, a bead filter (PolyGeyser, Model DF-6, Aquaculture Systems Technologies, L.L.C., New Orleans, LA, USA), a protein skimmer and UV-light disinfection. Water quality parameters were measured on a daily basis (NH<sub>4</sub><sup>+</sup> and NO<sub>2</sub><sup>-</sup> biweekly) and kept in a suitable range for Atlantic salmon (13.5 ± 0.4°C, 7.3 ± 0.1 pH, 10.3 ± 0.2 mg/L O<sub>2</sub>, 0.2 ± 0.1 mg/L NH<sub>4</sub><sup>+</sup>, 0.2 ± 0.04 mg/L NO<sub>2</sub><sup>-</sup>, (Microquant test kit for NH<sub>4</sub><sup>+</sup> and NO<sub>2</sub><sup>-</sup>, Merck, Darmstadt, Germany). Salinity was set to 13.0 ± 0.8 psu (HI 96822 Seawater Refractometer, Hanna Instruments Inc., Woonsocket-RI, USA) by mixing freshwater and seawater. Light was provided for 24 h throughout the experimental period. Prior to the start of the experiment Atlantic salmon smolts (mean body weight 82.32 ± 1.96 g) were randomly divided into six different groups in triplicate, each consisting of 28 fish and stocked into 18 tanks (300 L) of the RAS (Figure 1). Following rearing the fish for eight weeks in brackish water, all of the fish were transferred into a new RAS system, which was identical to the other, but operated with full strength seawater (salinity of 31.8 ± 0.5 psu). Water parameters for this system were as followed: 13.4 ± 0.3°C, 7.2 ± 0.1 pH, 10.4 ± 0.3 mg/L O<sub>2</sub>, 0.2 ± 0.1 mg/L NH<sub>4</sub><sup>+</sup>, 0.2 ± 0.07 mg/L NO<sub>2</sub><sup>-</sup>. The fish were kept under these conditions for additional two weeks before the experiment was terminated. The fish were fed manually twice per day (8 a.m. and 2 p.m.) until apparent satiation during the entire experiment. Leftover pellets were collected, counted and used to calculate feed intake.

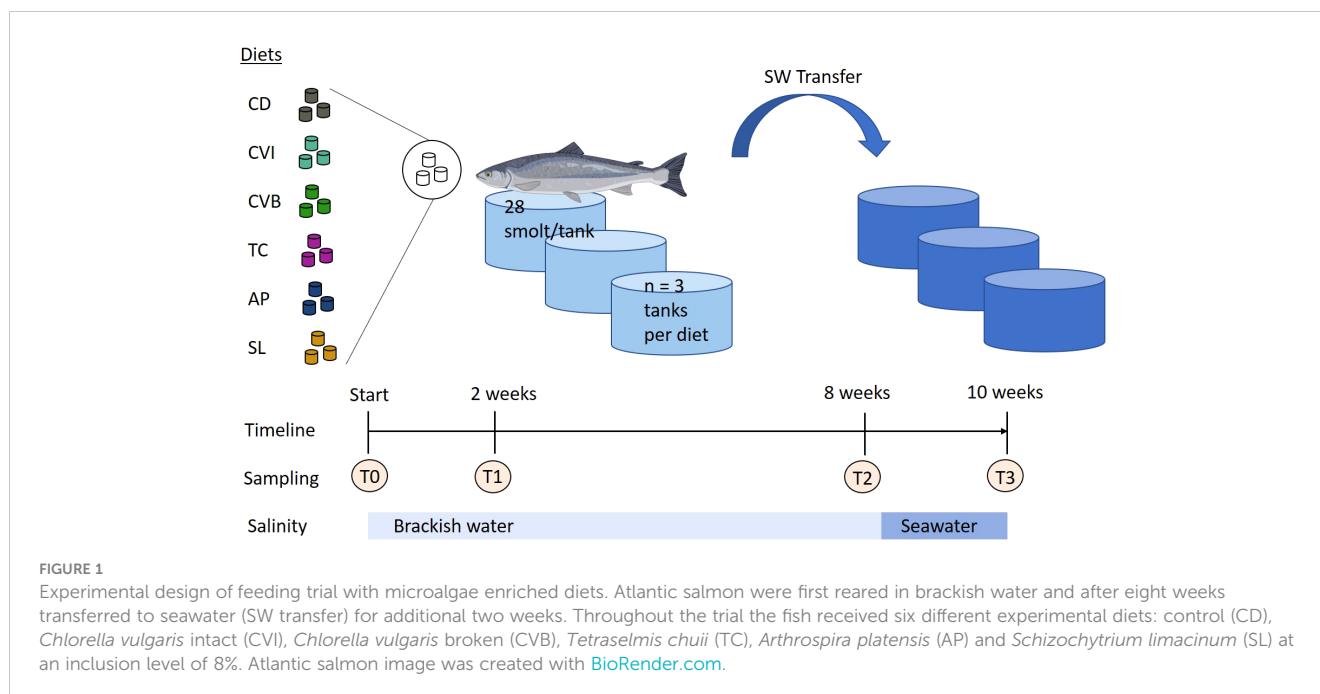
### 2.3 Fish sampling

Samples were collected before the onset of the experiment (T0), after two weeks (T1) and eight weeks of feeding the experimental

TABLE 1 Feed formulation of experimental diets in g/100g dry matter (DM).

Ingredients (g/100g DM)	CD	CVI	CVB	TC	AP	SL
Fish meal <sup>1</sup>	15	15	15	15	15	15
Microalgae	0	8	8	8	8	8
Blood meal <sup>2</sup>	6	6	6	6	6	6
Gelatine <sup>3</sup>	5	5	5	5	5	5
Pea protein isolate <sup>4</sup>	14	14	14	14	14	14
Soy protein concentrate <sup>5</sup>	11	11	11	11	11	11
Wheat gluten <sup>6</sup>	12	7.17	6.53	8.44	5.25	9.44
Wheat starch <sup>6</sup>	21.4	18.45	18.85	17.86	20.2	19.3
Canola oil <sup>7</sup>	5.5	5.5	5.5	6.1	5.5	2.5
Fish oil <sup>1</sup>	6	6	6	6	6	6
Methionine <sup>8</sup>	0.1	0.1	0.1	0.1	0.1	0.1
Vitamin & mineral premix <sup>4</sup>	0.5	0.5	0.5	0.5	0.5	0.5
CaHPO <sub>4</sub> <sup>9</sup>	2	2	2	2	2	2
Bentonite <sup>10</sup>	1.5	1.28	1.52	0	1.45	1.16

<sup>1</sup>Bioceval GmbH & Co. KG, Cuxhaven; Germany; <sup>2</sup>Saria SE & Co. KG, Selm, Germany; <sup>3</sup>Gustav Ehlert GmbH & Co. KG, Verl, Germany; <sup>4</sup>Emsland-Aller Aqua GmbH, Gollßen, Germany; <sup>5</sup>EURODUNA Rohstoffe GmbH, Barmstedt, Germany; <sup>6</sup>Kröner-Stärke GmbH, Ibbenbüren, Germany; <sup>7</sup>Cargill GmbH, Riesa, Germany; <sup>8</sup>Evonik Industries AG, Essen, Germany; <sup>9</sup>Lehmann & Voss & Co. KG, Hamburg, Germany; <sup>10</sup>Del Lago Bentonite, Castiglioni Pes y Cia., Buenos Aires, Argentina.



diets in brackish water (T2) and two weeks following transfer into seawater (T3; Figure 1). At each sampling nine fish per treatment (three per tank) were randomly sampled. The fish were quickly netted from the experimental tanks and euthanized by an overdose of buffered MS-222 (0.3 mg/L). For each fish total length and total weight was recorded. 2 ml of blood was collected in heparinized syringes by caudal vein puncture. The blood was transferred into 2 ml Eppendorf tubes and centrifuged at 4000 g for 8 min. Aliquots of the plasma were flash-frozen on dry ice and stored at  $-80^{\circ}\text{C}$  for the determination of plasma metabolites, total carotenoid content, and enzyme activities.

The liver and spleen were carefully removed and weighed for the calculation of organ specific indices. At the end of the brackish water phase (T2) a piece of the liver, anterior intestine and the spleen was placed in an RNase free tube and flash frozen in liquid nitrogen for gene expression analysis. In addition, after two (T1) and eight weeks (T2) of feeding the experimental diets a piece of the liver was flash-frozen on dry ice for later protein analysis using western blots.

At T0, T2 and T3 both fillets from every fish were taken, de-skinned, homogenized by means of a knife-mill (Grindomix GM200, Retsch GmbH, Haan, Germany) and stored at  $-40^{\circ}\text{C}$  for later analysis of proximate composition, fatty acid profile, as well as carotenoid content. At T0 and T2 three additional fish per tank were sampled and pooled for the analysis of whole-body proximate composition.

## 2.4 Proximate composition of whole body and diets

Proximate composition was analyzed in microalgae (Supplementary Table 2), diets (Table 2) and whole-body

homogenates in duplicates using the same methods. Whole body samples were freeze-dried (Alpha 1-2 LD plus and Alpha 1-4 LSC, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) until a stable weight was achieved and homogenized using a knife mill (GM 200, Retsch GmbH). Nutrients and gross energy were analyzed according to EU guideline (EC) 152/2009. Dry matter content was determined following drying of samples at  $103^{\circ}\text{C}$  in a drying oven for 4 h (ED 53, Binder GmbH, Tuttlingen, Germany). Ash content was determined after combustion in a combustion oven at  $550^{\circ}\text{C}$  (P300, Nabertherm, Lilienthal, Germany). Crude protein content was analyzed following the Kjeldahl method (InKjel 1225M, WD30, Behr, Düsseldorf, Germany). Crude lipid content was extracted with petroleum ether in a Soxhlet extraction system (Soxtherm, Hydrotherm, Gerhardt Königswinter, Germany) and quantified gravimetrically. Gross energy was determined using a bomb calorimeter (C 200, IKA, Staufen, Germany).

## 2.5 Diet and microalgae fatty acid composition

To physically break down the material, 10 g of each diet and microalgae (Supplementary Table 3) was ground with a mortar, mixed with 20 ml of distilled water and homogenised for 2 min with an Ultra-Turrax disperser (IKA). The slurry was lyophilised and mortared again. To determine the fatty acid composition, 20 mg sample (weighed to the nearest 0.1 mg) was then subjected to direct transesterification according to Griffiths et al. (2010). However, boron trifluoride-methanol was replaced by 3 M methanolic HCl (Sigma-Aldrich, Taufkirchen, Germany) and distilled water by 1 M aqueous NaCl (Merck, Germany). All other reagents were from Sigma-Aldrich. Each diet was transesterified in triplicate and the

TABLE 2 Crude composition (percent dry matter) as well as fatty acid composition (mg/100g dry matter) and pigment composition ( $\mu\text{g}/100\text{g}$  dry matter) of the experimental diets given as mean of two and pigment concentrations as mean of four replicate analyses.

Chemical composition (% DM)	CD	CVI	CVB	TC	AP	SL
Dry matter (%)	90.03	91.52	91.59	92.90	92.08	93.02
Crude protein (%)	51.04	51.26	51.00	50.93	51.11	51.38
Fat (%)	16.18	16.38	16.60	16.87	16.19	15.11
Ash (%)	7.47	8.15	8.28	8.61	8.07	7.72
Crude energy (MJ/kg)	22.72	22.71	22.68	22.72	22.69	22.70
<b>Fatty acids (mg/100 g DM)</b>						
C14:0	321	317	320	320	312	336
C15:0	30	37	40	40	36	34
C16:0	1570	1600	1601	1645	1672	1943
C17:0	26	27	29	28	26	28
C18:0	335	335	339	347	333	317
C20:0	57	55	56	59	56	47
C22:0	30	30	29	30	26	25
C24:0	28	30	25	27	27	26
<b>total SFA</b>	<b>2397</b>	<b>2431</b>	<b>2438</b>	<b>2496</b>	<b>2488</b>	<b>2757</b>
C16:1n11	21	30	24	21	20	20
C16:1n9	21	32	37	27	27	21
C16:1n7	332	342	367	336	346	337
C16:1n5	18	18	17	17	18	18
C16:1n3	13	13	14	13	14	14
C17:1n8	21	23	24	22	25	19
C18:1n9	5128	5014	5011	5490	5000	3543
C18:1n7	395	402	443	448	390	331
C18:1n5	21	20	20	20	21	21
C20:1n9	550	538	537	560	531	513
C20:1n7	32	29	33	33	33	29
C22:1n11	687	679	678	689	668	688
C22:1n9	83	76	74	85	73	71
C24:1n9	58	56	57	58	56	55
<b>total MUFA</b>	<b>7380</b>	<b>7272</b>	<b>7337</b>	<b>7817</b>	<b>7222</b>	<b>5677</b>
C16:2n6	n.d.	37	51	13	11	11
C18:2n6	2409	2360	2371	2409	2307	1836
C18:3n6	11	11	11	23	93	13
C20:2n6	41	40	39	42	40	39
C20:3n6	13	13	12	14	14	18
C20:4n6	41	40	41	43	41	45
C22:5n6	22	22	21	22	21	401
<b>total n6 PUFA</b>	<b>2537</b>	<b>2524</b>	<b>2545</b>	<b>2567</b>	<b>2527</b>	<b>2362</b>

(Continued)

TABLE 2 Continued

Fatty acids (mg/100 g DM)	CD	CVI	CVB	TC	AP	SL
C16:3n3	n.d.	71	96	n.d.	n.d.	n.d.
C16:4n3	n.d.	n.d.	12	89	n.d.	n.d.
C18:3n3 ALA	622	727	792	747	627	452
C18:4n3 SDA	143	144	146	178	142	154
C20:3n3	18	19	20	18	19	19
C20:4n3	48	47	50	49	48	70
C20:5n3 EPA	415	410	414	441	410	440
C22:5n3	86	84	86	88	85	89
C22:6n3 DHA	613	604	609	614	607	2518
<b>total n3 PUFA</b>	<b>1946</b>	<b>2107</b>	<b>2226</b>	<b>2223</b>	<b>1939</b>	<b>3742</b>
C16:2n4	24	24	28	24	26	26
C16:3n4	53	52	51	54	51	54
<b>total PUFA</b>	<b>4560</b>	<b>4706</b>	<b>4850</b>	<b>4868</b>	<b>4543</b>	<b>6184</b>
<b>n3 HUFA</b>	<b>1181</b>	<b>1165</b>	<b>1180</b>	<b>1209</b>	<b>1169</b>	<b>3136</b>
<b>Carotenoids (µg/100 g DM)</b>						
Lutein	29.8	3887	13251	3346	n.d.	36.9
Violaxanthin	n.d.	n.d.	n.d.	2186	n.d.	n.d.
Neoxanthin	n.d.	406	1548	4277	n.d.	n.d.
Zeaxanthin	n.d.	133	358	n.d.	3339	n.d.

n.d., not detected; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; HUFA, highly unsaturated fatty acids with 20 or more carbon atoms and 3 or more double bonds; ALA,  $\alpha$ -Linolenic acid; SDA, stearidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

fatty acid methyl ester (FAME) extracts used for separate gas chromatography (GC) analysis. Fatty acid contents were calculated as mg FAME/100 g dry matter (Table 2). For GC conditions, see the section on muscle proximate and fatty acid composition.

## 2.6 Proximate and fatty acid composition of muscle

Moisture and ash content were determined by drying samples of around 5 g for 12 h at 105°C, followed by ashing at 550°C. The nitrogen content was measured by Dumas using a LECO TruSpecN (Leco Instruments GmbH, Mönchengladbach, Germany). Lipids were extracted according to Smedes (1999) using cyclohexane and 2-propanol (VWR, Darmstadt, Germany) with modifications by Karl et al. (2012). For GC analysis of fatty acids, lipids were obtained in a separate extraction without final drying at 105°C and transesterified into FAME using methanolic potassium hydroxide (ISO-IDF, 2002).

Fatty acid analysis was performed using a 7890A gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with a 7683B autosampler, a split injection port (injection volume 1 µL, split 1:100), flame ionisation detection and a 100 m x 0.25 mm i.d. x 0.20 µm - CP-Sil 88 column (Agilent Technologies).

Hydrogen was used as the carrier gas with a constant flow of 1.6 mL min<sup>-1</sup>. Two min after injection, the initial oven temperature of 175°C was increased by 1°C per minute to 190°C, held constant for 1 min, then increased by 5°C per minute to 225°C, held constant for 7 min, and finally increased by 1°C per minute to 237°C (1 min constant). Chromatograms were evaluated using EZChrom Elite 3.3.2 (Agilent Technologies). Identification of individual FAME was achieved by comparison to known standards (Supelco<sup>TM</sup> 37 Component FAME mix, PUFA No. 1, PUFA No. 3; all obtained from Sigma-Aldrich) in the range from C14:0 to C22:6n3. Fatty acid contents for fillet samples were calculated as weight percentage (g FA/100 g FA) and are given as means of duplicate analyses.

## 2.7 Carotenoid content

Carotenoids were extracted from the diets and microalgae with methanol using an Ultra-Turrax (IKA) at 24,000 rpm for two cycles of 45 s (Schüler et al., 2020). Samples were centrifuged and the supernatants of three repetitions were combined. During extraction the samples were kept on ice. The extraction of the fish fillet homogenate was done according to Ostermeyer and Schmidt (2004). After an initial evaluation of carotenoids in individual fish samples (n = 18), muscle samples were pooled on a tank level (n = 3).

For the quantitative determination of the carotenoids an aliquot of the extract was evaporated and the residue dissolved in mobile phase. HPLC was carried out using a C30 analytical column (5  $\mu\text{m}$ , 250 x 4.6 mm i.d., YMC Europe, Dinslaken, Germany) preceding by a C30 guard column (5  $\mu\text{m}$ , 10 x 4.0 mm i.d.) and a gradient of methyl tert-butyl ether, methanol with a small amount of an ammonium acetate buffer (pH 4.6), similar to (Rasmussen et al., 2012). A flow rate of 1.0 mL min<sup>-1</sup> at 25°C with an injection volume of 100  $\mu\text{L}$  was used. The detection was performed with a photodiode array detector (UV 6000 LP; Thermo Finnigan, San Jose, CA, USA) at 450 nm and 470 nm. Peaks were identified by comparison of the retention times and the absorption spectra (between 380 nm and 700 nm) with those of synthetic standards. The carotenoids were quantified using an external standard containing lutein, fucoxanthin, violaxanthin, neoxanthin, astaxanthin, zeaxanthin and canthaxanthin (ChromaDex, Irvine, CA, USA; Sigma-Aldrich; Dr. Ehrenstorfer, Augsburg, Germany).

## 2.8 Plasma metabolites and enzyme activities

Plasma glucose, triglycerides, total protein, alkaline phosphatase (ALP), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activity were measured on a Fuji Dry Chem NX500i (Fujifilm, Ratingen, Germany) using commercial kits and following the manufacturer's instructions.

Total carotenoid content in plasma samples (Supplementary Figure 1) was measured after Donaldson (2012) with slight modifications. Briefly, 100  $\mu\text{L}$  of plasma was mixed with 100  $\mu\text{L}$  of 70% ethanol in a 1.5 mL reaction tube wrapped with aluminium foil and vortexed for one minute to precipitate the proteins. Then 300  $\mu\text{L}$  of n-heptane (Roth, Karlsruhe, Germany) was added and the mixture was vortexed for four minutes at maximum speed. The mixture was centrifuged at 2000 g for two minutes. Following separation of both layers 290  $\mu\text{L}$  of the heptane layer including the dissolved carotenoids were decanted and added to a 10 mm quartz cuvette (type 104-QS, Hellma, Müllheim, Germany) and further diluted with 410  $\mu\text{L}$  of heptane. The absorbance was measured at 448 nm using a spectrophotometer (SPECORD210, Analytik Jena GmbH, Jena, Germany). The carotenoid content was then calculated according to Donaldson (2012). Spectral profiles of every sample (300 – 600 nm) confirmed the presence of a carotenoid peak at ~ 448 nm (Supplementary Figure 1B). Preparation of extracts and measurements were performed under reduced light conditions to minimize pigment degradation in the samples.

## 2.9 Western blots of liver proteins

Total protein from liver samples (n = 3 pool per tank) was extracted with Radioimmunoprecipitation (RIPA) lysis buffer according to the manufacturer's protocol (RIPA Lysis Buffer System, Santa Cruz Biotechnology, Dallas, Texas, USA). The proteins Cu, Zn superoxide dismutase (Sod1) and

myeloperoxidase (Mpo) were analyzed in the salmon liver per SDS-PAGE and Western Blot. The protein  $\beta$ -actin served as loading control. A no template control and one positive control per antibody were included, *Danio rerio* liver for Mpo, Bovine liver for Sod1 and HEK-293 cells for  $\beta$ -actin. 20  $\mu\text{g}$  total protein was processed in reducing conditions with SDS sample and reducing buffer (both TruPAGE, Sigma-Aldrich, Schnellendorf, Germany) at 70°C for 10 min. SDS-PAGE was performed in a Xcell SureLock Mini-Cell (Thermo Fisher Scientific, Waltham, Massachusetts, USA) using precast 4-12% gradient gels, TruPAGE running buffer and antioxidant (Sigma-Aldrich). Proteins were electro-transferred to a PVDF membrane. For parallel protein detection of Mpo and Sod1 the membrane was horizontally cut. Primary antibody incubation, with Mpo antibody (ab210563, Abcam, Cambridge, UK) in 1:5000 dilution in PBS-T containing 2.5% skim milk and Sod1 antibody (NBP2-24915, Novus Biologicals, Bio-Techne Ltd., Abingdon, UK) in 1:500 dilution in PBS-T containing 2.5% skim milk, was performed at 4°C overnight. Secondary antibody anti-rabbit IgG conjugated HRP (sc-2357, Santa Cruz Biotechnology) was incubated in a 1:5000 dilution for 90 min at room temperature. Detection was performed using ECL detection reagents (Amersham, Global Life Sciences Solutions USA LLC, Marlborough, MA, USA) and chemiluminescence film (Amersham, GE Healthcare Ltd, Little Chalfont, UK) with 40 sec exposure time for both Mpo and Sod1. For the subsequent detection of  $\beta$ -actin the antibodies were stripped using 100 mM Glycin buffer (pH 2.5). The membrane was incubated in 1:5000 dilution of  $\beta$ -actin antibody (NB600-503, Novus Biologicals, Bio-Techne Ltd., Abingdon, UK) at 4°C overnight. Quantification of protein expression was done following densitometric analysis of the protein bands using GIMP and normalized to housekeeping ( $\beta$ -actin) protein expression.

## 2.10 Gene expression in liver, spleen and intestine

Total RNA was extracted using TRIzol (ThermoFisher Scientific, Waltham, MA, USA) and further purified with the ISOLATE II RNA Micro Kit (Meridian Bioscience Inc., Cincinnati, OH, USA). The concentration and integrity of the extracted RNA was measured by NanoDrop One (Thermo Fisher Scientific). Subsequently, cDNA synthesis was performed using Reverse Transcription Master Mix (Fluidigm, San Francisco, CA, USA). The samples were preamplified by the PreAmp Master Mix (Fluidigm) and at last treated with exonuclease I (New England BioLabs, Frankfurt/Main, Germany). All steps have been carried out according to the manufacturer's instructions.

45 genes with tissue specific regulation were selected from an established gene set composed of key immune and stress regulated genes in Atlantic salmon (Krasnov et al., 2020; Lund et al., 2022; Supplementary Table 4). We extended this set by three immunogene-specific primers derived for *hamp*, *saa5* and *sod1* (Supplementary Table 4). The same 48 genes (45 target and 3 reference genes) were measured in the tissue anterior intestine, liver and spleen.

The 48.48 gene expression biochips were primed in the MX IFC Controller (Standard BioTools, San Francisco, CA, USA). The pre-amplified cDNA samples were pipetted to the sample inlets and the primers were loaded on the assay and finally, analyzed with the Biomark HD using the manufacturer's thermal protocol "GE Fast 48 × 48 PCR+Melt v2.pcl" (application type: gene expression; passive reference: ROX; assay: single probe).

The raw qPCR Ct values were obtained using the Fluidigm real-time PCR analysis software v. 3.0.2 (Munich, Germany). Relative expression was calculated based on  $\Delta\Delta Ct$  where three reference genes coding for b-actin, ribosomal protein L4 and ribosomal protein S20 (*actb*, *rpl4*, *rps20*) were used as internal normalisers. The mean Ct per gene for all samples was used as a calibrator during the calculation. Relative expression values were log2 transformed prior to statistical analysis. Four individuals with abnormal phenotypic signs and subsequently abnormally high gene expression profiles were removed from the dataset. The genes *cxcl8* and *il1b* in the liver and *cxcl8* in the spleen were removed from the dataset, as to many missing values hampered analysis of the data.

## 2.11 Statistical analysis

Statistical analysis and data visualization were conducted using the software R (R version 4.1.0) in the environment RStudio. For all test  $\alpha = 0.05$  was used as the level of significance. Data is presented as mean  $\pm$  standard error of mean (SEM). For the performance parameters, protein concentrations in the liver as well as carotenoid concentrations in the muscle an appropriate statistical model based on generalized least squares was defined (Carroll and Ruppert, 1988) which included the factor diet as well as timepoint for the latter two. The residuals were assumed to be normally distributed and to be heteroscedastic, which was based on a graphical residual analysis. Analysis of variance (ANOVA) was conducted, followed by multiple contrast tests for heteroscedastic data (Hasler and Hothorn, 2008) in order to compare the several diets with the control diet. For plasma parameters, proximate and fatty acid composition of the muscle, as well as gene expression data, mixed effect models (Laird and Ware, 1982; Carroll and Ruppert, 1988; Pinheiro and Bates, 2000) were used. The model included diet, timepoint and their interaction as fixed factors and tank as a random factor. The residuals were assumed to be normally distributed and to be heteroscedastic. Based on this model, a Pseudo  $R^2$  was calculated (Nakagawa and Schielzeth, 2013) and an ANOVA was conducted, followed by multiple contrast tests in order to compare the several diets with the control diet, and the timepoints, respectively. If the factors diet and timepoint had no significant interaction, then corresponding multiple contrast tests were pooled over the levels of the remaining factor. Spearman correlation analysis was employed to relate muscle lutein and plasma carotenoid concentrations, since muscle lutein concentrations were not normally distributed. Pearson correlation analysis was applied to relate the increase in DHA with a decrease in fat content in the muscle.

## 3 Results

### 3.1 Fish performance and proximate body composition

Feeding the experimental diets for eight weeks to the salmon in brackish water revealed no difference in growth and feed intake among groups (Table 3). Feed conversion ratio (FCR) was highest in the control group (CD), and including broken *C. vulgaris* (CVB) in the feed significantly improved the feed conversion ratio ( $p = 0.03$ ). Furthermore, feeding CVB slightly improved the protein efficiency ratio ( $p = 0.11$ ) but reduced body condition ( $p = 0.01$ ). Hepatosomatic index and spleen somatic index were not affected by the diet (Table 3). Mortality was low and not different among diet groups. Furthermore, the diet did not affect proximate whole-body composition of the salmon (Table 4) but feeding CVB slightly increased ash content ( $p = 0.06$ ). After the fish were transferred to full strength seawater voluntary feed intake decreased to one third of the levels prior transfer but was not different among groups (Table 3).

### 3.2 Proximate and fatty acid composition of muscle

The diet did not affect protein, water and ash content of the muscle in brackish water (T2) and following transfer to seawater (T3; Tables 5; S5). Fat content was significantly reduced in brackish water in fish fed *A. platensis* (AP; 12% reduction) and *S. limacinum* (SL; 13.5% reduction) compared to CD (Table 5). Fat content decreased on average by 29% after transfer to seawater across all groups (Table 5). It decreased most in groups receiving intact and broken *C. vulgaris*, CVI (38%) and CVB (32.9%), but markedly less in fish fed AP (18.5%).

Generally, fatty acid composition in the muscle lipids reflected that of the diet (Table 5; Table 2). Both diet and timepoint significantly influenced the fatty acid composition, however, an interaction of both factors was absent in most cases (Tables 5; S5). *Alpha*-linolenic acid (LA) was significantly enriched in muscle lipids of CVB, CVI and *T. chuii* (TC) fed fish in brackish water and in CVB and CVI fed fish after transfer to seawater (Table 5). Steraidonic acid (SDA) content was higher in TC compared to CD at both timepoints. Eicosapentaenoic acid (EPA) levels were affected by an interaction of diet and timepoint and levels increased in CD by 11.3%, CVB by 16.2% and CVI 18.1% following transfer to seawater (Tables 5; S5). Docosahexaenoic acid (DHA) levels were significantly increased in SL compared to CD fed fish at both timepoints and relative levels increased following transfer to seawater in all groups by 23.8% (Figure 2A, Table 5). A significant negative correlation between the relative reduction in total fat content in the muscle and the relative increase in DHA based on diet group means was detected ( $R = 0.89$ ,  $p < 0.001$ ; Figure 2B).



**TABLE 3** Growth performance and organ specific indices of Atlantic salmon after eight weeks of feeding the experimental diets in brackish water and feed intake for the period of two weeks in seawater.

	CD	CVI	CVB	TC	AP	SL	ANOVA
IBW [g]	81.9 ± 0.5	80.6 ± 0.7	83.6 ± 0.6	80.6 ± 1.2	82.5 ± 0.5	81.6 ± 0.8	ns
FBW [g]	149.0 ± 2.1	149.4 ± 4.9	156.0 ± 2.3	147.7 ± 5.6	153.3 ± 1.8	147.9 ± 2.6	ns
WG [g]	67.1 ± 1.6	68.8 ± 4.6	72.5 ± 1.9	67.1 ± 6.1	70.7 ± 1.3	66.3 ± 1.8	ns
SGR	1.07 ± 0.01	1.1 ± 0.05	1.11 ± 0.02	1.08 ± 0.08	1.11 ± 0.01	1.06 ± 0.01	ns
DFI	1.18 ± 0.02	1.14 ± 0.08	1.14 ± 0.03	1.11 ± 0.05	1.19 ± 0.07	1.15 ± 0.03	ns
FCR	1.11 ± 0.01	1.03 ± 0.03	1.03 ± 0.01*	1.04 ± 0.03	1.08 ± 0.06	1.08 ± 0.04	0.01
PER	1.96 ± 0.02	2.07 ± 0.07	2.09 ± 0.02	2.05 ± 0.06	1.99 ± 0.11	1.94 ± 0.08	0.05
PRE	38.1 ± 0.1	40.3 ± 1.4	39.8 ± 0.6	39.0 ± 1.0	37.4 ± 1.7	37.0 ± 0.9	ns
CF	0.96 ± 0.01	0.90 ± 0.02	0.86 ± 0.01*	0.97 ± 0.02	0.95 ± 0.02	0.90 ± 0.02	0.01
HSI [%]	1.46 ± 0.10	1.42 ± 0.08	1.25 ± 0.04	1.35 ± 0.08	1.45 ± 0.09	1.28 ± 0.07	ns
SSI [%]	0.09 ± 0.00	0.09 ± 0.00	0.1 ± 0.01	0.1 ± 0.01	0.09 ± 0.01	0.09 ± 0.01	ns
Survival [%]	100 ± 0.0	97.6 ± 1.0	96.4 ± 1.7	95.2 ± 2.6	98.8 ± 1.0	95.2 ± 1.0	ns
DFI SW	0.44 ± 0.04	0.31 ± 0.04	0.39 ± 0.01	0.40 ± 0.05	0.53 ± 0.07	0.52 ± 0.04	ns

Data is presented as mean ± SEM, with n = 3 tanks per treatment for performance parameters and n = 9 individuals for organ specific indices. A significant difference ( $p < 0.05$ ) compared to the control diet (CD) was assessed by Dunnett's multiple comparisons and indicated with a \*; ns, not significant. Note that presented ANOVA results do not fully agree with results from multiple comparisons due to heteroscedasticity. IBW (initial body weight); FBW (final body weight); WG (weight gain); SGR (specific growth rate) =  $(\ln(\text{FBW}) - \ln(\text{IBW}))/\text{experimental days} * 100$ ; DFI (daily feed intake) = daily feed intake in % body weight; FCR (feed conversion ratio) = total feed intake (g)/weight gain (g); PER (protein efficiency ratio) = weight gain (g)/crude protein intake (g); PRE (protein retention efficiency) = crude protein gained (g)/crude protein intake (g) \* 100; CF (Fulton's condition factor) =  $\text{weight}/\text{fish length}^3 * 100$ ; HSI (hepatosomatic index) = liver weight (g)/fish weight \* 100; SSI (spleen somatic index) = spleen weight (g)/fish weight (g) \* 100; SW seawater.

### 3.3 Muscle and plasma carotenoid content

Lutein was the main carotenoid present in all groups and significantly enriched in the muscle at both timepoints of fish fed CVI, CVB and TC (Table 5) reflecting the content of lutein in the respective algae (Supplementary Table 3). Lutein content in fish fed CVB was more than two times higher than in fish fed CVI and TC. Zeaxanthin was detected in muscle of fish fed AP and TC and violaxanthin was only detected in fish fed TC (Table 5).

The total carotenoid content in plasma of CVB fed fish was two times higher than in fish fed CVI ( $p = 0.08$ ) and TC ( $p = 0.05$ ; Figure 3A), which was also visible when comparing plasma samples of the respective groups directly (Supplementary Figure 1A). No carotenoid content was detectable in groups fed CD and SL (Supplementary Figures 1A, B), while values for AP were below the calculated standard curve and were subsequently excluded from

further analysis. Muscle lutein concentrations significantly correlated with total carotenoid concentration in plasma samples (Spearman  $R = 0.52$ ,  $p = 0.03$ ; Figure 3B).

### 3.4 Plasma metabolites and enzyme activities

CVI inclusion significantly lowered aspartate aminotransferase and alanine aminotransferase activity in microalgae fed groups compared to CD ( $p \leq 0.046$ ; Figures 4A, B). Furthermore, SL lowered aspartate aminotransferase activity ( $p = 0.06$ ; Figure 4A). The highest variance in aspartate aminotransferase and alanine aminotransferase activity was found among fish fed CD, while it was lowest among fish fed CVI. Total plasma protein and alkaline phosphatase activity were not influenced by the diet but showed an

**TABLE 4** Proximate body composition (percent OS) of Atlantic salmon after eight weeks of feeding the experimental diets.

[%] OS	CD	CVI	CVB	TC	AP	SL	ANOVA
Moisture	70.41 ± 0.44	70.23 ± 0.23	70.47 ± 0.04	70.38 ± 0.17	70.54 ± 0.13	70.60 ± 0.08	ns
Ash	2.33 ± 0.01	2.47 ± 0.05	2.54 ± 0.03(*)	2.22 ± 0.05	2.34 ± 0.07	2.39 ± 0.04	0.004
Crude protein	18.23 ± 0.10	18.3 ± 0.17	18.11 ± 0.07	18.09 ± 0.01	18 ± 0.06	18.11 ± 0.11	ns
Crude lipid	9.04 ± 0.37	9.00 ± 0.27	8.88 ± 0.06	9.31 ± 0.14	9.12 ± 0.13	8.90 ± 0.22	ns
Energy[MJ/kg]	7.88 ± 0.16	7.82 ± 0.09	7.71 ± 0.02	7.90 ± 0.04	7.82 ± 0.04	7.77 ± 0.06	ns

Data is presented as mean ± SEM as original substance (OS), with n = 3 tanks per treatment. A trend ( $p < 0.1$ ) compared to the control diet (CD) was assessed by Dunnett's multiple comparisons and indicated with (\*); ns, not significant. Note that presented ANOVA results do not fully agree with results from multiple comparisons due to heteroscedasticity.

TABLE 5 Proximate, fatty acid and carotenoid composition of Atlantic salmon muscle fed microalgae enriched diets eight weeks in brackish water (T2) and two weeks following seawater transfer (T3).

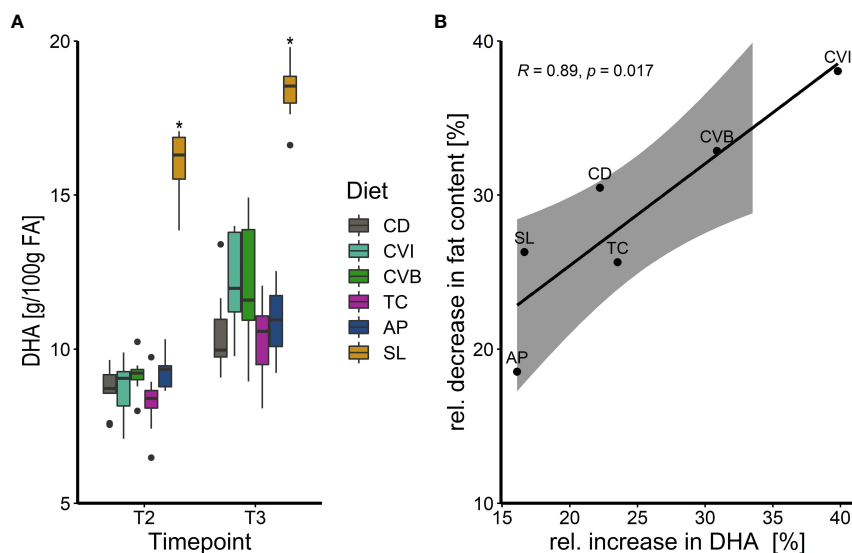
Timepoint	T2						T3					
Diet	CD	AP	CVB	SL	CVI	TC	CD	AP	CVB	SL	CVI	TC
<b>Proximate composition [% OS]</b>												
protein [%]	19.93 ± 0.19	20.41 ± 0.10	19.86 ± 0.23	20.21 ± 0.17	19.73 ± 0.14	20.28 ± 0.09	20.90 ± 0.35	20.68 ± 0.17	20.60 ± 0.15	20.43 ± 0.21	20.57 ± 0.18 <sup>b</sup>	20.71 ± 0.13
water [%]	74.71 ± 0.22	74.99 ± 0.24	75.11 ± 0.27	75.31 ± 0.22	75.10 ± 0.21	74.43 ± 0.19	75.65 ± 0.35	75.97 ± 0.18	76.32 ± 0.16	76.48 ± 0.33	76.49 ± 0.27 <sup>b</sup>	75.89 ± 0.12
ash [%]	1.46 ± 0.03	1.46 ± 0.04	1.54 ± 0.05	1.43 ± 0.02	1.38 ± 0.02	1.39 ± 0.02	1.49 ± 0.02	1.44 ± 0.01	1.46 ± 0.01	1.46 ± 0.02	1.48 ± 0.01	1.48 ± 0.01
fat [%]	4.66 ± 0.12	<b>4.1 ± 0.06<sup>a</sup></b>	4.29 ± 0.19	<b>4.03 ± 0.14<sup>a</sup></b>	4.60 ± 0.26	4.68 ± 0.26	3.24 ± 0.15 <sup>b</sup>	3.34 ± 0.12 <sup>b</sup>	2.88 ± 0.17 <sup>b</sup>	2.97 ± 0.12 <sup>b</sup>	2.85 ± 0.16 <sup>b</sup>	3.48 ± 0.15 <sup>b</sup>
<b>[% Fatty Acids]</b>												
total SFA	17.87 ± 0.16	17.82 ± 0.27	<b>17.11 ± 0.07<sup>a</sup></b>	18.12 ± 0.21	17.76 ± 0.21	17.8 ± 0.24	17.67 ± 0.24	17.53 ± 0.32	17.23 ± 0.23	18.74 ± 0.23	17.06 ± 0.08	17.69 ± 0.22
total MUFA	49.25 ± 0.27	48.31 ± 0.12	48.51 ± 0.32	<b>42.16 ± 0.45<sup>a</sup></b>	48.71 ± 0.28	49.29 ± 0.21	47.71 ± 0.39 <sup>b</sup>	47.27 ± 0.24 <sup>b</sup>	46.3 ± 0.55 <sup>b</sup>	<b>39.89 ± 0.41<sup>a, b</sup></b>	46.33 ± 0.53 <sup>b</sup>	47.93 ± 0.43
LA	11.99 ± 0.07	11.9 ± 0.09	12.26 ± 0.04	<b>10.8 ± 0.12<sup>a</sup></b>	12.15 ± 0.12	11.9 ± 0.06	11.67 ± 0.11 <sup>b</sup>	11.65 ± 0.13	11.53 ± 0.03 <sup>b</sup>	<b>10.02 ± 0.11<sup>a, b</sup></b>	11.65 ± 0.09 <sup>b</sup>	11.49 ± 0.11 <sup>b</sup>
total n6 PUFA	14.86 ± 0.07	14.99 ± 0.06	<b>15.15 ± 0.05<sup>a</sup></b>	<b>14.41 ± 0.10<sup>a</sup></b>	14.98 ± 0.12	14.69 ± 0.07	14.77 ± 0.11	14.9 ± 0.12	14.56 ± 0.04 <sup>b</sup>	<b>13.99 ± 0.09<sup>a</sup></b>	14.75 ± 0.07	14.46 ± 0.08
ALA	2.66 ± 0.05	2.79 ± 0.04	<b>3.16 ± 0.06<sup>a</sup></b>	2.54 ± 0.06	<b>3.04 ± 0.04<sup>a</sup></b>	<b>2.97 ± 0.02<sup>a</sup></b>	2.57 ± 0.06	2.72 ± 0.06	<b>2.96 ± 0.03<sup>a</sup></b>	<b>2.26 ± 0.05<sup>a, b</sup></b>	2.81 ± 0.03 <sup>b</sup>	2.73 ± 0.04 <sup>b</sup>
EPA	1.59 ± 0.03	1.63 ± 0.03	1.67 ± 0.03	1.7 ± 0.04	1.6 ± 0.04	1.59 ± 0.03	1.77 ± 0.05 <sup>b</sup>	1.74 ± 0.04	1.94 ± 0.05 <sup>b</sup>	1.75 ± 0.03	1.89 ± 0.06 <sup>b</sup>	1.79 ± 0.07
DHA	8.65 ± 0.22	9.32 ± 0.18	9.16 ± 0.19	<b>16.08 ± 0.34<sup>a</sup></b>	8.69 ± 0.27	8.28 ± 0.29	10.58 ± 41 <sup>b</sup>	10.83 ± 0.36 <sup>b</sup>	11.98 ± 0.67 <sup>b</sup>	<b>18.76 ± 0.45<sup>a, b</sup></b>	12.14 ± 0.51 <sup>b</sup>	10.23 ± 0.42 <sup>b</sup>
total n3 PUFA	15.36 ± 0.28	16.26 ± 0.23	16.63 ± 0.27	<b>22.83 ± 0.35</b>	15.86 ± 0.35	15.43 ± 0.35	17.36 ± 0.52 <sup>b</sup>	17.8 ± 0.45	19.54 ± 0.72 <sup>b</sup>	<b>25.16 ± 0.43<sup>a, b</sup></b>	19.43 ± 0.56 <sup>b</sup>	17.31 ± 0.51
total PUFA	30.96 ± 0.29	31.96 ± 0.26	32.49 ± 0.29	<b>37.91 ± 0.31<sup>a</sup></b>	31.53 ± 0.43	30.81 ± 0.39	32.83 ± 0.53	33.38 ± 0.54	34.74 ± 0.71	<b>39.8 ± 0.43<sup>a, b</sup></b>	34.84 ± 0.51 <sup>b</sup>	32.46 ± 0.51
n3 HUFA	11.97 ± 0.26	12.73 ± 0.20	12.73 ± 0.21	<b>19.64 ± 0.40<sup>a</sup></b>	12.1 ± 0.32	11.6 ± 0.35	14.16 ± 0.47 <sup>b</sup>	14.42 ± 0.44 <sup>b</sup>	15.92 ± 0.73 <sup>b</sup>	<b>22.34 ± 0.47<sup>a, b</sup></b>	15.96 ± 0.57 <sup>b</sup>	13.81 ± 0.51 <sup>b</sup>
<b>[ng/g muscle]</b>												
Lutein	11.1 ± 0.3	13.8 ± 1.6	670.6 ± 101.0	11.6 ± 0.4	<b>315.2 ± 3.7<sup>a</sup></b>	<b>229.2 ± 29.8<sup>a</sup></b>	15.4 ± 1.3	15.3 ± 0.7	<b>743.1 ± 64.9<sup>a</sup></b>	15.9 ± 1.7	<b>282.4 ± 17.9<sup>a</sup></b>	<b>203.1 ± 7.6<sup>a</sup></b>
Violaxanthin	n.d.	n.d.	n.d.	n.d.	n.d.	86.0 ± 14.9	n.d.	n.d.	n.d.	n.d.	n.d.	88.8 ± 5.0
Zeaxanthin	n.d.	68.9 ± 7.5	n.d.	n.d.	n.d.	14.8 ± 2.2	n.d.	98.2 ± 14.6	n.d.	n.d.	n.d.	14.9 ± 1.2

Data is presented as mean ± SEM, with n = 9 individuals per treatment for proximate and fatty acid composition and n = 3 (pooled on tank level) for carotenoids. Two-way ANOVA was used to assess the effect of diet and timepoint, as well as their interaction on the response variable. For ANOVA results see [Supplementary Table 5](#). A significant difference (p < 0.05) compared to the control diet (CD) within one timepoint was assessed by Dunnett's multiple comparisons and indicated in bold with a, while differences between timepoints within one diet were assessed by Tukey's multiple comparison test and indicated with b; ns, not significant, n.d. not detected. Note that presented ANOVA results do not fully agree with results from multiple comparisons due to heteroscedasticity.

overall increase between T1 and T2 (p < 0.001; [Supplementary Figure 2](#)). Total cholesterol increased significantly between the first two samplings for fish fed CVI, CVB and TC (p < 0.05; [Supplementary Figure 2](#)). Glucose furthermore increased over time only in fish fed SL (p = 0.02; [Supplementary Figure 2](#)).

### 3.5 Liver proteins

Abundance of myeloperoxidase (Mpo) in the liver of Atlantic salmon fed a microalgae-enriched diet was lower in most cases at both timepoints compared to CD ([Figure 5A](#)). This effect was

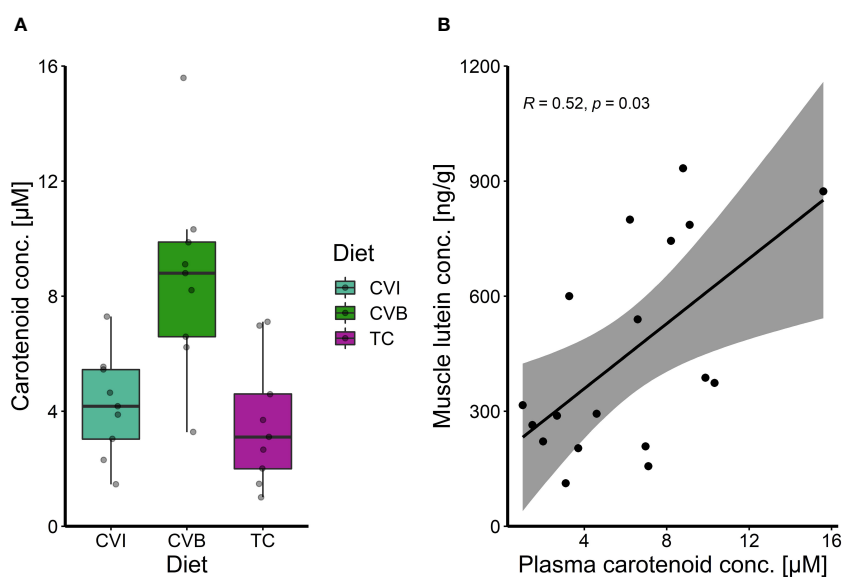


**FIGURE 2** (A) Boxplot of docosahexaenoic acid (DHA) concentration in g/100g fatty acids (FA) of Atlantic salmon muscle fat following feeding the experimental diets for eight weeks in brackish water (T2) and transferred to seawater for two weeks (T3), n = 9. The fish received six different experimental diets: control (CD), *Chlorella vulgaris* intact (CVI), *Chlorella vulgaris* broken (CVB), *Tetraselmis chuii* (TC), *Arthrospira platensis* (AP) and *Schizochytrium limacinum* (SL) at an inclusion level of 8%. A significant difference (p < 0.05) compared to the control diet (CD) was assessed by Dunnett’s multiple comparisons and indicated with a \* (B) Linear relationship between the relative increase in muscle lipid DHA content in % in relation to the relative decrease (%) of muscle fat content following transfer to seawater (T3). Note that for Pearson’s correlation analysis group means per diet were used (n = 6) and diet groups are indicated next to each datapoint.

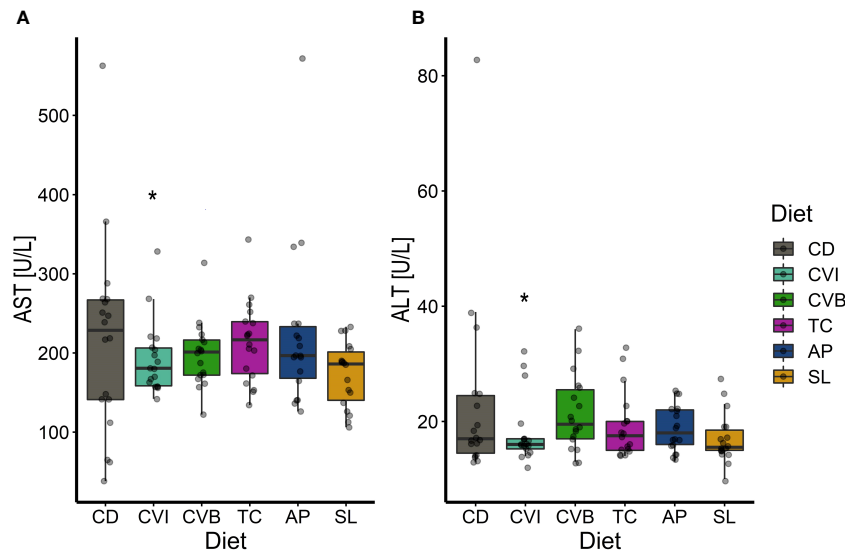
however only significant for fish fed AP at T1 (p = 0.049) with a 35% reduction compared to CD due to a large within group variation (Figure 5A). Though, AP at both timepoints showed the lowest variation. Sod1 protein level was induced by 3-fold in SL at T1 compared to CD (p = 0.10; Figure 5B).

### 3.6 Gene expression

Only few genes were significantly differentially expressed between microalgae fed groups compared to CD fish, due to a large overall variability in expression (Figures 6–8) although some



**FIGURE 3** (A) Boxplot of carotenoid concentration in plasma of Atlantic salmon after eight weeks of feeding experimental diets enriched with *C. vulgaris* intact (CVI), broken (CVB) or *T. chuii* (TC) and (B) Spearman correlation among plasma total carotenoid concentration and muscle lutein concentration after eight weeks of feeding experimental diets for fish where both parameters were measured on an individual level (n = 18).

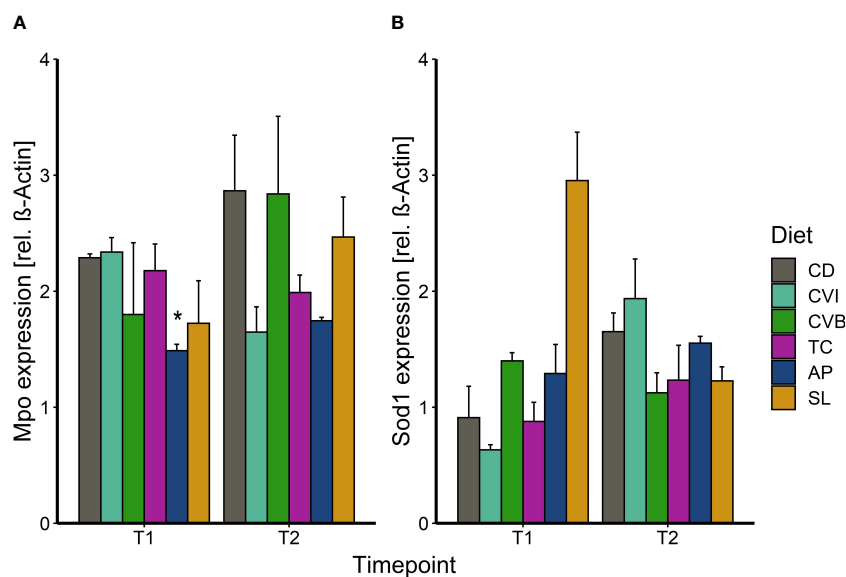


**FIGURE 4** Boxplot of aspartate aminotransferase (AST) activity (A) and alanine aminotransferase (ALT) activity (B) in plasma of Atlantic salmon fed microalgae enriched diets for eight weeks (T2, n = 18). The fish received six different experimental diets: control (CD), *Chlorella vulgaris* intact (CVI), *Chlorella vulgaris* broken (CVB), *Tetraselmis chuii* (TC), *Arthrospira platensis* (AP) and *Schizochytrium limacinum* (SL) at an inclusion level of 8%. A significant difference (p < 0.05) compared to the control diet (CD) was assessed by Dunnett's multiple comparisons and indicated with a \*.

large fold-changes were evident (Supplementary Figure 3). In the anterior intestine, increased *drtp1*-transcript levels were found in CVI- (2.8-fold, p = 0.065; Figure 6A) and CVB-fed salmon (7-fold; p = 0.023; Figure 6A). *isg15* transcript levels were induced in all microalgae fed groups except for CVI (Figure 6B), although not statistically significant. *il1r2* transcript levels were reduced by 2.7-

fold in fish fed CVI (p = 0.039; Figure 6C) and *il10rb* levels were reduced in fish fed CVI (p = 0.028; Figure 6D) and TC (p = 0.039).

In the liver, the transcript levels of the acute-phase gene *saa5* were significantly reduced in fish fed CVB (0.37-fold; p = 0.016; Figure 7A) and SL (0.29-fold; p = 0.012, Figure 7A). *c1ql2* transcripts were significantly induced in fish fed CVI (p = 0.032)



**FIGURE 5** Protein expression of myeloperoxidase (A) and superoxide dismutase 1 (B) in liver tissue of Atlantic salmon following feeding the microalgae enriched diets for two weeks (T1) and eight weeks (T2) in brackish water. The fish received six different experimental diets: control (CD), *Chlorella vulgaris* intact (CVI), *Chlorella vulgaris* broken (CVB), *Tetraselmis chuii* (TC), *Arthrospira platensis* (AP) and *Schizochytrium limacinum* (SL) at an inclusion level of 8%. Data is presented as mean + SEM, n = 3 (pooled on tank level). A significant difference (p < 0.05) compared to the control diet (CD) was assessed by Dunnett's multiple comparisons and indicated with a \*.

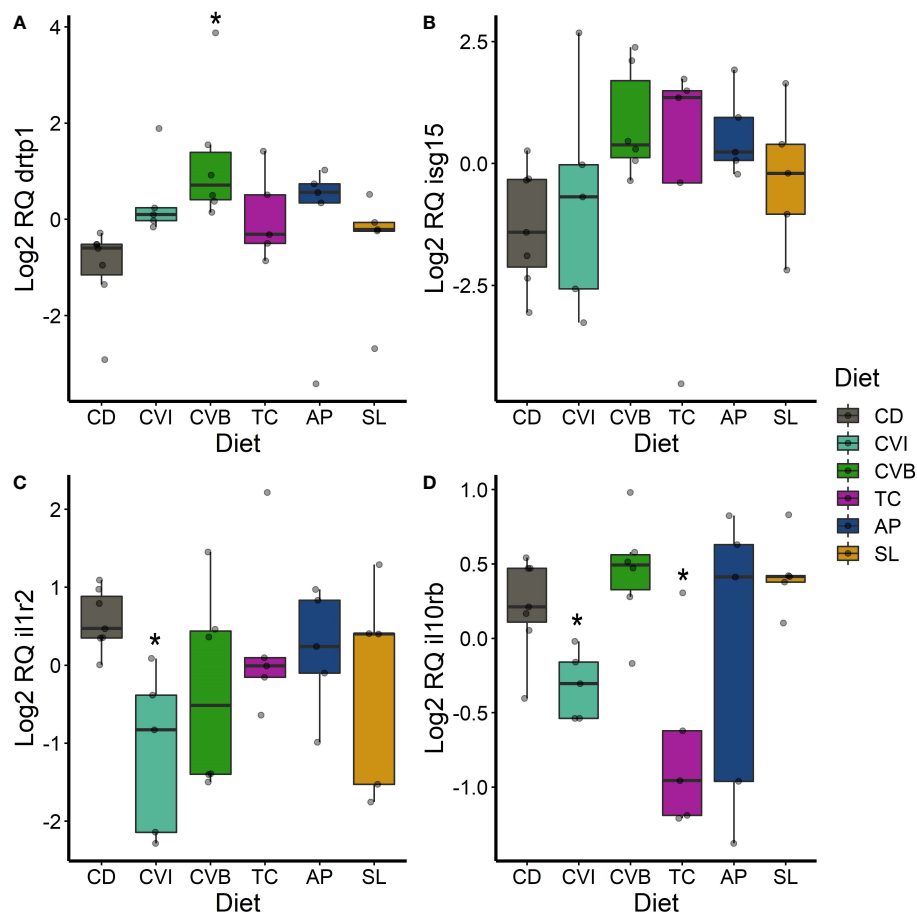


FIGURE 6

Boxplot of the gene expression in the anterior intestine of *drtp1* (A), *isg15* (B), *il1r2* (C) and *il10rb* (D) of fish fed microalgae enriched diets for eight weeks. The fish received six different experimental diets: control (CD), *Chlorella vulgaris* intact (CVI), *Chlorella vulgaris* broken (CVB), *Tetraselmis chuii* (TC), *Arthrospira platensis* (AP) and *Schizochytrium limacinum* (SL) at an inclusion level of 8%. Expression values were normalized relative to the mean expression of all samples and log<sub>2</sub> transformed ( $n = 5 - 7$ ). A significant difference ( $p < 0.05$ ) compared to the control diet (CD) was assessed by Dunnett's multiple comparisons and indicated with a \*.

and SL ( $p = 0.03$ ; Figure 7B). *hamp* transcripts were 2.2-fold higher concentrated in the liver of fish fed CVB ( $p = 0.066$ ; Figure 7C) and *lyzc2* transcripts were even 12-fold increased in fish fed TC ( $p = 0.04$ ; Figure 7D) compared to the control group.

In the spleen, *c1ql2* transcripts were slightly 1.5-fold induced in fish fed CVB ( $p = 0.095$ ) and TC ( $p = 0.097$ ; Figure 8A). *ikba* transcripts were 0.59-fold reduced in the spleen of fish fed CVI ( $p = 0.06$ ; Figure 8B). Furthermore, transcript abundance of *saa5* was 2.1-fold increased in fish fed CVI ( $p = 0.034$ ; Figure 8C). The transcript level of *cd209d* was reduced across all microalgae-fed fish, although not statistically significant (Figure 8D).

Since the levels of plasma markers and selected transcripts varied largely, we conducted a correlation analysis to identify connections and validate the overall utility of the used health parameters. However only alkaline phosphatase (ALP) activity in plasma significantly correlated with *clra* ( $R = 0.46$ ,  $p = 0.008$ ; Supplementary Figure 4A) and *c4b* transcript levels in the liver ( $R = 0.45$ ,  $p = 0.008$ ; Supplementary Figure 4B).

## 4 Discussion

Microalgae are gaining attention as a sustainable ingredient to replace fishmeal or oil in aquaculture diets (Shah et al., 2018; Kousoulaki et al., 2020; Sarker et al., 2020a; Sarker et al., 2020b) and further as a functional supplement, prebiotic and immunostimulant for farmed fish (Reyes-Becerril et al., 2013; Rahimnejad et al., 2017; Messina et al., 2019; Sun et al., 2019; Teimouri et al., 2019). In this study microalgae inclusion did not negatively affect performance of Atlantic salmon reared in recirculating aquaculture systems. However, we found that health, immunity as well as fatty acid and pigment deposition were influenced in an algae specific manner.

Inclusion of microalgae in fish feed has been shown to affect the growth performance via increasing feed intake or improving feed conversion in a variety of fish species (Table 6). Including 5% *Chlorella sorokiniana* in the diet increased feed intake and thus growth in rainbow trout (Chen et al., 2021) and including *Chlorella*

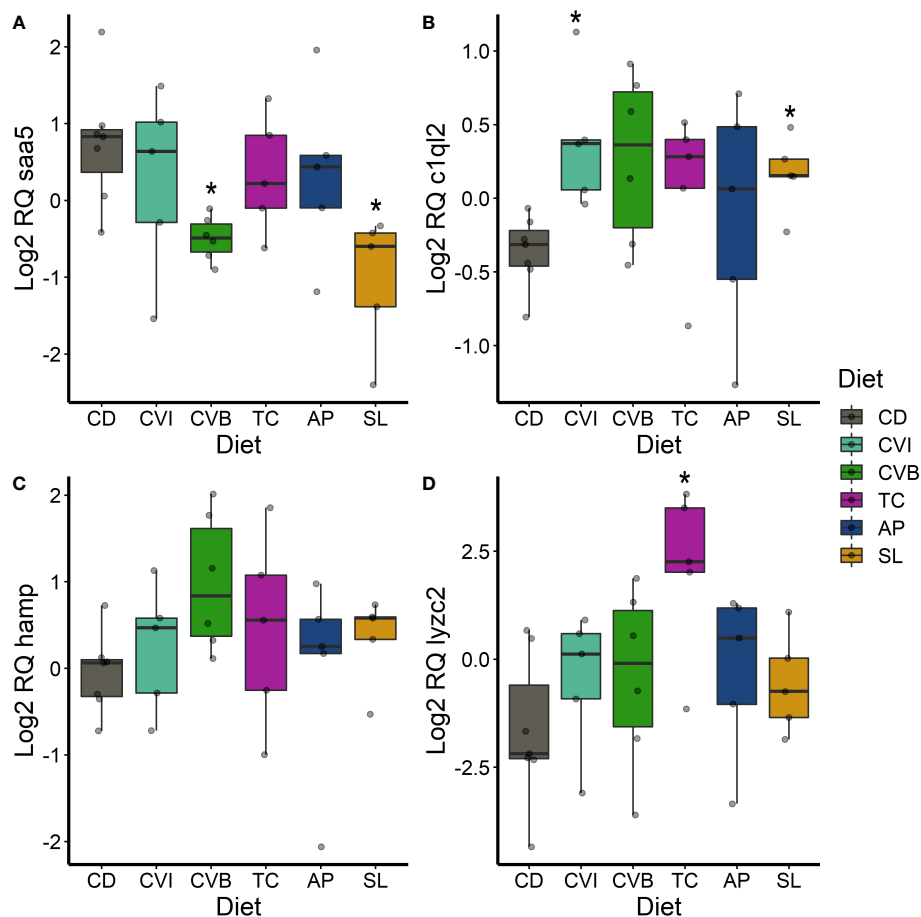


FIGURE 7

Boxplot of the gene expression in the liver of *saa5* (A), *c1ql2* (B), *hamp* (C) and *lyzc2* (D) of fish fed microalgae enriched diets for eight weeks. The fish received six different experimental diets: control (CD), *Chlorella vulgaris* intact (CVI), *Chlorella vulgaris* broken (CVB), *Tetraselmis chuii* (TC), *Arthrospira platensis* (AP) and *Schizochytrium limacinum* (SL) at an inclusion level of 8%. Expression values were normalized relative to the mean expression of all samples and log2 transformed ( $n = 5 - 7$ ). A significant difference ( $p < 0.05$ ) compared to the control diet (CD) was assessed by Dunnett's multiple comparisons and indicated with a \*.

*vulgaris* at levels of 10 to 15% improved feed intake and conversion in olive flounder (Rahimnejad et al., 2017). In this line including broken *C. vulgaris* in our study improved feed conversion efficiency but not feed intake in Atlantic salmon. Nevertheless at 8% inclusion level the palatability of the feeds in our study was not negatively affected, likely because these microalgae do not contain high amounts of anti-nutritional factors, as observed for other plant-based ingredients (Nagel et al., 2012; von Danwitz and Schulz, 2020). Voluntary feed intake decreases in many fish species in response to stress (Kulczykowska & Sánchez Vázquez, 2010). Transferring the salmon into seawater reduced appetite as previously described (Usher et al., 1991). Although microalgae were shown to increase feed intake in many species (Table 6) and mitigate acute stress (de Mattos et al., 2019), we found no indications that microalgae could increase feed intake during the critical time period of the first weeks in seawater. In contrast, a diet enriched with the feeding stimulant squid extract was able to improve feed intake of Atlantic salmon in this time period (Toften et al., 2003) and other ingredients which act as feed attractants might be explored in the future. The overall lower

growth performance compared to other studies (Kousoulaki et al., 2020; Ytrestøyl et al., 2020) is likely attributed to the use of pelletized feeds (Kiron et al., 2012), as compared to high performance extruded feeds.

Many microalgae species such as *Chlorella* and *Tetraselmis* contain a rigid cell wall, with a rigid cell wall fraction build of chitin- or chitosan-like polysaccharides (Domozych et al., 2012; Weber et al., 2022), which can reduce nutrient digestibility. Digestibility was reduced in Atlantic salmon fed with *Chlorella vulgaris* already at 6% inclusion (Tibbetts et al., 2017), but pre-extruded *Nanochloropsis* included at 10% improved dry matter digestibility and did not change protein digestibility in Atlantic salmon (Gong et al., 2020). The improved PCR found for fish fed broken *C. vulgaris* in our study, a species with a particularly rigid cell wall, is probably related to the destructed cell wall (Weber et al., 2022). The actual mechanism for improvements in feed conversion efficiency by microalgae is however not well understood, but seems to be related to promoting growth of beneficial intestinal bacteria (Ma et al., 2022), as well as improving intestinal health and nutrient uptake (Perera et al., 2020; Molina-Roque et al., 2022). Although

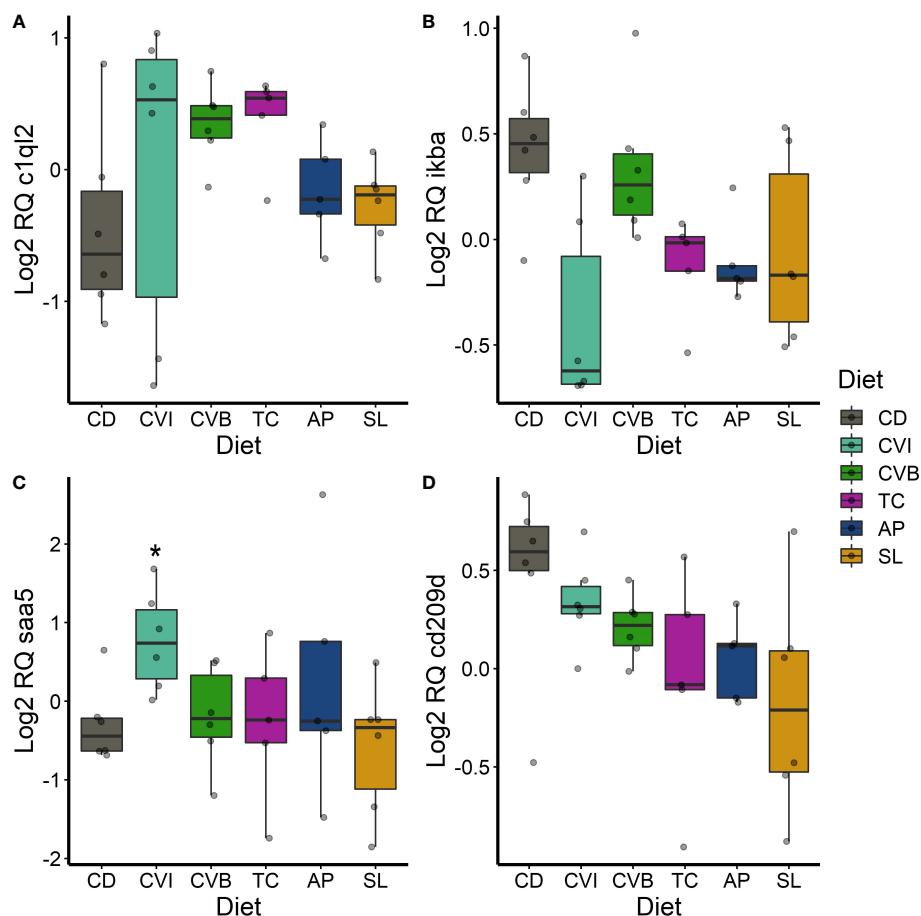


FIGURE 8

Boxplot of the gene expression in the spleen of *c1ql2* (A), *ikba* (B), *saa5* (C) and *lyzc2* (D) of fish fed microalgae enriched diets for eight weeks. The fish received six different experimental diets: control (CD), *Chlorella vulgaris* intact (CVI), *Chlorella vulgaris* broken (CVB), *Tetraselmis chuii* (TC), *Arthrospira platensis* (AP) and *Schizochytrium limacinum* (SL) at an inclusion level of 8%. Expression values were normalized relative to the mean expression of all samples and log2 transformed ( $n = 5 - 7$ ). A significant difference ( $p < 0.05$ ) compared to the control diet (CD) was assessed by Dunnett's multiple comparisons and indicated with a \*.

inclusion levels of microalgae in diets vary greatly (Table 6) there seems to be a threshold at 10 – 15% inclusion for carnivorous fish, upon where growth performance is negatively affected likely because of reduced digestibility.

Besides its direct effects on growth, microalgae as a functional feed additive can influence body proximate composition and somatic indices by modulating energy metabolism. Low inclusion levels of microalgae derived nutraceuticals increased body proximate protein content, hepatosomatic index and glucose uptake capacity of the liver of gilthead sea bream, *Sparus aurata* (Perera et al., 2020). *C. vulgaris* in the diet was found to influence lipid metabolism in rats given a high fat diet, where it was able to lower triglycerides, total cholesterol and LDL cholesterol (Cherng and Shih, 2005). Similar results have been found in a study with humans (Ebrahimi-Mameghani et al., 2014). Reduced condition, hepatosomatic index and whole-body fat content in salmon fed diets containing broken *C. vulgaris* in our study also indicates interference with the lipid metabolism. It is however unclear whether increased metabolization or decreased deposition of lipid and glycogen took place as a response to the diet. However, in

contrast to the above-mentioned studies, total cholesterol levels in plasma were unaffected.

Microalgae supplementation had a clear effect on the fatty acid profile of the fish muscle and levels found in the muscle of the salmon mirrored levels in the diets (Bell et al., 2001; Caballero et al., 2002). Increased levels of *alpha*-linolenic acid in the muscle of fish fed *C. vulgaris* (intact and broken) and *T. chuii*, as well as higher levels of DHA in fish fed *S. limacinum* resulted from higher contents of these specific fatty acids in the respective microalgae. This was evident although the experimental period of eight plus two weeks was rather short compared to other studies (Bell et al., 1993; Ruyter et al., 2000; Sissener et al., 2016). Atlantic salmon need to take up essential fatty acids via the diet and dietary requirements for EPA + DHA for Atlantic salmon post-smolts have been found to be ~ 0.5% of dry matter (Bou et al., 2017). All diets contained sufficient EPA and DHA, but high levels of DHA (2.5% of DM) in the diet containing *S. limacinum* were not reflected to the same degree in the muscle of the fish. The results must, however, be interpreted carefully, as preferential retention of specific fatty acids can influence the results in the muscle (Bell et al., 2003). Retention

TABLE 6 Growth and immune effects of microalgae species incorporated in the diet and fed to different fish species.

Microalgae species	Fish species	Days	Inclusion rate [%]	SGR	FCR	DFI	Antioxidant activity	Immune cells and markers	Antibacterial activity	Immune signaling	Source
<i>A. platensis</i>	<i>O. niloticus</i>	84	0.125 - 1	↑	↓	↑	↑	↑↓	↓		Abdel-Tawwab and Ahmad, 2009
<i>A. platensis</i>	<i>S. aurata</i>	30	42.6				↓	↑			de Mattos et al., 2019
<i>A. platensis</i>	<i>C. carpio</i>	40	1					↑			Khalil et al., 2017
<i>A. platensis</i>	<i>C. gariepinus</i>	84	12.5 -18.75	↑	↓		↑	↑			Raji et al., 2018
<i>A. platensis</i>	<i>O. mykiss</i>	70	2.5 -10.0	↓↑	↓		↑↓				Teimouri et al., 2019
<i>A. platensis</i>	<i>O. mykiss</i>	70	2.5 -10.0					↑			Yeganeh et al., 2015
<i>C. vulgaris</i>	<i>S. salar</i>	28	20						↑↓		Grammes et al., 2013
<i>Chlorella</i> sp.	<i>C. auratus</i>	56	1.0 - 4.0	↑	↓	↑		↑		↑	Luo et al., 2018
<i>C. vulgaris</i>	<i>O. niloticus</i>	60	5	↑	↓		↑	↑	↑		Mahmoud et al., 2020
<i>C. vulgaris</i>	<i>C. gariepinus</i>	84	12.5 - 18.75	↑	↓		↑	↑			Raji et al., 2018
<i>Chlorella</i> sp.	<i>C. auratus</i>	60	0.4 - 2.0	↑				↑			Zhang et al., 2014
<i>C. sorokiniana</i>	<i>O. mykiss</i>	90	0 - 10			↑	↓↑	↑	↑↓		Chen et al., 2021
<i>D. salina</i>	<i>D. rerio</i>	30	15							↑	Fan et al., 2022
<i>I. galbana</i>	<i>D. rerio</i>	30	15				↑			↑	Fan et al., 2022
<i>L. incisa</i>	<i>D. rerio</i>	30	7.50 -15.0				↑		↑	↑	Nayak et al., 2020
<i>N. gaditana</i>	<i>S. aurata</i>	28	5.0 -10.0	↓					↑		Cerezuela et al., 2012b
<i>N. gaditana</i>	<i>S. salar</i>	84	10.0 - 20.0	↓	↑	↑	↑			↑	Sørensen et al., 2017
<i>Navicula</i> sp.	<i>L. peru</i>	28/ 56	10				↑	↑	↑		Reyes-Becerril et al., 2014
<i>Navicula</i> sp.	<i>S. aurata</i>	14/ 28	10							↑	Reyes-Becerril et al., 2013
<i>P. tricornutum</i>	<i>S. aurata</i>	28	10					↓			Cerezuela et al., 2013
<i>P. tricornutum</i>	<i>S. aurata</i>	14/ 28	10				↑	↑	↑	↑	Cerezuela et al., 2012b
<i>P. tricornutum</i>	<i>S. aurata</i>	28	5.0 - 10.0	↓↑			↑		↑	↑	Cerezuela et al., 2012b
<i>P. tricornutum</i>	<i>D. rerio</i>	30	15				↑			↑	Fan et al., 2022

(Continued)



TABLE 6 Continued

Microalgae species	Fish species	Days	Inclusion rate [%]	SGR	FCR	DFI	Antioxidant activity	Immune cells and markers	Antibacterial activity	Immune signaling	Source
<i>S. almeriensis</i>	<i>O. niloticus</i>	30	25	–			↑		↑		<a href="#">García-Márquez et al., 2020</a>
<i>S. almeriensis</i>	<i>O. niloticus</i>	60	5.0 - 22.0	↑	↑	–	↑	↑	↑		<a href="#">Abdel-Tawwab et al., 2022</a>
<i>Schizochytrium</i> sp.	<i>M. salmoides</i>	84	5.7 - 14.1				↑↓	↑↓		↑	<a href="#">Habte-Tsion et al., 2020</a>
<i>Schizochytrium</i> sp.	<i>O. niloticus</i>	105	1.2					↓			<a href="#">de Souza et al., 2020</a>
<i>S. limacinum</i>	<i>S. salar</i>	330	2.62 - 6.25		↓	↓	↑	↓	↓	↑	<a href="#">Kousoulaki et al., 2020</a>
<i>S. limacinum</i>	<i>C. altivelis</i>	30	1	↑			↑			↑	<a href="#">Sun et al., 2019</a>
<i>S. limacinum</i>	<i>T. ovatus</i>	56	3	↑	↓	↑				↓	<a href="#">Xie et al., 2019</a>
<i>T. chuii</i>	<i>S. aurata</i>	28	10					↓			<a href="#">Cerezuela et al., 2013</a>
<i>T. chuii</i>	<i>S. aurata</i>	14/ 28	10				↑	↑	↑	↑	<a href="#">Cerezuela et al., 2012b</a>
<i>T. chuii</i>	<i>S. aurata</i>	28	5.0 - 10.0	↓				↑	↑		<a href="#">Cerezuela et al., 2012b</a>
<i>T. suecica</i> & <i>T. lutea</i>	<i>D. labrax</i>	105	6.0 - 18.0						↑		<a href="#">Messina et al., 2019</a>
<i>T. ultriculosum</i>	<i>O. mykiss</i>	90	5.0 -10.0	↑	↑	↑		↑↓	↑		<a href="#">Chen et al., 2021</a>

Note that only studies were included which reported effects on immunity and were not solely based on performance parameters. Responses marked with arrows are shown when at least one inclusion level caused a significant increase or decrease. In case different inclusion levels or diets caused divergent responses both arrows are shown. SGR, specific growth rate; DFI, daily feed intake; FCR, feed conversion ratio.

efficiency of DHA was indicated to be dose dependent (Glencross et al., 2014; Emery et al., 2016) and high dietary DHA in the *S. limacinum* diet could have reduced the need for an efficient retention of DHA in the muscle. Furthermore diets rich in saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) improve long-chain polyunsaturated fatty acid (LC-PUFA) metabolism efficiency (Xu et al., 2014; Emery et al., 2016). The diet enriched with *S. limacinum* had a lower MUFA content due to reduced inclusion of canola oil. Together with a lower digestibility of palmitic acid, which is present in high concentrations in *Schizochytrium* (Kousoulaki et al., 2020; Hart et al., 2021), this could have led to an overall lower fat content in the muscle of fish fed with this microalgae. Although palmitic acid was enriched in the diet containing *S. limacinum*, it was only slightly increased in the muscle at the end of the trial and its metabolic fate requires more attention in future studies. Muscle fat content was also reduced in salmon fed *A. platensis* for eight weeks in our study and *Arthrospira platensis* has been found to reduce hyperlipidaemia in the rat model (Hua et al., 2018; Li et al., 2019). However, no effect was detected in our study on cholesterol levels in plasma, which has been described by Hua et al. (2018).

Seawater transfer results in increased energy demands and coupled with reduced feed intake caused a significant reduction in the muscle fat content, primarily in the relative abundance of specific fatty acids of salmon smolts (Woo et al., 1978; Sheridan, 1989; Usher et al., 1991). Since monounsaturated fatty acids are preferentially used as metabolic fuel (Henderson, 1996) polyunsaturated fatty acids such as DHA were protected from being metabolized and not oxidized during the early time window in seawater. Hence, relative abundance of DHA in the muscle of all groups increased significantly after transfer to seawater and was linearly related to the decrease in total fat content of the muscle.

Besides polyunsaturated fatty acids also pigments are important in maintaining health and immune function in fish (de Carvalho & Caramujo, 2017). Lutein, the dominating pigment present in all muscle samples, was found to improve growth and antioxidant status of whiteleg shrimp *Litopenaeus vannamei* (Fang et al., 2021) and improve survival of goldfish *Carassius auratus* (Besen et al., 2019). Differences in the carotenoid profile among the diet groups were directly related to its feed origin, with lutein found in highest concentrations in fish fed broken *C. vulgaris*, followed by intact *C. vulgaris* and *T. chunii*. Sørensen et al. (2023) also found high concentrations of lutein in the muscle of salmon fed *T. chunii* biomass, but in contrast to their study, we did not detect any astaxanthin in the muscle samples. This could be explained by the lower inclusion of fishmeal in our diet, which is a natural source of astaxanthin (Lim et al., 2018) and further by a difference in the pigment profile of the used microalgae products. Lutein concentrations in the muscle of the salmon in our study were linearly related to total carotenoid concentration found in plasma samples, which could be caused by a dynamic equilibrium of carotenoids between the bloodstream and muscle. However, it could also imply that fish that accumulated more carotenoids over the entire trial also ingested more during the days before sampling. Aside from lutein, the xanthophylls zeaxanthin and violaxanthin were present in the muscle of fish fed *A. platensis*

and *T. chunii* respectively. All detected carotenoids have important functions in maintaining eye-health in mammals (Giordano and Quadro, 2018) and could be investigated for the prevention of eye health disorders such as cataract in fish, a common problem in salmonid aquaculture (Waagbø et al., 2003; Bjerås and Sveier, 2004).

While several studies have investigated health and immune effects of microalgae in fish, detected responses seem to depend on both microalgae species as well as fish species investigated (Table 6). We detected microalgae specific influences on health and immunity in Atlantic salmon at both local (intestine) and systemic (plasma, liver, spleen) scales. Adding broken *C. vulgaris* to the diet in this study lowered aspartate aminotransferase and alanine aminotransferase activity levels in the salmon plasma which may indicate improved liver health. Activity of these two enzymes is primarily considered an indicator of liver damage, as higher levels result from destructed or damaged liver cells (Huang et al., 2006). A meta-analysis on the effect of *Chlorella* supplementation on liver health in humans found an overall reduction of aspartate aminotransferase levels, while no effect was detected on alanine aminotransferase serum levels (Yarmohammadi et al., 2021).

Oxidative stress resulting from the increased formation of reactive oxygen species was shown to be reduced by the dietary intake of antioxidants in various fish species (reviewed by Hoseinifar et al., 2021). Superoxide dismutase 1, which catalyses the breakdown of superoxide radicals (Fukai and Ushio-Fukai, 2011), was induced in salmon fed *S. limacinum* after two weeks of feeding the diets. High amounts of DHA from *S. limacinum* are prone to peroxidation resulting in the formation of 4-hydroxyhexenal (4-HHE) which in turn can activate the Nrf2 antioxidant pathway inducing expression of sod (Yang et al., 2019). Several studies with mammalian cell lines have indicated that DHA is able to increase GSH content (Arab et al., 2006) as well as intracellular Sod and Gpx concentrations (Clementi et al., 2019). Our results indicate an early effect of the diet; however, protein levels after eight weeks were similar to the control diet. This suggests that beneficial effects of a functional diet may change over administration time and the potential temporal “habituation effect” requires more attention in future studies. Myeloperoxidase is a characteristic enzyme of neutrophil granulocytes which is involved in the oxidative burst response, where it catalyses the oxidation of chloride ions (Klebanoff, 1999; Frijhoff et al., 2015). It has been further associated with inflammatory processes and various diseases in humans (Davies & Hawkins, 2020). Reduced protein concentrations in the liver of salmon fed *A. platensis* could be caused by a lower abundance of neutrophil granulocytes in the liver, indicating no acute inflammatory response of the liver. Granulocytes in the blood of Nile tilapia *Oreochromis niloticus* fed diets containing different levels of *A. platensis* decreased strongly after 12 weeks of feeding (Abdel-Tawwab and Ahmad, 2009), but no inferences about the concentration or activity of Mpo has been made in this study.

The liver furthermore produces acute phase proteins and levels of transcripts encoding the acute phase protein serum amyloid A (*saas5*) were downregulated in fish fed broken *C. vulgaris* and *S. limacinum*. This highlights the potential anti-inflammatory role of

these microalgae with described effects of their main chemical components in the literature; lutein in case of *C. vulgaris* (Chung et al., 2017; Demmig-Adams et al., 2020) and DHA in case of *S. limacinum* (Li et al., 2005; Mullen et al., 2010).

However, microalgae addition increased the expression of *c1ql2* in the liver and spleen. *C1ql2* is closely related to the first subcomponent of the complement system and might be therefore involved in the response to a variety of environmental conditions in Atlantic salmon (Krasnov et al., 2020; Beemelmanns et al., 2021). The precise function of *c1ql2* is still unknown (Lao et al., 2008; Köbis et al., 2017). In addition to *c1ql2*, the upregulated levels of transcripts coding for the anti-microbial peptide hepcidin (*hamp*) and lysozyme (*lyzc2*) in the liver indicate a potentially enhanced anti-microbial defense by microalgae-enriched diets (Messina et al., 2019; García-Márquez et al., 2020).

Functional feed additives are thought to modulate the local immune response in the intestine and several of the investigated genes in the anterior intestine were modulated by the diet. The increased expression of *drtp1* in all microalgae supplemented groups may be linked to a general response of the intestine towards novel antigens in the diet, but was also found to be induced after an acute phase response (Martin et al., 2006; Talbot et al., 2009). Interferon-stimulated gene 15 (*isg15*) was upregulated in the anterior intestine in response to the microalgae diets. This gene is induced by type 1 interferon and acts like a cytokine (Perng & Lenschow, 2018). Interestingly, other cytokine receptors, namely interleukin 1 receptor (*il1r2*) as well as interleukin 10 receptor (*il10rb*) were downregulated in most microalgae diets. This may indicate a reduced sensitivity of the intestine towards pro-inflammatory signals or a general reduction of pro-inflammatory signals present in the intestine. Grammes et al. (2013) showed that including *Chlorella vulgaris* in the diet had anti-inflammatory action and protected Atlantic salmon from developing a soybean meal-induced enteritis (SBMIE). In zebrafish *Danio rerio* feeding diets with PUFA-rich microalgae increased the expression of the anti-inflammatory cytokine *il10* (Nayak et al., 2020). Interference of the diet with pro-inflammatory signaling was also detected in the spleen where expression of NFκB inhibitor alpha (*ikba*; Wang et al., 2009) was reduced in fish fed intact *C. vulgaris*.

The future of using microalgae as a functional feed ingredient largely depends on its production cost and economic benefits when incorporated into diets for Atlantic salmon. Although we investigated microalgae which are already cultivated at commercial scale, the current price (~20 – 30€ per kg) permits its use at higher inclusion levels only in restricted time periods. These might be during the production of juveniles, where feed costs are generally lower or in the final stage of production. Enhancing the product quality before slaughtering by increasing the fillet DHA and carotenoid content, with known benefits for human health can represent an economically viable strategy which should be further explored.

## Conclusion

Our study revealed that microalgae addition of 8% to the diet could have positive effects on the health of Atlantic salmon reared in

RAS without affecting its growth performance. We confirmed the transfer of important functional components of microalgae (polyunsaturated fatty acids and pigments) into the fish muscle, but the role and function of many of the functional compounds present in microalgae remains elusive and needs further investigation. Our results further indicate that microalgae enriched diets induce a local anti-inflammatory response in the intestine, improve oxidative stress response and stimulate complement and antibacterial responses in liver and spleen. Based on our comprehensive data, we encourage future studies to provide a holistic view on the health status of fish when evaluating functional feeds in aquaculture and investigate the use of microalgae enriched diets in other economically important production phases.

## Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## Ethics statement

The experiment was approved by the animal welfare officer of the “Fraunhofer IMTE Büsum” and the local authority of Schleswig-Holstein, according to the German animal welfare law (NTP – ID 00043858-1-0). The study was conducted in accordance with the local legislation and institutional requirements.

## Author contributions

JMu: Conceptualization, Data curation, Formal Analysis, Investigation, Visualization, Writing – original draft. MP: Data curation, Investigation, Writing – review & editing. JMo: Data curation, Methodology, Writing – review & editing. UO: Data curation, Methodology, Writing – review & editing. DRvM: Data curation, Writing – review & editing. AR: Data curation, Writing – review & editing. TG: Funding acquisition, Project administration, Writing – review & editing. JL: Writing – review & editing, Data curation, Methodology. TS: Data curation, Methodology, Writing – review & editing. HS: Conceptualization, Supervision, Writing – review & editing. CS: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2023.1273614/full#supplementary-material>

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