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Farmed Sea Lettuce (*Ulva fenestrata*) as a Promising Ingredient for Rainbow Trout (*Oncorhynchus mykiss*)

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ABSTRACT

Introducing novel and sustainable feed ingredients is essential to improve the environmental impact of fish aquaculture. Some species of seaweed, such as the green alga sea lettuce (*Ulva fenestrata*), constitute a promising novel ingredient due to their suitable amino acid profile and favourable growth traits. However, the effects of farmed *Ulva* in feed for cold-water salmonids have not yet been studied. Here we show that rainbow trout (*Oncorhynchus mykiss*) growth was improved by including *Ulva* meal in the feed while maintaining intestinal health and fish welfare. The study lasted for 12 weeks, and experimental diets included a control feed, and two feeds with 3% and 15% *Ulva* meal, respectively. The growth rate was highest for fish given the 15% *Ulva* diet during the first 6 weeks and the 3% *Ulva* diet for the last 6 weeks. This was likely due to an increased feed intake (FI) and improved palatability resulting from the inclusion of *Ulva*. Analysis of intestinal health parameters, primary barrier function and histomorphology, revealed no impaired barrier or inflammatory responses to any of the diets. Similarly, other primary and secondary stress indicators including plasma cortisol levels and oxidative stress markers in the liver and muscle tissues, were unaffected by dietary treatments. Interestingly, the fillet colour became more yellow with increased *Ulva* inclusion, which could affect the acceptance of the product to customers. Nevertheless, the results demonstrate that sea-farmed *Ulva* can contribute to improving the sustainability of future aquafeeds for rainbow trout.

1 | Introduction

New sustainable ingredients in fish feed, such as seaweeds, are a key factor to improve the environmental impact of aquaculture as over 70% of the greenhouse gas emissions for farmed fish originates from the feed production [1, 2]. Fishmeal and fish oil have traditionally been the main ingredients in fish feed, but over time factors such as an increased demand together with stagnated supplies and increased prices have led to the search for alternative ingredients [3, 4]. Soybean meal emerged as a low-cost protein replacement, and although there are certified and responsibly sourced options, parts of the industry still use toxic insecticides, pesticides and practises that can causes deforestation and biodiversity reduction [5–7]. A potentially more sustainable alternative ingredient is seaweeds, which absorbs and assimilates dissolved inorganic nutrients directly from the seawater they are farmed in, resulting in a low environmental impact [7, 8]. Sea-farmed seaweeds, in fact, have one of

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the lowest greenhouse gas emissions of all blue foods at ca. 2000 kg $CO_2e\ t^{-1}$ [8].

Green seaweed (Chlorophyta) are currently the least produced and harvested seaweed phylum, but recent increases in production and diversification have raised expectations about their future potential both as human food, and in biotechnological applications [9]. *Ulva*, also known as sea lettuce, is a green seaweed genus identified as a promising candidate for upscaled farming in the global seaweed industry [10]. *Ulva fenestrata* in particular is a suitable aquaculture species in Northern Europe due to its high growth rate under the temperature and nutrient conditions typical of the region [11]. The nutrient content of *U. fenestrata* varies depending on the time of year due to the influence of environmental parameters such as irradiance, temperature and available nutrients [12]. The content of protein, fatty acids and bioactive phytochemicals is typically highest in the early spring and declines towards summer in sea-based cultivations, while the carbohydrate and ash content increase later in the season [13]. Cultivating *U. fenestrata* in nutrient rich waste streams from the fish industry can increase the seaweed protein content further, thereby enhancing their potential as feed ingredients [14–16]. *Ulva* species have an estimated protein content up to 29% dry weight with the traditional Kjeldahl conversion factor of 6.25 (protein content is 23.2% with a conversion factor of 5 according to Angell et al. [17]) with values typically around 20% [18–20]. The protein content is lower than that of soybean meal at ca. 45%–50% and fish meal at ca. 70%–76% [21]. The proportion of essential amino acids is around 30%–40% of the total amino acid content, which is similar to that of soybean meal [15, 22–24]. Two of the most important essential amino acids for salmonids are methionine and lysine, as deficiencies can lead to various pathological conditions such as inflammation and fatty liver [2, 25, 26]. The content in *Ulva* from Swedish and Norwegian waters ranges from 3.3%–5.8% for lysine and 0.5%–2.6% for methionine [15, 22, 24, 27]. *Ulva* is also a source of bioactive compounds, such as sulphated polysaccharides including ulvan, sulfolipids, glycolipids, photosynthetic pigments and betaine [9, 28, 29]. These substances include antioxidant compounds that counteract the formation and negative effects of radical oxygen species (ROS) along with antimicrobial agents to combat infections in fish [30–32]. Improvements in oxidative stress response, stress resistance, and immunostimulatory effects have been observed on fish when fed supplementations including these compounds for gilthead sea bream (*Sparus aurata*), European sea bass (*Dicentrarchus labrax*), Nile tilapia (*Oreochromis niloticus*) and Atlantic salmon (*Salmon salar*; [33–36]). The nutritional content, therefore, makes the genus *Ulva* a promising candidate for future aquafeeds.

In feed formulations, various raw materials are combined in different proportions to meet the nutritional requirements of the farmed species [4]. Novel ingredients are studied both at higher inclusion rates and as additives in smaller quantities depending on their nutritional properties [7]. The relatively high protein content in *Ulva*, for a seaweed, may reduce reliance on other bulk protein sources in feed formulations while the presence of bioactive compounds also makes it interesting as an additive ingredient [9]. When including such novel ingredients in feed, it is important to verify that the nutritional requirement of the animal is met but also that it will not hamper their health and welfare [7]. To explore the full effects of a novel feed, beyond measures of growth, various in-depth mechanistical physiological parameters can be used [37, 38]. For example, intestinal health and morphology are good indicators in feed studies as imbalanced feeds have been shown to reduce health and welfare by causing long term stress, enteritis and increased epithelial permeability [39–41]. Similarly, expressions of appetite regulating genes and hormones can be important indicators to explain variations in feed intake (FI) and growth rates [42]. Since seaweed can have positive effects on the immune status and oxidative metabolism of the fish, measuring oxidative stress responses is of high relevance as well [35]. Beyond ensuring fish health and welfare, it is important that aquaculture products developed with novel feeds are also appealing to consumers. To evaluate this, quality parameters such as proximate composition and fillet colour can be assessed. Previous research using *Ulva* spp. in aquafeeds report differing effects. Including *Ulva* in varying levels (3%–30%) in feed for gilthead sea bream and European sea bass have shown mixed results on growth, ranging from negative [35] to positive [43–45] or lack of effects [46, 47]. Including up to 10% *Ulva* in the diet of rainbow trout (*Oncorhynchus mykiss*) also had varying results with either reduced [48] or no effects on growth [49]. Rainbow trout is one of the main aquaculture fish species in the European Union [50] and dominates the Swedish aquaculture finfish production [51]. It is therefore a relevant study species for feed trials investigating local and novel alternative ingredients. The prior rainbow trout feed studies used wild harvested *Ulva rigida* and *Ulva lactuca* from Turkey [48, 49]. There is, however, a lack of studies evaluating the effects of including sea-farmed *U. fenestrata*, rather than wild harvested *U. rigida* and *U. lactuca*, in the feed of rainbow trout. Studying the inclusion of a locally farmed seaweed ingredient is of high relevance to improve the development of sustainable feeds in the Nordic aquaculture industry.

The aim of this study was therefore to evaluate sea-farmed dried *U. fenestrata* as an ingredient in experimental diets for rainbow trout. This was investigated through the following main objectives: (1) Evaluate effects of using *Ulva* biomass both at a functional additive level (3% inclusion) and as a bulk ingredient fully replacing the soybean concentrate (15% inclusion) on fish growth, intestinal health, systemic and oxidative stress, appetite regulation, and product quality parameters and (2) Evaluate the seasonality of nutritional potential in *Ulva* biomass by studying effects of spring and summer harvests on the abovementioned physiological and product quality parameters.

2 | Materials and Methods

2.1 | Experimental Animals and Holding Conditions

On December 5, 2022, 153 rainbow trout of 73.9 ± 15.7 g (mean ± standard deviation) were transported to the animal facility at the Department of Biological and Environmental Sciences, University of Gothenburg (Gothenburg, Sweden) from the fish farm Vänneåns Fiskodling (Knäred, Sweden). The fish were randomly distributed into nine conical circular tanks with dimensions of 61 × 56 cm (diameter × height), each holding 100 L of water. Each tank was covered with a plastic lid that was half dark and half transparent, equipped with a powerhead (Eheim Powerhead 650, 650 L h^{-1}, EHEIM GmbH & Co. KG, Deizisau, Germany) to reinforce a circular current and a ø 3 cm aeration stone connected to pressurised air. The fish were acclimated for two and a half weeks until any experimental treatment was performed on them. Both acclimation and experimental periods were conducted in the described nine experimental tanks supplied with water from a freshwater recirculating aquaculture system (RAS) with a total water volume of 10.8 m^3. The photoperiod was set to 06:30–19:00, with dimming up and down during 30 min at the start and end. During the acclimation period, fish were fed to satiation daily with Spirit Trout 4.5 mm pellets (Skretting, Stavanger, Norway).

On 22 December 2022, fish were lightly sedated in MS-222 (100 mg L^{-1}, Ethyl 3-aminobenzoate methanesulfonate, Sigma–Aldrich, St. Louis, United States) buffered with sodium bicarbonate (Sigma–Aldrich, St. Louis, United States) and tagged intraperitoneally with passive integrated transponder (PIT) tags (12 mm, Biomark, Boise, USA). The fish were immediately returned to their previous tanks after tagging and were left undisturbed for another two and a half weeks until further handling.

Throughout the study, water quality parameters were measured bi-weekly. The water temperature was kept at 9.61 ± 0.05°C, pH at 7.31 ± 0.06, and oxygen at 88.67 ± 2.97% saturation. The concentrations of dissolved inorganic nitrogen were analysed using a DR2800 laboratory spectrophotometer with ammonia (NH_3-N) reagent powder pillows (salicylate method, 8155), nitrite (NO_2-N) reagent powder pillows (diazotisation, method 8507) and nitrate (NO_3^--N) LCK 339 kit for analysis (Hach Lange, Dusseldorf, Germany). The NH_3-N concentration was kept at 0.10 ± 0.02 mg L^{-1}, NO_2^--N concentration at 0.03 ± 0.00 mg L^{-1}, and NO_3^--N at 7.73 ± 0.76 mg L^{-1}.

2.2 | Feeding Trial

The feeding trial started on January 9, 2023. The fish were lightly sedated with MS-222, following the previously described dose and buffering, and their body weight (bw) and fork length were recorded. They were redistributed between the previously described tanks (17 fish per tank) to achieve a balanced weight with respect to total tank biomass (1255.8 ± 22.7 g, mean ± SEM). Each tank was randomly assigned one of three diets in triplicates. The experiment lasted for 12 weeks, during which the fish were hand-fed twice daily at 9:00 and 14:30 with a fixed ration. The initial feeding ratio was set to 1.5% of their bw per day. Excess feed was collected with a sieve 20 min after each feeding by flushing the tanks, dried in an oven overnight, and weighted. This pellet mass was used to calculate the total feed consumption for the previous day. If fish in a tank consumed more than 95% of their feed for three consecutive days, the feeding ratio was increased with 10%. The feeding ratio was readjusted to 1.5% bw per tank after the half-way sampling and the above correction of ration, if needed, was applied also for the last half of the experimental period.

2.3 | Experimental Diets

Biomass of *U. fenestrata* was cultivated at the Tjärnö Marine Laboratory (Strömstad, Sweden). Fertilisation was induced in adult *U. fenestrata* and the gametes and juvenile thalli were grown under land-based hatchery conditions for 7 weeks before they were deployed to an offshore sea farm in the Koster archipelago (Skagerrak, Sweden, N58.859901, E11.068660) in October 2021 [11]. The spring and summer biomasses were harvested at the beginning of March and May 2022, respectively. The seaweeds were freeze-dried, ground into meals separately and stored in −40°C (Table 1). The feed production was done at the Feed Technology Laboratory (FTL, Department of Animal Nutrition and Management, Swedish University of Agricultural Sciences, Uppsala, Sweden). Three experimental diets were formulated (Table 2) with the aim to be isoenergetic (20.4–20.8 MJ kg^{-1} gross energy), isonitrogenous (43.5%–44% crude protein [CP]), and isolipidic (22.5% crude fat). The first experimental diet was the control diet which contained the following protein sources; 40% fish meal (low temperature fish meal, North Atlantic origin), 15.3% soy protein concentrate, 8% wheat gluten and 13.6% wheat meal. The lipids in the diet were commercially obtained fish oil (12%, North Atlantic origin) and rapeseed oil (5%). The second experimental diet (3% *Ulva*) was formulated to include 3% *Ulva* meal from the summer-harvested biomass, partially substituting the soy protein concentrate, to evaluate if biomass from the summer could have beneficial functional effects. The third experimental diet (15% *Ulva*) was formulated to include 15% *Ulva* meal from the spring biomass, fully substituting the soy protein concentrate and designed to evaluate potential as a bulk ingredient. Wheat gluten and wheat meal were used to balance diets (Table 2). All three diets were extruded at the FTL using a Twin-Screw Extruder (3 mm screen; model: Ketse 20/40, Brabender, Duisburg, Germany) followed by vacuum coating of the lipid fraction (Vacuum Coater, Amandus Khal, Reinbek, Germany). Directly after the production, diets were shipped to the animal facility at the University of Gothenburg and stored at 4°C until the start of the feeding trial.

2.4 | Chemical Analysis

The composition of experimental ingredients (Table 1) and the calculated composition of the experimental feeds (Table 3) were analysed by a certified laboratory (Eurofins Food & Agro Testing Sweden AB, Linköping, Sweden). The reference methods used were NMKL 6:2003 for crude protein, NMKL 160 for crude lipid, NMKL 173 for ash, NMKL 23 for water content, EU nr 1169/2011 for carbohydrate and gross energy, ISO 13903:2005 for amino acids, ISO 5498 for fibre, SS-EN ISO 17294-2:2016 /SS-EN 13805:2014 for calcium, cupper, zink, iron, manganese, magnesium, phosphorous, potassium, sodium, and SS-EN ISO 11885:2009 /SS-EN 13805:2014 for sulphur. Fatty acids were analysed using gas chromatography coupled with a flame ionisation detector (Eurofins Food & Agro Testing Sweden AB, Linköping, Sweden).

2.5 | Sampling

The feeding trial was divided into two growth periods through a weight and length assessment half-way through the trial. On 20 February 2023, after 6 weeks, all fish were lightly sedated in MS-222 (see above for dose and buffering) and their PIT tag number, individual weight and fork length was recorded to the closest 0.1 g or cm.

The final sampling was performed after six more weeks, between April 3 and 5, 2023, with four fish sampled per tank for all physiological and product quality parameters ($n = 12$ per treatment, 36 fish sampled in total). Fish were deeply anesthetised in 12 mg L^{-1}

TABLE 1 | The proximate composition, amino acids, fatty acids and mineral profile of the dried spring and summer biomass of *U. fenestrata* (g 100 g^{-1} if not otherwise specified).

Proximate composition	Spring biomass *U. fenestrata*	Summer biomass *U. fenestrata*
Crude protein	27.60	6.88
Crude lipid	3.05	1.15
Ash	19.90	17.10
Water content	8.95	40.80
Nitrogen-free extract	40.50	34.10
Essential amino acids		
Arginine	1.50	0.18
Histidine	0.39	0.05
Isoleucine	0.81	0.13
Leucine	1.59	0.23
Lysine	1.10	0.16
Methionine	0.48	0.05
Phenylalanine	1.06	0.17
Threonine	1.09	0.20
Valine	1.24	0.22
Non-essential amino acids		
Alanine	1.68	0.31
Aspartic acid	2.19	0.39
Cysteine and cystine	0.30	0.05
Glutamic acid	3.45	0.40
Glycine	1.27	0.24
Hydroxyproline	0.20	0.20
Ornithine	0.05	0.05
Proline	1.18	0.14
Serine	1.05	0.20
Tyrosine	0.69	0.09
Tryptophan	0.36	0.04
Fatty acids (% of total fatty acids)		
Saturated fatty acids	17.6	27.6
Monounsaturated fatty acids	12.8	17.1
Polyunsaturated fatty acids	45.4	38.1
Omega n-6 fatty acids	6.1	10.7
Omega n-3 fatty acids	39.3	27.4
C 20:5 n-3 (EPA)	0.5	1.5
C 22:6 n-3 (DHA)	<0.1	1.1
Ratio omega n-6/omega n-3 fatty acids	0.16	0.39
Minerals and fibres		
Fibre	3.5	3.1
Calcium	2.8	7.8
Copper	0.0044	0.0022
Zinc	0.0110	0.0073
Iron	0.26	0.20
Manganese	0.014	0.015

of metomidate hydrochloride (Aquacalm, Syndel Laboratories, Ltd., Nanaimo, Canada) and subsequently killed by cutting the spine. A 2 mL blood sample was taken from the caudal vein using heparinised syringes and centrifuged at 4°C for 5 min at 10,000 g before plasma was extracted. The plasma was stored at −80°C prior to analysis. The fish were individually identified through reading of their PIT tag numbers, and their individual weight and length was measured. The head was then excised, and the hypothalamus was dissected out, placed on dry ice and transferred to −80°C for later qPCR analyses. Two muscle samples of 1 × 1 × 1 cm were taken from the dorsal side, just anterior of the dorsal fin from each fish, and immediately placed in liquid nitrogen for later analyses of oxidative stress markers lipid peroxidation (LPO) and glutathione.

The body cavity of the fish was opened, and the liver was dissected out and weighed. Two liver samples of 0.5 × 0.5 × 0.5 cm were then cut and placed in liquid nitrogen for later analysis of oxidative stress markers. The entire intestine was then excised between the last pyloric caeca and the anus, and divided into a proximal and a distal segment, separated by the ileo-rectal valve. One histological section, 3 mm ring, was cut from the first part of each intestinal segment and placed in 4% formaldehyde buffer for 24 h, then transferred to 70% ethanol until analysis. The remaining segments were opened longitudinally, washed, and placed on ice in ice-cold Ringer's solution for FW rainbow trout (mmol L^{-1}: NaCl 140, KCl 2.5, $CaCl_2$ 1.5, $MgCl_2 \cdot 7H_2O$ 0.8, $NaHCO_3$ 15.0, $NaH_2PO_4 \cdot 2H_2O$ 1.0, HEPES 5.0, Glucose 10.0, Glutamine 20.0, according to Sundell et al. [52]). The intestinal segments, proximal and distal from each fish, were subsequently mounted in Ussing chambers for electrophysiological analyses as described below. All visceral fat was collected and weighed and finally a Norweigan Quality Cut (NQC) was taken, folded in aluminium foil, placed on dry ice and stored at −80°C prior to analyses of product quality parameters.

On April 5, all remaining fish were sedated in a lethal dose of MS-222 and killed with a sharp blow to the head. Their PIT tag

TABLE 2 | Feed formulation (g 100 g^{-1}) and calculated composition (% unless otherwise specified) of the three experimental diets control, 3% *Ulva*, and 15% *Ulva*.

	Diet		
	Control	**3% *Ulva***	**15% *Ulva***
Ingredients			
Fish meal	38	38	38
Soy protein concentrate	15	12	0
Wheat gluten	8	10	10
Wheat meal	14	12	12
Potato starch	4	4	4
Fish oil	12	12	12
Rapeseed oil	5	5	5
Premix of vitamins and minerals	1	1	1
Spring biomass *U. fenestrata*	—	—	15
Summer biomass *U. fenestrata*	—	3	—
Choline chloride	0.5	0.5	0.5
Titanium dioxide	0.5	0.5	0.5
Monocalcium phosphate	2	2	2
Calculated composition			
Crude protein	44.0	43.7	43.5
Digestible protein	39.4	39.1	39.0
Gross energy (MJ kg^{-1})	20.4	20.8	20.4
Digestible energy (MJ kg^{-1})	17.9	18.3	18.0
Crude lipid	22.5	22.5	22.5
Lysine (g kg^{-1})	28.9	24.9	27.9
Methionine (g kg^{-1})	10.9	10.6	10.8
Phosphorus	1.3	1.2	1.2
Digestible phosphorus	0.8	0.8	0.8
Dry mash oil content	5.9	6.0	5.9

TABLE 3 | Proximate composition (g 100 g^{-1} if not otherwise specified) and energy (kJ 100 g^{-1}) of the three experimental diets control, 3% *U. fenestrata* and 15% *U. fenestrata*.

	Diet		
Proximate composition	**Control**	**3% *Ulva***	**15% *Ulva***
Crude Protein	40.8	45.9	44.9
Crude lipid	19.7	20.4	21.5
Ash	11.7	10.0	10.2
Water content	3.63	3.68	3.62
Carbohydrates	24.2	20.1	19.8
Gross energy	1834	1877	1895
Essential amino acids			
Arginine	2.26	2.54	2.64
Histidine	0.73	0.87	0.91
Isoleucine	1.49	1.71	1.81
Leucine	2.90	3.23	3.40
Lysine	2.49	2.78	2.91
Methionine	0.94	0.94	1.01
Phenylalanine	1.79	1.92	2.04
Threonine	1.58	1.68	1.79
Valine	1.77	1.89	2.03
Non-essential amino acids			
Alanine	2.26	2.30	2.46
Aspartic acid	3.28	3.83	4.01
Cysteine	0.51	0.51	0.58
Glutamic acid	7.80	8.23	8.93
Glycine	2.34	2.46	2.62
Hydroxyproline	0.33	0.26	0.24
Ornithine	<0.01	<0.01	<0.01
Proline	2.44	2.66	2.94
Serine	1.80	1.98	2.12
Fatty acids (% of total fatty acids)			
Saturated fatty acids	20.4	20.1	20.3
Monounsaturated fatty acids	41.8	42.0	41.9
Polyunsaturated fatty acids	33.2	33.6	33.4
Omega n-6 fatty acids	10.1	10.7	10.7
Omega n-3 fatty acids	23.1	22.9	22.8
C 20:5 n-3 (EPA)	5.7	5.8	5.7
C 22:6 n-3 (DHA)	9.6	9.9	9.8
Ratio omega n-6/omega n-3 fatty acids	0.44	0.47	0.47

(Continues)

numbers were recorded, and weight and length were measured. One fish per tank ($n = 3$) were frozen whole in −20°C for later analysis of proximate composition. The three fish from each experimental diet were pooled for this analysis.

2.6 | Growth and Feed Utilisation

Specific growth rates (SGR) were calculated for individual fish from the start until the half-way sampling and from the half-way sampling until the end of experiment according to:

$$SGR = 100 \times \frac{\ln(bw_{final}) - \ln(bw_{initial})}{d}, \quad (1)$$

TABLE 3 | (Continued)

Proximate composition	Control	3% *Ulva*	15% *Ulva*
Minerals (g 100 g^{-1})			
Calcium	20	20	20
Magnesium	4.6	1.8	2.7
Phosphorous	17	17	17
Potassium	11.0	9.0	9.6
Sodium	0.87	0.53	0.63
Sulphur	11.0	5.4	7.1

where bw_{final} is body weight at the final measuring point of the period, $\text{bw}_{\text{initial}}$ is body weight at the first measuring point of the specific period and d is the duration of the period in days.

The condition factor (CF) was calculated according to:

$$\text{CF} = 100 \times \frac{\text{bw}}{\text{forklength}^3}. \tag{2}$$

FI was calculated per fish as:

$$\text{FI} = \frac{\text{feed consumption} - \text{uneaten pellets}}{n_{\text{tank}}}, \tag{3}$$

where feed consumption is the dried feed (g) consumed in the tank per day, uneaten pellets is the dried mass of collected pellets after feedings and n_{tank} is the number of fish per tank.

Feed conversion ratio (FCR) was calculated as:

$$\text{FCR} = \frac{C_T}{w_{\text{final}} - w_{\text{initial}}}, \tag{4}$$

where C_T is the total feed consumption in the tank, w_{final} is the fish weight at the end of experiment and w_{initital} is the fish weight at the start of the experiment, per tank.

Hepatosomatic index (HSI) was calculated as

$$\text{HSI} = 100 \times \frac{l_w}{\text{bw}}, \tag{5}$$

where l_w is liver weight (g). Visceral fat index (VFI) was calculated using the same equation with the visceral fat weight replacing liver weight.

2.7 | mRNA Expression of Appetite Regulating Genes

The hypothalamus was analysed for transcription of selected appetite regulatory genes (Table 4). The total RNA was extracted with the RNeasy Lipid Tissue Mini Kit (Qiagen N.V., Hilden, Germany). In brief, the hypothalamus was placed in tubes with 1 mL QIAzol lysis reagent and a steel bead, then homogenised and lysed in a TissueLyser II homogenizer (Qiagen N.V., Hilden, Germany). 200 µL chloroform was added to the lysate, then samples were shaken and centrifuged at 12,000 g and 4°C for 15 min. The supernatant was extracted and 500 µL of 70% ethanol was added. The RNA was repeatedly washed in a spin column with multiple buffers and extracted with RNAse-free water. The purity and concentration of RNA were measured in a NanoDrop One/OneC micro volume spectrophotometer (Thermo Fisher Scientific, Waltham, USA). To obtain equal levels of RNA, samples were diluted using RNase-free water. The iScript cDNA Synthesis Kit (Bio-Rad Laboratories Inc., Richmond, USA) was used for reversed transcription of the extracted RNA to complementary cDNA.

For qPCR reactions, samples were analysed in duplicates with 10 µmol L^{-1} of forward and reverse primers using SYBR Green as the fluorescent intercalating agent (SsoAdvanced Universal SYBR Green Supermix, Bio-rad Laboratories Inc., Richmond, USA). The efficiency of the primer pairs for the target genes was verified with cDNA dilution series (0.39–12.5 ng µL^{-1}) from four random samples. The primers were approved in the efficiency pairs 90%–110%. The qPCR programme was run for 40 cycles (10 s denaturation at 95°C and 30 s annealing and extension at 60 or 61°C with the CFX Connect Real-time PCR Detection System; Bio-Rad Laboratories Inc., Richmond, USA). The generated melt curves were analysed to assess quality. The gene expressions were compared to that of reference genes β-actin and ELF1α, and calculated as relative expression according to the $2^{\Delta CT}$ method [56]:

$$\text{Relative expression} = 2\wedge\left(C_T^{(\text{reference})} - C_T^{(\text{target})}\right), \tag{6}$$

where C_T (reference) is the threshold cycle for reference genes and C_T (target) is the threshold cycle for the respective target genes.

2.8 | Hormone and Ion Plasma Levels

The radioimmunoassay (RIA) for measuring insulin-like growth factor-I (IGF-I) was done according to Shimizu et al. [57] based on the original protocol from Moriyama et al. [58]. The antibody, originally developed for barramundi IGF-I, was obtained from GroPep (GroPep Ltd., Thebarton, Australia) and made in rabbits (Agrisera, Vännäs, Sweden). The growth hormone (GH) RIA was performed according to Björnsson et al. [59], with modifications given in Gong et al. [60]. In the GH and IGF-I RIAs, [^{125}I]-labelled tracers were used, and radioactivity measurements were done using a gamma counter (Wallac 1417, LKB Instruments, Turku, Finland). The RIA for measuring the plasma cortisol levels was performed with a [^{3}H]-labelled tracer as described by Young [61] using a previously validated sheep cortisol antibody (Code: S020; Lot: 1014-180182) from Guildhay Ltd. (Guildford, Surrey, UK, no longer active; Sundh et al. [62]). A beta counter (Wallac 1409 DSA Liquid Scintillation Counter, LKB Instruments, Turku, Finland) was used to measure the radioactivity. Plasma levels of Na$^+$, Cl$^-$ and K$^+$ were measured with a Convergys ISE comfort Electrolyte Analyzer (Convergent Technologies GmbH & Co. KG, Coelbe, Germany).

TABLE 4 | The nucleotide sequences of primer pairs to reference and target genes for the appetite regulatory RNA expressions analysed with qPCR.

Gene	Direction	Sequence	Accession number	References
CART	F	GTCCATCGTTCTTAGTGCTGAA	AB455538	Jørgensen et al. [53]
	R	CAGTTGCTTTTCGTTGGTCAA		
CRF	F	ACAACGACTCAACTGAAGATCTCG	NM001124286	Jørgensen et al. [53]
	R	AGGAAATTGAGCTTCATGTCAGG		
MC4R	F	TTCTCACACTGGGGATAGTCA	AY534915.1	Jørgensen et al. [53]
	R	CACAGCCAAAGAACAGATGAAT		
NPY	F	AGAATTGCTGCTGAAGGAGAG	AF203902	Jørgensen et al. [53]
	R	GGGACAGACACTATTACCACAA		
POMCA1	F	CTCGCTGTCAAGACCTCAACTCT	TC86162	Leder and Silverstein [54]
	R	GAGTTGGGTTGGAGATGGACCTC		
EF1α	F	AGGCATTGACAAGAGAACCATT	AF498320.1	Striberny et al. [55]
	R	TGATACCACGCTCCCTCTC		
β-actin	F	ATGGAAGATGAAATCGCC	AF157514	—
	R	TGCCAGATCTTCTCCATG		

Abbreviations: CART, cocaine- and amphetamine-regulated transcript; CRF, corticotropin-releasing factor; F, forward; MC4R, melanocortin 4 receptor; NPY, neuropeptide Y; POMCA1, proopiomelanocortin A1; R, reverse.

2.9 | Intestinal Electrophysiology and Barrier Function

The Ussing chamber technique was used to assess the barrier function and electrophysiology of the intestinal epithelium [63]. The proximal and distal intestinal regions were carefully peeled using forceps to remove the serosal layer, mounted into the Ussing chambers, and 4 mL Ringer's solution was added to each half-chamber. The analysis was performed according to Sundell et al. [52] with modifications from Sundell and Sundh [63]. Mounted intestinal regions were acclimated for 60 min in Ringer's solution before measurements began. A gas lift using pressurised air was continuously bubbling in both half-chambers to assure stable Ringer pH, tissue oxygenation and prevent unstirred layers. Temperature was kept constant by water supplied cooling mantles set at the acclimation temperature of the fish, 10°C.

Upon start of experiment, electrical parameters were measured every 5 min until the end at 150 min. Paired KCl electrodes in 3 mol L^{-1} KCl solution were connected through agar bridges, placed close to the intestinal epithelium, to the half-chambers and measured the transepithelial potential difference (TEP). Platinum electrodes, placed in each half-chamber, applied alternating DC voltages (U) across the epithelium, set to create alternating currents (I) between −30 and 30 µA. This generated TEP/I pairs, and the calculated slope when fitting them to a straight line represents the transepithelial electrical resistance (TER). Using Ohms law, the short circuit current (SCC) was calculated.

The TEP values represent the potential difference across the intestinal epithelium generated by active and passive ion transfer. The TER values in the fish intestine, which is a leaky epithelium [64, 65], mainly reflects the paracellular permeability to ions. The SCC values indicate the active transport of ions across the intestine. An inert hydrophilic marker, [^{14}C]-mannitol (PerkinElmer, Waltham, USA), was used as a further indicator of paracellular epithelial permeability. A radiolabelled essential and in fish limiting amino acid, [^{3}H]-L-lysine (PerkinElmer, Waltham, USA), was used to assess active amino acid uptake. The [^{14}C]-mannitol (spec. act. 1.29×10^{14} DPM mol^{-1}) and [^{3}H]-L-lysine (spec. act. 5.73×10^{16} DPM mol^{-1}) was added to Ringer's solution (also containing 0.5 mmol L^{-1} cold L-Lysine). This Ringer's with radioactive markers replaced the initial Ringer's solution in the mucosal half-chamber after the acclimation period. In parallel, the Ringer's in the serosal half-chamber was replaced by fresh Ringer's. At timepoints 0, 20, 25, 30, 60, 80, 85, and 90 min, 50 µL samples were taken from the serosal Ringer's. 4.5 mL Ultima Gold scintillation cocktail (Perkin Elmer, Waltham, USA) was added to each marker sample, and the radioactivity was measured in a beta counter (Wallac 1409 DSA Liquid Scintillation Counter, Turku, Finland) using a dual label protocol. The calculation for apparent permeability (Papp) of mannitol was as follows

$$P_{\mathrm{app}} = \frac{dQ}{dt} \times \frac{1}{(A \times C_0)}, \qquad (7)$$

where dQ/dt is the rate of which [^{14}C]-mannitol moved to the serosal half-chamber, A is the exposure area of the intestine in the chamber (0.75 cm^2), and C_0 is the [^{14}C]-mannitol concentration in mucosal half-chamber at start. The lysine transport was calculated as follows

$$L - \mathrm{Lysine\ transport} = \frac{dQ}{dt} \times \frac{1}{A}. \qquad (8)$$

With dQ/dt as the rate of which [^{3}H]-L-lysine moved to the serosal half-chamber and A as the exposure area of the intestine in the chamber (0.75 cm^2).

2.10 | Histological Analyses

To assess the microscopic morphology of the intestinal sections, the fixed tissue samples were dehydrated using an ethanol gradient and treated with clearing agent Histolab Clear (Histolab Products AB, Askim, Sweden). The samples were embedded in paraffin and sections of 5 µm were made from each sample using a Shandon finesse microtome (Thermo Fisher Scientific, Waltham, USA). Six cross-sections at ≥50 µm distance apart were taken from each sample for two fish per tank ($n = 6$). The sections were mounted on APES treated microscope slides (3-Aminoprepyl triethoxysilane, Sigma–Aldrich, St Louis, USA). The slides were dewaxed with Histolab Clear (Histolab Products AB, Askim, Sweden) and rehydrated using a reverse ethanol gradient. The tissues were stained with haematoxylin-eosin (Histolab Products AB, Askim, Sweden) and alcian blue 8 GX (pH 2.5, Merck, KGaA, Darmstadt, Germany). For each section, two photographs were taken with a DXM1200 camera (Nikon, Tokyo, Japan) mounted to an Olympus BX60 microscope (Nikon, Tokyo, Japan). In total, 12 pictures were taken per fish and intestinal section. For each picture, two measurements of lamina propria width, villi height, and the number of goblet cells per µm were made with ImageJ software (Wayne Rasband, National Institutes of Health, Maryland, USA) according to Baeverfjord and Krogdahl [66].

2.11 | Oxidative Stress Markers

The liver and muscle samples were analysed for two oxidative stress markers, LPO, and the glutathione redox system through assessment of reduced glutathione (GSH) and oxidised glutathione (GSSG). LPO shows the degradation of lipids caused by oxidative damage and was measured using the LPO (malondialdehyde, MDA) Assay Kit from Sigma–Aldrich (St. Louis, United States) on 10 mg of tissue sample. For the glutathione analysis, 50 mg tissue samples were homogenised using ultra sonication in four times weight:volume of 5% SSA (5-Sulfosalicylic acid dihydrate; Sigma–Aldrich, St. Louis, United States). The samples rested 15 min on ice and were then centrifuged at 10,000 g for 20 min at 4°C before supernatant was collected. The measurements of glutathione redox system were analysed according to Baker et al. [67] with some adjustments by Vandeputte et al. [68]. The reaction mixture comprised of 143 mmol L^{-1} $Na_2H_2PO_4$ with 6.3 mmol L^{-1} EDTA buffer set to pH 7.4, along with 1 mmol L^{-1} DTNB (for GSSG) and 10 mmol L^{-1} DTNB (for GSH), and 2 mmol L^{-1} NADPH. The kinetic assay was initiated with 20 mL GR-solution (Glutathione reductase, 17 IU mL^{-1}, from baker's yeast *Saccharomyces cerevisiae*, Sigma–Aldrich, St. Louis, United States). Absorbance was measured at 415 nm for 7 min. The assay measures the total glutathione content (tGSH) and the production of oxidised glutathione (GSSG), enabling also the calculation of the ratio between GSH and GSSG as %GSSG.

2.12 | Metabolic and Product Quality Analysis

Three whole fish per treatment were pooled, ground into a homogenous mass and analysed for their chemical composition by a certified laboratory (Eurofins Food & Agro Testing Sweden AB, Linköping, Sweden), using the methods outlined in Section 2.4. Three technical replicates were made per treatment. Fillet colour was measured as CIELAB colour space ($L^* =$ dark to light; $a^* =$ green to red; and $b^* =$ blue to yellow), using a Minolta Chroma Meter CR-300 (Konica Minolta, Tokyo, Japan). Six measurements were taken per sample from the dorsal half of the cutlet. pH was measured in triplicate for each sample using a WTW 340 pH metre (WTW, Weilhem, Germany).

2.13 | Ethical Statement

The ARRIVE guidelines were followed for all experimental procedures and husbandry protocols [69]. The study followed the regulations from the Swedish National Board for Laboratory Animals and had been approved by the Gothenburg's animal ethics committee (Dnr: 5.8.18-22192/2023).

2.14 | Statistics

SPSS (IMB SPSS Statistics for Windows, Version 24.0, IMB Corp, Armonk, USA) was used for statistical analyses and GraphPad Prism version 10.0.0 for Windows (GraphPad Software, Boston, USA) was used to make graphs.

One-way ANOVAs were performed to assess differences between treatment groups (experimental diets), with Tukey's HSD (Honestly Significant Difference) as the post hoc test. All data were first analysed for normality of residuals using histograms, QQ plots, and for homogeneity of variances using Levene's test. If the data failed to meet the criteria for normality of residuals or variances, logarithmic, square root or exponential transformations were applied and they were tested once more. If the requirement for normality of variances remained unmet, data were analysed using the non-parametrical Kruskal–Wallis tests (KW). Significant differences were further tested using the Mann–Whitney test (MW). Data is presented as mean ± standard error of the mean (SEM) unless otherwise stated. For all statistical tests, alpha was set to 0.05.

3 | Results

3.1 | Growth and Feed Utilisation

The fish had similar weights at the experimental start (73.8 ± 2.0 g for control, 70.8 ± 2.2 g for 3% *Ulva*, 77.2 ± 2.4 g for 15% *Ulva*), but the weights after the first 6-week period and the final 6 week period differed significantly depending on fish diet. The fish receiving the 15% *Ulva* diet grew best during the first 6 weeks, while the best growth was found for the 3% *Ulva* group during the final 6-week growth period. These growth differences were caused by differences in FI and FCR.

The WG for the first 6 weeks of the feeding trial was significantly higher for fish given the 15% *Ulva* diet compared to the control and 3% *Ulva* diets (KW, $H\ (2) = 8.619$, $p = 0.013$; Figure 1A1; MW, $Z = -2.721$, $p = 0.007$; MW, $Z = -2.296$, $p = 0.022$; Supporting Information 1: Table 1). The SGR of the fish during these first 6 weeks differed significantly in the same way, with higher SGR for fish given the 15% *Ulva* diet compared to both the control and 3% *Ulva* diets (KW, $H\,(2) = 8.672$, $p = 0.013$; Figure 1B1; MW, $Z = -2.694$, $p = 0.007$; MW, $Z = -2.347$, $p = 0.019$). The FI during this period tended to be higher for the fish given the 15% *Ulva* diet compared to the control and the 3% *Ulva* diet, although not statistically significant ($p = 0.051$; Figure 1C1).

The WG for the final 6 weeks of the feeding trial was significantly higher for fish given the 3% *Ulva* diet compared to the control and

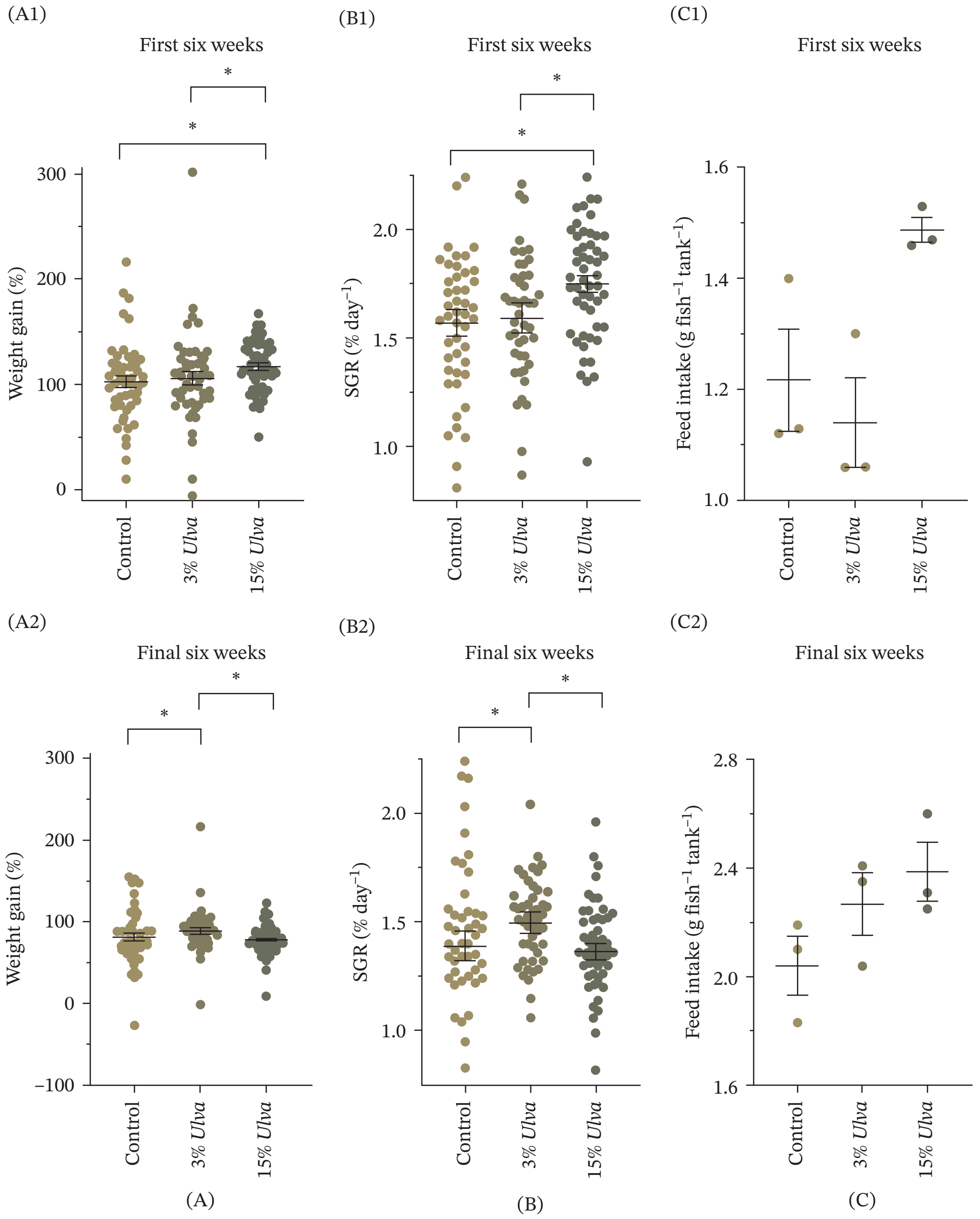


FIGURE 1 | Subfigures (A1–C1) show results from the first 6-weeks of the feeding trial and Subfigures (A2–C2) show results from the final 6-weeks of the feeding trial. Subfigures (A1, A2) illustrate the weight gain ($n = 50, 51, 50$; $n = 50, 51, 49$) and Subfigures (B1, B2) illustrate the specific growth rate (SGR), while Subfigures (C1, C2) illustrate the feed intake per tank ($n = 3$). Bars represent mean ± standard error of mean (SEM). Significant differences ($p < 0.05$) were found for the main effect (Kruskal–Wallis) for subfigures (A1, A2, B1, and B2). These groups were further tested with group specific analyses (Mann–Whitney, $p < 0.05$) and significant results are annotated by stars.

15% *Ulva* diets (KW, H (2) = 9.500, $p = 0.009$; Figure 1A2; MW, $Z = -2.0492$, $p = 0.040$; MW, $Z = -3.130$, $p = 0.002$; Supporting Information 1: Table 1). The same pattern was measured for SGR during the final 6 week growth period, with significantly higher SGR for fish given the 3% *Ulva* diet compared to the control and 15% *Ulva* diets (KW, H (2) = 9.096, $p = 0.011$; Figure 1B2; MW, $Z = -1.956$, $p = 0.050$; MW, $Z = -3.081$, $p = 0.002$). The FI during this period tended to be higher for fish given the 15% *Ulva* diet compared to the control and 3% *Ulva* diets, although not statistically significant ($p = 0.158$; Figure 1C2). The CF did not differ between treatment for either experimental period (Supporting Information 1: Table 1).

The FCR did not differ between fish given the different diets for the first 6 weeks (Figure 2A, Supporting Information 1: Table 1). During the final 6 weeks, the FCR for fish given the 15% *Ulva* diet was significantly higher compared to control diet (one-way ANOVA, F (2,6) = 5.379, $p = 0.046$; Figure 2B).

The HSI was significantly higher for fish given the 15% *Ulva* diet compared to the control and 3% *Ulva* diets (KW, H (2) = 13.544, $p = 0.001$; Figure 3; MW, $Z = -3.298$, $p < 0.001$; MW, $Z = -2.855$, $p = 0.004$). The VFI did not differ between treatments (Supporting Information 1: Table 1).

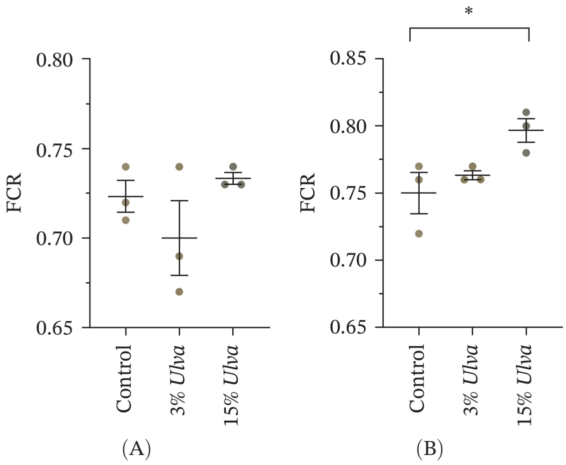


FIGURE 2 | Feed conversion ratio (FCR) per tank for (A) the first 6 weeks of the feeding trial ($n = 3$) and (B) the final 6 weeks of the feeding trial. Bars represent mean ± standard error of mean (SEM). Significant differences ($p < 0.05$) were found for the main effect (Kruskal–Wallis) for (B). This group was further tested with group specific analyses (Mann–Whitney, $p < 0.05$) and significant results are annotated by stars.

3.2 | mRNA Expression of Appetite Regulating Genes

The hypothalamic mRNA expressions of Cart, Crf, NPY, PomC and MC4R did not differ between treatments (Supporting Information 2: Table 2).

3.3 | Hormone and Ion Plasma Levels

The plasma cortisol levels were significantly lower for fish given the 3% *Ulva* diet compared to the control diet (KW, $H(2) = 7.880$, $p = 0.019$; Table 5; MW, $Z = -2.608$, $p = 0.009$). The plasma IGF-1 and GH levels did not differ between treatments. The plasma Ca^{2+} levels were significantly lower for fish given the 15% *Ulva* diet compared to the control and 3% *Ulva* diets (one-way ANOVA, $F(2,33) = 8.487$, $p = 0.001$; Table 5; Tukey, $p = 0.003$; $p = 0.003$). The plasma Cl^- levels were significantly higher for fish given the 15% *Ulva* diet compared to the control and 3% *Ulva* diets (one-way ANOVA, $F(2,33) = 16.930$, $p < 0.001$; Tukey, $p < 0.001$; $p = 0.003$). The plasma K^+ levels were significantly higher for fish given the 3% *Ulva* diet compared to control diet, with no further differences with fish given the 15% *Ulva* diet (one-way ANOVA, $F(2,33) = 4.975$, $p = 0.013$; $p = 0.011$). The plasma Na^+ levels did not differ between treatments.

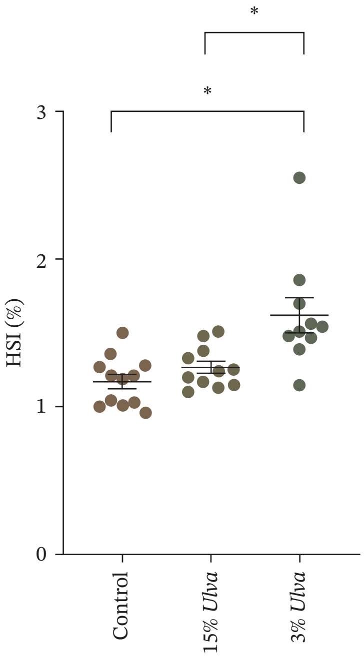


FIGURE 3 | Hepatosomatic index (HSI) at final sampling ($n = 12, 11, 10$). Bars represent mean ± standard error of mean (SEM). Significant differences ($p < 0.05$) were found for the main effect (Kruskal–Wallis). The group was further tested with group specific analyses (Mann–Whitney, $p < 0.05$) and significant results are annotated by stars.

3.4 | Intestinal Electrophysiology and Barrier Function

None of the electrical parameters of the intestinal epithelia, TER, TEP or SCC, showed any statistical difference in response to dietary treatment. There was a tendency for higher TER in the proximal intestine for fish given the control diet (90.56 ± 11.71 Ω) compared to the 15% *Ulva* diet (71.86 ± 11.43 Ω) and the 3% *Ulva*

TABLE 5 | The plasma levels of cortisol, IGF-I and GH (ng mL^{-1}), and of Ca^{2+}, Cl^-, K^+ and Na^+ (mmol L^{-1}).

	Diet		
Plasma level	**Control**	**3% *Ulva***	**15% *Ulva***
Cortisol	2.62 ± 1.00^a	1.00 ± 0.38^b	1.75 ± 0.63^{ab}
IGF-I	25.65 ± 10.36	34.54 ± 12.49	22.66 ± 5.86
GH	0.90 ± 0.32	0.92 ± 0.17	1.01 ± 0.37
Ca^{2+}	1.42 ± 0.04^a	1.42 ± 0.04^a	1.32 ± 0.04^b
Cl^-	128.37 ± 4.39^a	136.58 ± 4.48^a	152.21 ± 7.60^b
K^+	1.33 ± 0.27^a	2.14 ± 0.49^b	1.52 ± 0.27^{ab}
Na^+	162.8 ± 1.31	163.7 ± 3.77	161.6 ± 3.33

Note: Data are presented as mean ± standard error of mean (SEM). Significant differences ($p < 0.05$) were found for the main effect (ANOVA) for cortisol, Ca^{2+}, Cl^- and K^+. These groups were further tested with post hoc tests (Tukey, $p < 0.05$) and different superscripts within rows indicate significant results.

diet (53.38 ± 8.48 Ω), although not statistically significant ($p = 0.076$; Figure 4A). While the TER in the distal intestine tended to be higher for fish given the 15% *Ulva* diet (217.83 ± 12.71 Ω) compared to the control (179.79 ± 19.81 Ω) and 3% *Ulva* diet (142.86 ± 29.28 Ω) it was not statistically significant ($p = 0.079$; Figure 4B). The apparent permeability did not differ between treatments for either intestinal tissue.

The L-lysine transport, which assesses the active uptake of amino acids, did not differ between treatments.

3.5 | Histological Analyses

The histological parameters in either intestinal segment did not significantly differ depending on diet (Figure 5, Supporting Information 3: Table 3).

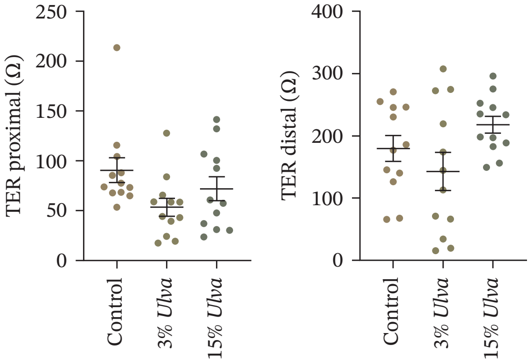
3.6 | Oxidative Stress Markers

The muscle GSSG concentrations were significantly higher for fish given the control diet compared to the 3% *Ulva* diet (one-way ANOVA, F (2,32) = 3.327, $p = 0.049$; Table 6; Tukey, $p = 0.046$). There were no further differences in muscle or liver glutathione. Also, the liver MDA concentrations were significantly higher for fish given the 3% *Ulva* diet compared to the 15% *Ulva* diet

TABLE 6 | Concentration of tGSH (µmol g^{-1}), GSSG (µmol g^{-1}), % GSSG (%) and MDA (nmol mL^{-1}) in muscle and liver tissues.

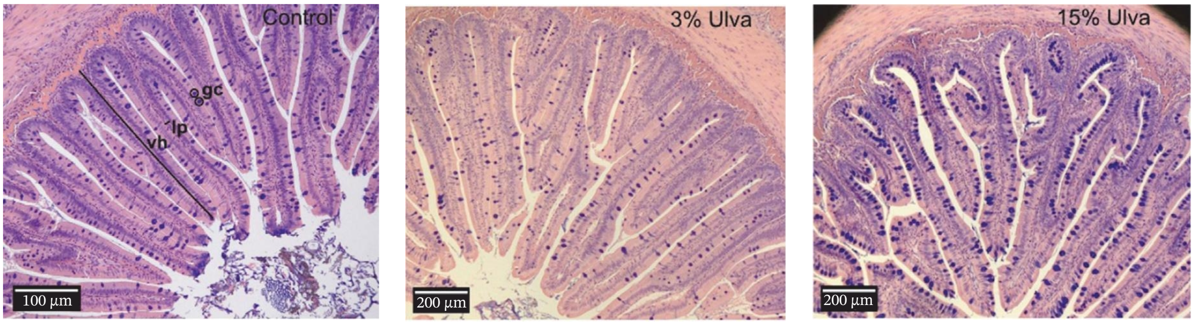
	Diet		
	Control	**3% *Ulva***	**15% *Ulva***
Glutathione in liver			
tGSH	757.4 ± 27.8	763.6 ± 22.3	802.7 ± 17.3
GSSG	6.9 ± 1.9	10.7 ± 3.3	7.6 ± 2.7
%GSSG	0.91 ± 0.24	1.46 ± 0.47	0.98 ± 0.36
Glutathione in muscle			
tGSH	1080.0 ± 88.5	875.1 ± 97.1	1118.7 ± 63.2
GSSG	1.7 ± 0.2^{a}	1.1 ± 0.1^{b}	1.4 ± 0.2^{ab}
%GSSG	0.16 ± 0.01	0.14 ± 0.01	0.13 ± 0.02
MDA			
Liver	4.01 ± 0.30^{ab}	5.09 ± 0.43^{a}	3.54 ± 0.31^{b}
Muscle	9.29 ± 1.69	9.45 ± 1.52	10.09 ± 0.93

Note: Data are presented as mean ± standard error of mean (SEM). Significant differences ($p < 0.05$) were found for the main effect (ANOVA) for muscle GSSG and liver MDA. These groups were further tested with post hoc tests (Tukey, $p < 0.05$) and different superscripts within rows indicate significantly different values.



(A) (B)

FIGURE 4 | The transepithelial resistance (TER) in (A) proximal intestine and (B) distal intestine. Bars represent mean ± standard error of mean (SEM). No significant differences ($p < 0.05$) were found for the main effect (ANOVA).



(A) (B) (C)

FIGURE 5 | Histological cuts of proximal intestine for fish given the (A) control diet, (B) 3% *Ulva* diet and (C) 15% *Ulva* diet. Measured parameters were villi height (vh), lamina propria width (lp), and goblet cell count (gc).

(one-way ANOVA, $F(2,31) = 4.485$, $p = 0.019$; Table 6; Tukey, $p = 0.019$). The muscle MDA levels did not differ between treatments.

3.7 | Metabolic and Product Quality

The fillet composition of whole fish, pooled per treatment, is presented in Table 7. Fish fed 3% and 15% *Ulva* diets had a lower omega n-6 to omega n-3 ratio compared to the control diet. The content of PUFAs, including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), was

The fish fillet colour changed depending on which diet the fish had been fed. The b^* colour parameter values, indicating yellow, differed depending on diet. The b^* values were significantly higher for fish given the 15% *Ulva* diet (33.62 ± 0.65) compared to the control (23.03 ± 0.31) and the 3% *Ulva* diet (25.13 ± 0.33; KW, $H(2) = 28.148$, $p < 0.001$; Figure 6C; MW, $Z = -4.157$, $p < 0.001$; MW, $Z = -4.157$, $p < 0.001$). Fish given the 3% *Ulva* diet also had significantly higher b^* values than the control diet (MW, $Z = -3.263$, $p < 0.001$). The L^* and a^* parameters, indicating dark to light and green to red, respectively, (Figure 6A, B) and fillet pH did not differ between treatments.

4 | Discussion

We showed that both spring and summer biomass of *U. fenestrata* are promising ingredients in rainbow trout diets. The resulting feeds had high nutritional content and improved the fish growth, probably due to a combination of nutritional content and increased FI and palatability by *Ulva* inclusion. The fish health and welfare responses were similar across diets, including the

TABLE 7 | Proximal composition (g 100 g^{-1} if not otherwise specified) of pooled whole fish ($n = 3$) fed the three diets and energy content (kJ 100 g^{-1}).

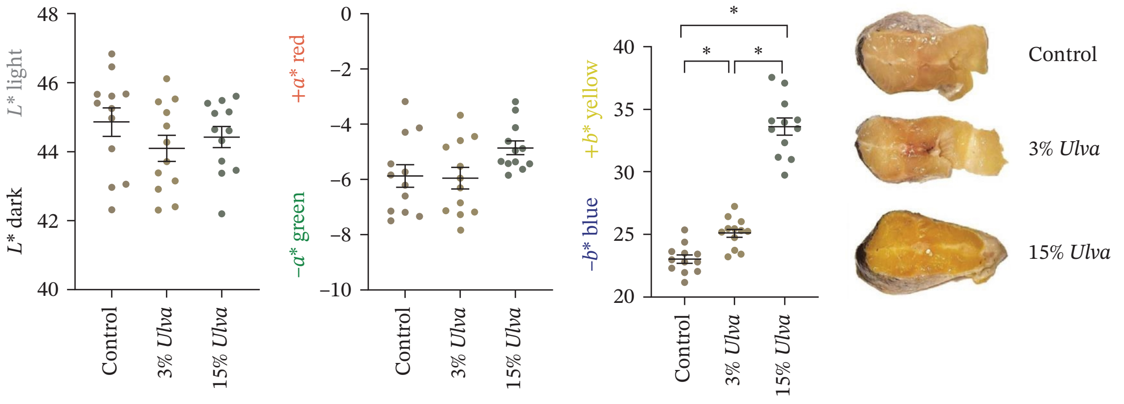
	Diet		
Proximate composition	**Control**	**3% *Ulva***	**15% *Ulva***
Water content	8.27 ± 0.23	8.14 ± 0.35	8.50 ± 0.27
Ash	6.41 ± 0.09	6.01 ± 0.12	6.63 ± 0.17
Crude protein (Kjelldahl)	49.23 ± 0.31	47.23 ± 0.57	46.47 ± 0.27
Crude fats	30.67 ± 1.59	34.13 ± 1.40	32.20 ± 1.77
Carbohydrates	5.42 ± 1.54	4.49 ± 1.88	6.21 ± 1.11
Gross energy	2064.00 ± 34.12	2142.33 ± 34.77	2087.00 ± 42.52
Essential amino acids			
Arginine	2.86 ± 0.04	2.77 ± 0.03	2.74 ± 0.03
Histidine	1.02 ± 0.03	1.04 ± 0.04	0.95 ± 0.02
Isoleucine	1.84 ± 0.03	1.76 ± 0.07	1.69 ± 0.03
Leucine	3.34 ± 0.07	3.22 ± 0.09	3.11 ± 0.03
Lysine	3.66 ± 0.11	3.57 ± 0.11	3.41 ± 0.06
Methionine	1.37 ± 0.03	1.39 ± 0.05	1.33 ± 0.01
Phenylalanine	1.91 ± 0.04	1.86 ± 0.05	1.78 ± 0.02
Threonine	2.11 ± 0.04	2.05 ± 0.07	1.98 ± 0.04
Valine	2.24 ± 0.04	2.14 ± 0.06	2.03 ± 0.04
Non-essential amino acids			
Alanine	2.96 ± 0.04	2.87 ± 0.04	2.77 ± 0.06
Aspartic acid	4.52 ± 0.07	4.40 ± 0.11	4.28 ± 0.10
Cysteine	0.47 ± 0.01	0.47 ± 0.02	0.42 ± 0.00
Glutamic acid	6.33 ± 0.07	6.15 ± 0.16	5.91 ± 0.13
Glycine	3.75 ± 0.07	3.71 ± 0.13	3.57 ± 0.06
Hydroxyproline	0.57 ± 0.01	0.50 ± 0.09	0.50 ± 0.04
Ornithine	<0.01	<0.01	<0.01
Proline	1.96 ± 0.02	2.08 ± 0.08	1.97 ± 0.06
Serine	2.02 ± 0.03	2.00 ± 0.04	1.91 ± 0.03

(Continues)

TABLE 7 | (Continued)

Proximate composition	Diet Control	 3% *Ulva*	 15% *Ulva*
Fatty acids (% of total fatty acids)			
Saturated fatty acids	23.73 ± 1.16	22.43 ± 0.93	24.37 ± 1.21
Monounsaturated fatty acids	52.43 ± 2.51	48.60 ± 1.59	50.87 ± 2.11
Polyunsaturated fatty acids	19.07 ± 3.90	24.50 ± 2.62	19.67 ± 3.65
Omega n-6 fatty acids	9.43 ± 0.61	10.17 ± 0.44	8.90 ± 0.60
Omega n-3 fatty acids	9.63 ± 3.26	14.30 ± 2.22	10.77 ± 3.09
C 20:5 n-3 (EPA)	1.40 ± 0.53	2.17 ± 0.36	1.57 ± 0.50
C 22:6 n-3 (DHA)	4.30 ± 1.84	6.83 ± 1.26	4.60 ± 1.70
Ratio omega n-6/omega n-3 fatty acids	1.23 ± 0.25	0.77 ± 0.11	1.06 ± 0.28
Minerals			
Calcium	11.00 ± 0.00	10.67 ± 0.27	11.70 ± 1.42
Magnesium	0.80 ± 0.01	0.77 ± 0.01	0.85 ± 0.02
Phosphorous	12.00 ± 0.00	12.00 ± 0.00	12.33 ± 0.72
Potassium	10.67 ± 0.27	9.37 ± 0.26	9.87 ± 0.11
Sodium	2.23 ± 0.03	2.10 ± 0.05	2.20 ± 0.00
Sulphur	5.93 ± 0.03	5.70 ± 0.05	5.83 ± 0.05

Note: Data are presented as mean ± standard error of mean (SEM) with SEM showing variation of technical replicates.



(A) (B) (C) (D)

FIGURE 6 | The fillet colour parameters from CIELAB ($n = 12$) with (A) illustrating $L^* =$ dark to light, (B) illustrating $a^* =$ green to red and (C) illustrating $b^* =$ blue to yellow. (D) shows representative pictures of the fillets for control diet, 3% *Ulva* diet, and15% *Ulva* diet. Bars represent mean ± standard error of mean (SEM). Significant differences ($p < 0.05$) were found for the main effect (Kruskal–Wallis) for b^*. This group was further tested with group specific analyses (Mann–Whitney, $p < 0.05$) and significant results are annotated by stars.

gastro-intestinal health and the oxidative stress status in the liver and muscle tissues. An apparent side effect was that increased *Ulva* inclusion in the diets corresponded to increased yellowness in the fish fillets.

All experimental diets were formulated to satisfy the nutrient requirements of rainbow trout, with the control diet mirroring commercial diets [70]. While the nutrient composition of *Ulva* markedly differed between the spring and summer biomass, both were included in the study to evaluate if, irrespective of harvest time, they could contribute functionally to fish health in addition to their role as a macro nutrients source. The crude lipid and protein contents, including both essential and non-essential amino acids, of the *Ulva* were higher in the spring than the summer and the omega n-6 to n-3 fatty acids ratio was lower. However, due to their overall low lipid content, the *Ulva* inclusion did not meaningfully affect the fatty acid composition, including the PUFA content, of the resulting diets. For protein, the nutritional requirements for rainbow trout suggests a digestible protein content of ca. 35%–50% depending on fish size [71, 72]. As a result, commercial feeds for grow-out rainbow trout typically contain a crude protein content around 40% [70]. All three experimental feeds in this study met these nutritional standards with crude protein content spanning 40%–45%.

The feed formulation was based on the nutrient composition of the raw materials and was balanced to achieve a crude protein content of ca. 44% across all experimental diets [73]. However, the proximate composition analysis of the produced diets revealed that the crude protein content was slightly higher in the two *Ulva*-containing feeds compared to the control feed. This discrepancy of ca. 5% is likely due to a combination of factors, including minor manual variations during meal weighing and mixing, and analytical variation caused by small sample sizes [74–76] The formulations of the 3% *Ulva* diet and the control diet were naturally very similar, differing only by the 3% *Ulva* biomass inclusion, yet still showed differences in crude protein content. In contrast, the 15% *Ulva* biomass inclusion, combined with balancing during formulation, resulted in a nutrient composition more similar to that of the 3% *Ulva* diet regarding the protein and lipid composition. This makes substantial manual errors during diet preparation less likely and suggests that analytical variability may be the more probable explanation. Such deviations are not uncommon in small-scale experimental feed production facilities; yet must be considered when interpreting the results of the feeding trial. The increased protein content in the *Ulva*-containing feeds could be one of the explanatory factors behind the improved growth performance, as increased dietary protein content can enhance growth in rainbow trout [77]. However, the diets were isocaloric, and the highly similar macro- and micronutrient compositions of the 3% and 15% *Ulva* inclusion diets still allows for meaningful conclusions to be drawn regarding the effects of replacing soy protein concentrate with *Ulva* on fish performance and feed efficiency. The amino acid profile is important to consider in novel feeds, with methionine and lysine as two of the most important and limiting essential amino acids for salmonids and deficiencies can lead to reduced growth, inflammation, oxidation, and fatty liver [25, 78]. Rainbow trout feeds should include 1.3%–2.9% lysine and 0.6%–1.0% methionine [72, 79]. Our feeds contained 2.5%–2.9% lysine and 0.9%–1.0% methionine, which is in the upper part of these optimal ranges. Regarding the content of essential fatty acids, the feed should include at least 1% 18:2 n-6 fatty acids, 1% EPA and 0.5% DHA, with commercial diets typically containing 4.1% n-6 fatty acids and 2.0% in total of EPA and DHA [70, 72, 80]. In the current study 18:2 n-6 fatty acid content was 2.0%–2.3%, EPA content was 1.1%–1.2%, and DHA content was 1.9%–2.1%, which reflects higher levels than in most commercial feeds. The whole fish proximate composition showed, overall, similar nutritional content between fish fed the different diets. The omega n-6 to n-3 ratio was lower in fish given the *Ulva* diets, particularly the 3% diet, compared to control. The increased omega n-3 content suggests that apart for being a source, *Ulva* also stimulates its uptake and assimilation. This is in line with previous studies on 5%–10% of *Ulva* meal inclusion, both raw and autoclaved, resulting in increased n-3 fatty acid content in rainbow trout [49]. The nutritional content of the feeds and fish overall show that both spring and summer *Ulva* biomass are useful aquafeed ingredients.

Fish given the 15% *Ulva* diet had the highest weight gain during the first half of the feeding trial, likely due to a higher FI than other diets. During the second half of the experiment, the fish given the 3% *Ulva* diet had the highest weight gain, coupled with an increased FI compared to the first 6 weeks. The same trends were reflected in the SGR results. The size of a fish can influence its SGR, with larger fish typically having lower SGRs than smaller ones [81]. Since the fish on the 15% *Ulva* diet were larger at the start of the second time period, it could have influenced their SGR and contributed to the relatively lower values during the second half of the feeding trial. The plasma levels of GH and IGF-I were similar across all diets. IGF-I has been suggested as the best growth biomarker in fish, but the correlation with growth is not always clear and can be influenced by multiple environmental and physiological parameters [82–84]. The stable levels of IGF-I may rather indicate an active endocrine system with high turn-over rate of hormone and receptor levels in balance also with the fish's FI and nutritional status [82]. Overall, growth responses to experimental diets with *Ulva* meal appear to vary depending on various factors, such as *Ulva* species, climate, and which ingredients that were replaced in the feed trials. Including 10% *U. lactuca* meal in diet for rainbow trout has been reported to reduce the growth and feed utilisation compared to control feed without seaweed [48]. Using a 10% inclusion of non-autoclaved *U. rigida* meal was found to improve rainbow trout growth while 10% inclusion of autoclaved *U. rigida* meal reduced growth [49]. Both studies substituted wheat meal with *Ulva* meal, and their feeds were isocaloric and had a similar proximate composition [48, 49]. The increased SGR in the present study, contradicting these previous studies, could be due to various reasons. Both *Ulva* diets contained ca. 5% higher crude protein content than the control, which could partly explain the improved growth performance. Nonetheless, the temporal pattern of the response, together with the observed differences in fatty acid assimilation and omega n-3 enrichment in the fish, suggests that the effect may not be solely attributable to protein level, but may also reflect beneficial properties of *Ulva* as a feed ingredient. The elevated growth in the present study was likely caused by a combination of higher protein content and improved palatability. Another potential suggestion is that we substituted soy protein concentrate, rather than wheat meal, with the *Ulva* meal, and that the absence of soy protein concentrate was beneficial. An alternative suggestion is that non-processed *Ulva* meal is preferable to processed *Ulva* meal. For example, high-temperature thermal treatment (140°C) of fish meal prior to extrusion negatively affected the protein structure and resulted in reduced digestibility in rainbow trout [85], and it's possibly that *Ulva* biomass is similarly affected by high temperature treatments. Varying responses on SGR have also been reported in previous literature on other species with experimental *Ulva* feeds. Improved SGR has been observed when including 25% *Ulva* biomass in diet of gilthead seabream, while inclusion of 15% *Ulva* reduced growth below control in the same species [43, 44]. Inclusions of 5% and 15% had no effects on growth rates, reports other literature on gilthead seabream, or that 5% inclusion had no effects while 8% decreased it [35, 46, 47]. Including 10% *Ulva* meal in Atlantic cod (*Gadus morhua*) feed was possible without compromising growth [86].

Despite the improved growth observed in this study, the 15% *Ulva* diet also had the highest FCR. That *Ulva* meal can increase FCR has been previously observed in Nile tilapia, with increased FCR compared to control feeds as *Ulva* meal content increased from 10%–20% [87] and 10%–30% [88]. The authors suggested it might result from imbalanced amino acid profiles, the presence of antinutritional factors, or a high inclusion of dietary fibres [87, 88]. The present *Ulva* diets had a similar, or higher, amino acid content compared to the control diet, which makes the explanation of an imbalanced amino acid profile unlikely. Anti-nutritional factors in seaweeds can include tannins, phytic acid, saponins and trypsin inhibitors, which can cause water retention and increased excretion [87, 89]. The fibre content, measured only in the dried *Ulva* meal, was likely elevated in the 15% *Ulva* diet compared to the other feeds and could therefore also have contributed to the reduced digestibility observed in this dietary group. The apparent nutrient digestibility in juvenile rainbow trout has been observed to decline when including 30% *Ulva* meal in diet, both for dry matter, proteins, lipids, and energy [90]. This could be due to the presence of anti-nutritional factors or fibres, which may have contributed to the elevated FCR observed also in the present study. Our results, taken together, suggest that the potential negative effects associated with reduced digestibility from *Ulva* inclusion were compensated by a higher voluntary FI and the elevated protein in the experimental diets, thus maintaining the overall growth rate. FI in fish is controlled by two systems, the homeostatic system and the hedonic system. The homeostatic system is modulated by energy stores and gives hunger signals to stimulate feeding, while the hedonic system encourages feeding based on high palatability beyond the homeostatic need [91]. The hypothalamus has a key signalling function in the homeostatic system and appetite regulation in fish, with NPY and AgRP causing orexigenic signalling by antagonising anorexigenic MC4R and POMC [53]. Other known anorexigenic factors in fish includes CART and CRF [92]. There was no difference in the mRNA expression of these genes in the present study. However, since gene expression results provides only a snapshot of the situation at the time of sampling, it is possible that these appetite-regulating genes were upregulated earlier in the feeding trial. Alternatively, the elevated FI in fish fed the *Ulva* diets is due to increased palatability, which would lead to feeding rates beyond the point of strict energy requirements and hunger expressions. Palatability in gilthead seabream has been shown to increase with inclusion of 20% and 35% *Ulva* in the diet [93], which is in line with the results of the present study. One of the main feed stimulants for fish is amino acids, although the exact amino acids that trigger responses varies between species [94, 95]. For example, for red seabream (*Pagrus major*) alanine, valine, glycine, serine, arginine and glutamine were found to be the most effective to trigger feeding behaviour [94]. In the present study the content of all amino acids was higher, except cysteine and hydroxyproline, in the two *Ulva* feeds and a combination of these likely enhanced the FI.

Another interesting effect of the experimental feeds was the higher HSI in fish given the 15% *Ulva* diet. An increased liver size is usually caused by excess lipid or glycogen accumulation typically due to high lipid or starch content in the diet [96]. Since the carbohydrate content was lower in the *Ulva* feeds compared to control, that is an unlikely explanation. The crude lipid level was slightly higher in the 15% *Ulva* feed, but this was not reflected in the whole-body proximate composition. If the high HSI had been caused by increased lipid content in feed, it is expected that the overall fat accumulation would be higher in the whole-body analysis [97]. Instead, the elevated HSI may be due to metabolic adjustments, or there may be an active substance in *Ulva* that stimulates fat or carbohydrate accumulation in the liver. For example, *Ulva* spp. have been found to contain saponins [98], which has been shown to cause lipid accumulation in the liver and increased HSI in rohu (*Lobeo rohita*; [99]). Targeted liver analyses such as hepatic lipid content and histology could further assess these effects and their possible correlation to *Ulva* saponin content.

When assessing novel feed ingredients, it is important to evaluate in-depth mechanistical parameters along with growth and FI measurements to ensure a more complete evaluation of fish health and welfare [100]. The analyses in our study included both primary and secondary stress responses. The primary stress response, here evaluated as plasma cortisol levels, showed levels below 10 ng mL^{-1} for all treatments, which is considered non-stress levels for rainbow trout and indicate a lack of acute stress response [101, 102]. The lower level in fish given the 3% *Ulva* diet compared to the control diet was statistically significant, but since the plasma cortisol levels were very low in all dietary groups, it does not suggest that this difference is of biological significance nor that there was any acute stress response for the other diets. Among the secondary stress responses investigated, the plasma ion levels were within previously reported ranges for rainbow trout; 1.09–3.16 mmol L^{-1} for Ca^{2+}, 125–162 mmol L^{-1} for Cl^{-}, 154–163 mmol L^{-1} for Na^{+} and 2.5–5.9 mmol L^{-1} for K^{+} [14, 38, 103, 104]. The plasma concentration of Ca^{2+} is tightly regulated in rainbow trout and thus, the variation between individuals in each group is low. Even though the present mean values of plasma Ca^{2+} was statistically different, the small difference in mean values, 1.32–1.42 mmol L^{-1}, is probably of no biological significance. As the Ca^{2+} content was similar in the experimental feeds, we suggest it is most likely an effect of individual variation. The plasma Cl^{-} levels were higher in fish given the 15% diet, and while the feed content Cl^{-} was not measured, we hypothesis that it could be due to elevated content in the seawater grown *Ulva*. Other secondary stress responses included effects on the gastrointestinal tract, which is a key organ in the evaluation of health effects by novel feeds [40, 105]. The distal and proximal intestines were analysed using histology, which can reveal negative health effects, including enteritis or pathological conditions caused by imbalanced feeds [41, 66, 106]. In the present study there were no differences between fish given the different diets for any of the measured pathological indicators, thus indicating equal epithelial properties. The electrical properties of the gastrointestinal tract, here assessed using an Ussing chamber methodology, evaluate the gut barrier functions [107]. The total electrical resistance, TER, reflects the trans epithelial permeability which is reduced by different stressors, including the presence of enteritis, for example, caused by imbalanced feeds [40]. The TER in the distal intestine tended to be higher for fish given the 15% *Ulva* diet compared to control, although not statistically significant, which may indicate a more robust barrier function.

Ulva spp. has a high content of antioxidant and antimicrobial substances, for example, sulphated polysaccharides, lipids, photosynthetic pigments and secondary metabolites [9]. Since these can counteract the harmful effects of ROS, *Ulva* meal was hypothesised to improve the fish antioxidant response [30–32]. There were, however, no consistent improvements in the evaluated markers for oxidative stress, neither in LPO nor the glutathione redox system, in liver or muscle tissues. Diets with 2.5%–8% *Ulva* meal inclusion have previously given different antioxidant responses in fish depending on which antioxidant indicator that was investigated. In European seabass there were no differences in MDA (LPO) or superoxide dismutase (SOD), whereas the glutathione peroxidase response was reduced and the catalase (CAT) response increased [35]. A combined stress and feed study in European seabass evaluated effects of a 7.5% dietary seaweed inclusion with mixed species (2.5% *Fucus* sp., 2.5% *Gracilaria* sp. and 2.5% *Ulva* sp.) and found that it did not mitigate negative effects on antioxidant responses [108]. The present results are inconclusive, and more oxidative stress markers, for example, CAT, would be needed to be able to conclude if inclusion of dried *Ulva* in the feed has an effect on antioxidant responses or not. An alternative explanation is that the feed production processing reduced the bioavailability of the bioactive compounds. For example, the stability and retention of bioactive carotenoids, which *Ulva* contains, can be compromised during extrusion processes and the bioavailability is typically reduced with ~5%–10% [109, 110]. In addition to considerations of fish health and welfare aspects, aquaculture products need to be attractive for consumers. Among several product quality parameters, colour is a key quality attribute, particularly for products such as salmonids where consumers have clear expectations [111]. We found a distinct difference for yellowness (b^*), based on the CIELAB results, with fillets from fish given the 15% *Ulva* diet having a more pronounced yellow colour. This was likely caused by accumulation of dietary carotenoid pigments found in *Ulva*, which have previously been shown to cause yellowing of the meat in several fish species including rainbow trout [112]. In the case of rainbow trout fillets, colour preferences can vary depending on the market but are often expected to range from white to salmon-pink [111]. Increased yellowness could, therefore, make the product less desirable and reduce the consumer acceptance. Feed optimisation may be needed in the future along with research on how to manage the formation of yellow colouring resulting from high carotenoid inclusions. It is possible that sustainability claims linked to the novel feed, even though resulting in off-colouration of the fillets, could improve consumers' willingness-to-pay when compared to conventionally coloured fillets, thereby compensating for the reduced visual attraction [113].

5 | Conclusions

This is the first study evaluating the potential of utilising farmed *U. fenestrata* from temperate waters in rainbow trout feed. The results demonstrate that *Ulva* meal inclusion at 3% and 15% levels, originating from both spring and summer biomass, was successful and did not compromise either the feed nutrient profile or the bioavailability of essential nutrients. The growth rates of fish fed the experimental *Ulva* diets were improved compared to fish given the control diet, likely caused by an increased FI and palatability. No adverse effects were observed on fish health and welfare, evaluated with both primary and secondary stress responses. Dietary *Ulva* content made the fillet more yellow, which may influence the consumer acceptance. Taken together, this study demonstrates that inclusion of up to 15% *Ulva* meal in rainbow trout diets was beneficial for fish growth without compromising their health and welfare.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supporting Information 1. Table S1: Shows the data (average ± SEM) on weight gain (WG), specific growth rate (SGR), feed intake (FI) and feed conversion ratio (FCR) for fish given the three diets during the two experimental periods. It also shows the condition factor (CF) during the initial, mid-point, and final sampling, the hepatosomatic index (HIS) and visceral fat index (VFI) at the final sampling, and survival rate throughout the experiment.

Supporting Information 2. Table S2: Shows data (average ± SEM) on the hypothalamic mRNA expressions of Cart, Crf, NPY, PomC and MC4R relative to reference genes EF1α and β-Actin.

Supporting Information 3. Table S3: Shows data (average ± SEM) on the histological parameters villi height, lamina propria width, and goblet cell count from both proximal and distal intestine.

Supporting Information 4. The graphical abstract illustrates the three different experimental feeds used in the trial and that it was performed with rainbow trout. It also shows the methods used to evaluate the health and welfare effects of the feed.