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Diets supplemented with *Saccharina latissima* influence the expression of genes related to lipid metabolism and oxidative stress modulating rainbow trout (*Oncorhynchus mykiss*) fillet composition

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Abstract

This study aimed to evaluate the impact of diets including increasing amounts (1, 2 and 4%) of an iodine-rich macroalgae, *Saccharina latissima*, on gene expression and fillet composition of commercial-sized rainbow trout. Liver and muscle expression of genes related to growth, iodine, oxidative stress, and lipid metabolism, and the fillet content of fatty acids, cholesterol, and vitamin D_3 were assessed. The highest kelp inclusion led to lower final body weight and HSI, without significant differences in mRNA transcription of genes involved in growth (*ghr1*, *ghr2* and *igf1*) or iodine metabolism (*dio1*, *thra*, and *thrb*). A significant downregulation of an oxidative stress marker, *gpx1b2*, was observed in fish fed 2% *S. latissima*, which might suggest the need for less endogenous antioxidants. Dietary inclusion of kelp impacted lipid metabolism, with a downregulation of fatty acid synthase, accompanied by a general decrease of fatty acids in fillet. The present study demonstrated that supplementation of diets with 1 or 2% *S. latissima* can be achieved without detrimental effects on rainbow trout final weight. Evidence suggest a lipid-lowering effect of diets that did not compromised fillet EPA and DHA concentrations, being 3.7 times above the recommended levels for human consumption.

Keywords: *Oncorhynchus mykiss*, sugar kelp, oxidative stress, lipid metabolism, fillet composition

1. Introduction

Continued growth of the world population, coupled with trends moving towards healthy and/or natural eating habits, has put pressure on the aquaculture industry to develop alternatives to the limited fisheries-based food products (FAO, 2018). Rainbow trout (*Oncorhynchus mykiss*) is one of the most important species produced by aquaculture in Europe, with production exceeding 351 million tonnes in 2016 (FEAP, 2017). Diets for this species already include high levels of sustainable and eco-friendly plant protein sources to replace fish meal, but such diets can compromise fish growth and flesh quality (De Francesco et al., 2004; Lazzarotto et al., 2018).

Seafood consumption is associated with healthy dietary behaviours and fish are the most important natural sources of omega-3 fatty acids, minerals and vitamins, particularly iodine and vitamin D_3, which are essential for human health (Lund, 2013). With increased seafood consumption, and the large amounts

of vegetable-based ingredients used in aquafeeds, it has become even more pertinent that functional fish diets are able to provide products with health-promoting nutrients. In this context, macroalgae have raised the interest of fish farmers due to their richness in bioactive compounds like carotenoids, vitamins and minerals (Wan et al., 2019) that could simultaneously enhance the nutritional value of end-products for human consumption and contribute to fish welfare. Some macroalgae, such as sugar kelp, *Saccharina latissima,* are commonly produced at an industrial scale for different purposes, including biofuels and feed supplements (Stévant et al., 2017). *S. latissima* is a large brown macroalgae known for its high concentrations in important minerals, particularly iodine, sugars (e.g. glucose and mannitol) and carotenoids such as fucoxanthin (Nielsen et al., 2016; Roleda et al., 2018), being therefore a promising algae to fortify aquafeeds. However, this macroalgae has variable and generally low protein (1–21% of DM) and lipid (0.5-3.4% DM) contents, that largely depend on harvesting season and geographic area (Holdt and Kraan, 2011; Marinho et al., 2015a; 2015b; Nielsen et al., 2016). In some countries, *S. latissima* is produced as part of an integrated multitrophic aquaculture (IMTA), taking up nutrients from open-cage salmon farming (Fossberg et al., 2018) and has great potential for a sustainable production with high biomass yield (Handå et al., 2013).

In fish, the beneficial effects of supplementing diets with different seaweeds have been demonstrated in a number of species (Ergün et al., 2008; Peixoto et al., 2016; Ribeiro et al., 2017a; Wassef et al., 2013). But the use of brown seaweeds, in particular *S. latissima*, has been little explored in animals' feeds (Øverland et al., 2019), and no information is available concerning its effects on fish metabolism, welfare and fillet composition. In rainbow trout fed diets supplemented with 0.4% of an iodine-rich brown algae *Laminaria digitata* improved growth was observed (Ribeiro et al., 2017a), whilst in gilthead seabream, *Sparus aurata*, higher supplementation (10%) did not affect growth performance (Ribeiro et al., 2015). Araújo et al. (2016) showed that the inclusion of *Gracilaria* sp. meal in diets for rainbow trout was possible up to 5 %, but a higher inclusion level impaired growth. These suggest that growth responses among fish species depend not only on the algae species but also on its inclusion level.

The impact of dietary macroalgae supplementation on lipid metabolism is a poorly explored topic, although evidence suggest that algae may mediate both lipid accumulation and mobilization (Nakagawa, 1997). Seaweeds were shown to modulate muscle lipid profile (Wan et al., 2019), with increased muscle

polyunsaturated fatty acids content being reported in rainbow trout fed brown algae *Macrocystis pyrifera* (Dantagnan et al., 2009). Xuan et al. (2013) observed a reduction in whole-body and liver lipid contents and hepatosomatic index in black seabream, *Acanthopagrus schlegelii,* fed diets with 20% *Gracilaria lemaneiformis* inclusion. In *Lates calcarifer*, dietary supplementation with increasing levels of *Gracilaria pulvinate* (3-9%) led to a decrease of serum triglycerides and cholesterol when compared with the control diet that contained a soybean meal and fishmeal blend (Morshedi et al., 2018).

As natural sources of compounds with free radical scavenger effects (carotenoids, certain polysaccharides and polyphenols), the potential of seaweeds as natural feed ingredients capable of improving the antioxidant status of fish has also been proposed (Gomez-Zavaglia et al., 2019). Increased activity of some antioxidant-related enzymes, such as catalase and superoxide dismutase (Sod), was observed in liver mitochondria of Atlantic salmon, *Salmo salar,* fed diets containing a brown seaweed meal, *Laminaria* sp. (Sotoudeh and Mardani, 2018). In contrast, the supplementation of diets for rainbow trout with a red seaweed, *G. pygmaea*, resulted in a reduction in the activity of glutathione peroxidase and Sod (Sotoudeh and Mardani, 2018), showcasing the potential of macroalgae to modulate antioxidant defense systems.

Iodine deficiency in humans is a global problem, which is driving the aquaculture sector to develop fortification strategies. Fish dietary supplementation with macroalgae *L. digitata*, *G. vermiculophylla* and *Undaria pinnatifida* has been successfully used as a strategy to increase iodine content in rainbow trout (Ribeiro et al., 2017a; Valente et al., 2015), Senegalese sole, *Solea senegalensis*, (Moutinho et al., 2018) and gilthead seabream (Ribeiro et al., 2015). The high amounts of iodine found in brown seaweeds (up to 10,000 $mg.kg^{-1}$ dry weight) might pose a barrier to its use by the food and feed industries (Holdt and Kraan, 2011; Lüning and Mortensen, 2015), even at low dietary levels. According to the European Commission Recommendation 2015/861, the current authorized maximum content of total iodine in feeds for fish in the European market is 20 mg $iodine.kg^{-1}$. The presence of high levels of toxic metals in brown macroalgae may also limit their use as feed ingredients (Biancarosa et al., 2018). Iodine is essential in the synthesis of thyroid hormones (Ths) that directly or indirectly regulate key metabolic processes related to growth, energy and lipid metabolism in both humans and animals (Lazarus, 2015; Mullur et al., 2014). Dietary iodine is absorbed via the small intestine and, in the thyroid, incorporated into thyroglobulin, ultimately

forming the pro-hormones, thyroxine (T_4) and triiodothyronine (T_3). T_4 is converted into the active form T_3 by deiodinase enzymes and the action of Ths exerted through nuclear thyroid hormone receptors (Milanesi and Brent, 2017). The growth hormone/insulin-like growth factor I (Gh/Igf1) system has been described as an useful tool to evaluate the impact of nutritional factors on fish growth (Pérez-Sánchez et al., 2018) and Ths act at both Gh and Igf1 levels (Rodriguez-Arnao et al., 1993). The impact of diets with high iodine content on fish metabolism has been poorly explored, and most studies focus in larvae. Atlantic cod larvae fed rotifers with high iodine concentrations (129 $mg.kg^{-1}$) showed signs of iodine-induced toxicity in thyroid follicles, without differences on thyroid hormone concentrations (Penglase et al., 2013). On the other hand, dietary inclusion of iodine-enriched rotifers and Artemia increased outer-ring deiodinase activity and prevented goiter in *S. senegalensis* larvae (Ribeiro et al., 2011). In mice, excess iodine intake in the form of potassium iodate has been associated with hepatic accumulation of triglycerides, accompanied by an upregulation of fatty acid synthase gene (Xia et al., 2013) and decreased expression of low-density lipoprotein receptor in liver (Zhao et al., 2010).

The aim of the present study was to evaluate the impact of diets including increasing amounts (1, 2 and 4%) of an iodine-rich macroalgae, *S. latissima,* on gene expression and fillet composition in commercial-sized rainbow trout. Expression of genes involved in growth, iodine metabolism, oxidative stress and lipid metabolic pathways were assessed in both liver and muscle. The fillet content of fatty acids, cholesterol and vitamin D_3 were also evaluated. The results facilitate a better understanding of the consequences of rainbow trout dietary supplementation with *S. latissima* on fish metabolism, as well as the impact of such diets on the fillet nutritional value for human consumption.

2. Materials and Methods

2.1. Animal husbandry

Female rainbow trout (*Oncorhynchus mykiss*) were transported from a commercial fish farm (Lundby fisk, Dybvadgårdsvej 1, DK-9240 Nibe, Denmark) to the DTU-Aqua facilities at the North Sea Research Centre (Niels Juelsvej 30, 9850 Hirtshals, Denmark). The fish trial was performed by experienced scientist/staff according to the recommendations of the Danish National Committee for the Protection of Animals used for scientific purposes, and in compliance with the guidelines of the European Union

(directive 2010/63/EU). Fish were held in quarantine for 3 weeks in large (3000 L) circular outdoor tanks (15-20ppt saltwater) and fed commercial diets (BioMar AS).

2.2. Experimental diets

Four iso-nitrogenous and iso-caloric diets were formulated containing increasing amounts of *S. latissima* (0%, 1%, 2% and 4%) and extruded by Sparos Lda, Olhão, Portugal. The experimental diets included dried *S. latissima* kelp, produced in an integrated multi-trophic aquaculture (IMTA), at the expense of wheat meal. The iodine concentration in the macroalgae was 5,415 ± 184 $mg.kg^{-1}$ dry weight, resulting in diets with iodine levels of 67, 124 and 255 mg $iodine.kg^{-1}$ dry weight for 1%, 2% and 4% diet, respectively. All diets were additionally enriched with selenium by adding selenised yeast. Feed ingredients and diet composition are summarised in Table 1.

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Table 1

Ingredients and proximate composition of the control and the three experimental diets.

	Dietary Treatments			
Ingredients, %	**Control**	**1% *S. latissima***	**2% *S. latissima***	**4% *S. latissima***
Fishmeal LT70 [1]	14.00	14.00	14.00	14.00
Krill meal [2]	3.00	3.00	3.00	3.00
Soy protein concentrate [3]	21.00	21.00	21.00	21.00
Wheat gluten [4]	9.00	9.00	9.00	9.00
Wheat meal [5]	6.34	5.34	4.34	2.34
Faba beans (low tannins) [6]	10.00	10.00	10.00	10.00
Fish oil [7]	8.96	8.96	8.96	8.96
Rapeseed oil [8]	24.24	24.24	24.24	24.24
Vitamin and Mineral Premix [9]	1.29	1.29	1.29	1.29
MAP (Monoammonium phosphate) [10]	1.20	1.20	1.20	1.20
Se yeast [11]	0.02	0.02	0.02	0.02
Astaxanthin [12]	0.04	0.04	0.04	0.04
L-Lysine [13]	0.30	0.30	0.30	0.30
L-Threonine [14]	0.20	0.20	0.20	0.20
DL-Methionine [15]	0.40	0.40	0.40	0.40
Yttrium oxide [16]	0.01	0.01	0.01	0.01
Saccharina latissima[17]		1.00	2.00	4.00
Proximate composition				
Dry matter, DM, %	94.80	94.20	94.20	93.80
Crude protein, % DM	41.67	40.98	40.45	39.98
Crude fat, % DM	36.29	37.47	37.47	37.63
Ash, % DM	3.48	3.57	3.78	4.48
Phosphorus, % DM	0.74	0.71	0.71	0.71
NFE, % DM	18.46	18.05	18.37	17.91
Energy, $kJ.g^{-1}$, % DM	26.12	26.35	26.30	26.18
Iodine, $mg.kg^{-1}$, % DM	3.85	66.72	123.67	255.33
Vitamin D_3, $\mu g.100\ g^{-1}$, % DM	7.07	6.90	7.22	6.82

[1] NORVIK LT: 70.6% crude protein (CP), 5.8% crude fat (CF), Sopropêche, France

[2] Krill meal: 61.1% CP, 17.4% CF, Aker Biomarine, Norway

[3] Soycomil P: 63% CP, 0.8% CF, ADM, The Netherlands

[4] VITEN: 82% CP, 2.1% CF, Roquette, France

[5] Wheat meal: 10.2% CP, 1.2% CF, Casa Lanchinha, Portugal

[6] Faba beans low tannins: 28% CP, 1.2% CF, Casa Lanchinha, Portugal

[7] Sopropêche, France

[8] Henry Lamotte Oils GmbH, Germany

[9] INVIVONSA Portugal SA, Portugal: Vitamins (IU or mg/kg diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 500 mg; inositol, 500 mg; biotin, 3 mg; calcium panthotenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg.kg^{-1} diet): copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulphate,7.5 mg; sodium chloride, 400 mg; excipient wheat middling's

[10] Windmill AQUAPHOS, 26% P, ALIPHOS, Belgium

[11] ALKOSEL R397: 2200 mg selenium/kg, Lallemand, France

[12] Carophyll Pink 10% CWS, DSM Nutritional Products, Switzerland

[13] Biolys: 54.6% Lysine, Evonik Nutrition & Care GmbH, Germany

[14] L-Threonine 98.5%, Ajinomoto EUROLYSINE S.A.S, France

[15] DL-METHIONINE FOR AQUACULTURE 99%, EVONIK Nutrition & Care GmbH, Germany

[16] Sigma-Aldrich, USA

[17] Dry *Saccharina latissima*: 5.9% CP; 0.5% CF; 5,415 ± 184 mg iodine.kg^{-1}

2.3. Growth trial and sampling

After the quarantine period, fish were transferred to a facility with 12 circular polyethylene rearing tanks (diameter 1m, ~600 L), supplied with aerated flow-through fresh seawater (7-11°C). A stable oxygen saturation was maintained during feeding hours and light conditions were 14.5L: 9.5D cycles (light on 07:30-22:00). At the beginning of the trial, 561 fish anaesthetized with benzocaine (200 mg/L) were weighed individually (196.1 ± 25.6 g) and distributed among the 12 tanks. Fish were fed approximately 0.9% of the biomass, with belt feeders, for 12 weeks, in triplicate tanks. Before sampling, fish were sacrificed by anaesthetic overdose. At the end of the 12-week period, all fish were weighed and measured individually. Liver weights were recorded for hepatosomatic determination (HSI) calculated as HSI = 100 x (liver weight/body weight). Muscle samples from 12 fish per tank were then allocated to one of three pools (n=3 pools of 4 fish each) for determination of flesh composition. For gene expression analysis, muscle and liver tissues (0.1 g) were collected from 18 fish per tank, chopped into smaller pieces, and immediately preserved in RNAlater™ stabilization solution (Thermo Fisher Scientific, Massachusetts, USA) before being stored at -80 ºC until RNA extraction.

2.4. RNA extraction and cDNA synthesis

Total RNA was extracted from 15 mg of tissue (n = 9) using NZYol reagent (Nyztech, Lisbon, Portugal) according to the manufacturer's recommendations with some modifications. These modifications

included disruption of samples with NZYol and glass beads using a Precellys® 24 homogenizer (Bertin Technologies, France), followed by centrifugation at 12,000 x g for 10 min at 5 ºC. The resulting supernatant was used for the phase separation step. After removal of the aqueous phase with RNA, a similar volume of ethanol 70% was added and samples mixed immediately. Subsequently, RNA was purified using NZY Total RNA Isolation kit (Nyztech, Lisbon, Portugal) following the manufacturer's guidelines. RNA quantity was determined by spectrophotometry, and its quality was assessed based on the absorbance ratio 260:280 nm, using a Take 3 Micro-Volume plate on a Synergy™ HT Multi-Detection Reader and the Gen5™ software (BioTek Instruments, Winooski, VT, USA). RNA integrity was evaluated based on gel electrophoresis in a 1% (w/v) agarose TAE gel stained with GelRed™ nucleic acid stain (Biotium, Hayward, CA, USA). All samples with A_{260}/A_{280} ratios of 1.80-2.10 and clear banding pattern of 28S:18S ribosomal RNA were used for further analysis. Subsequently, 1 µg of total RNA was reverse transcribed to cDNA using NZY First-Strand cDNA Synthesis Kit (Nyztech, Lisbon, Portugal) following the standard protocol.

2.5. Gene expression analysis

Expression of genes related to a) growth: growth hormone receptor 1 and 2 (*ghr1* and *ghr2*), insulin-like growth factor I (*igf1*); b) iodine metabolism: iodothyronine deiodinase 1 (*dio1*), thyroid hormone rector alpha (*thra*), thyroid hormone receptor beta (*thrb*); c) oxidative stress: glutathione peroxidase 1 isoform b2 (*gpx1b2*), glutathione peroxidase 4 isoform a1 (*gpx4a1*), superoxide dismutase 1 (*sod1*); and d) lipid metabolism: acetyl-CoA carboxylase (*acc*), carnitine palmitoyl transferase 1 isoform beta 1 (*cpt1b1*), elongation of very long chain fatty acids protein 2 (*elovl2*), fatty acid synthase (*fas*), low density lipoprotein receptor (*ldlr*) was assessed by reverse transcription q-PCR (Table 2). PCR was performed on an Eppendorf Mastercycler ep Gradient S (Eppendorf, Germany) with NZYSpeedy qPCR Green Master Mix (2x) (Nyztech, Lisbon, Portugal). Reactions were carried out with 400 nM of each primer (forward and reverse), 5 µL of Green Master mix and 2 µL of cDNA, in a total reaction volume of 10 µL. Thermal cycling conditions were 95 ºC for 2 min, followed by 35 cycles at 95 °C for 5 s and 58-62 °C for 28 s (annealing temperatures are presented in Table 2). At the end of each run, a post-amplification dissociation curve (60 to 95, 0.5 °C in each cycle) was obtained to ensure reaction specificity. PCR efficiency for the target genes

was obtained based on the slope of a standard curve, using a 5-fold serial dilution of cDNA from a sample pool of all experiments. Efficiency ranged between 90 and 102% (Table 2), and each unknown sample and standard curves were run in duplicate, as well as a negative control without cDNA.

The stability of four reference genes (*β-actin*, *ef1α*, *gapdh* and *hprt1*) was determined using geNorm algorithm (Vandesompele et al., 2002) implemented in qbase+ software, version 3.2 (Biogazelle, Zwijnaarde, Belgium - www.qbaseplus.com). For liver samples, *hprt1* was the most stable followed by *β-actin*, *ef1α* and *gapdh*. For muscle samples, the stability was as follows: *β-actin* > *hprt1* > *gapdh* > *ef1α*. For each tissue, the geometric mean of the three most stable reference genes was used to calculate a gene expression normalization factor for each sample. Data were analysed based on a relative method, where the relative abundance of target genes was calculated using the comparative critical threshold ($\Delta\Delta C_T$) method (Livak and Schmittgen, 2001). The average C_T across all samples for a given gene was used to transform the average C_T of each sample to relative quantities normalized using the factor calculated for each sample. The relative quantities were standardised against the control diet.

Table 2

Oligonucleotide primers used in the reverse transcription-qPCR analysis.

Gene		Primer Sequence 5'-3´	Annealing T (ºC)	PCR Efficiency (%)		Accession Number	Reference
β-actin	F	TCCTTCCTCGGTATGGAGTCT	62	Liver	96	NM_001124235	Dong et al. (2017)
	R	TTACGGATGTCCACGTCACAC		Muscle	92		
ef1α	F	TCCTCTTGGTCGTTTCGCTG	62	Liver	91	NM_001124339	Wang et al. (2018)
	R	ACCCGAGGGACATCCTGTG		Muscle	93		
gapdh	F	TTCACCACTACAACCCAATCAAC	60	Liver	92	NM_001124246	Wang et al. (2018)
	R	CACAATCAGCTTCCCGTCC		Muscle	98		
hprt1	F	GCCTCAAGAGCTACTGCAATG	60	Liver	92	XM_020502063	Pacitti et al. (2016)
	R	GTCTGGAACCTCAAATCCTATG		Muscle	92		
ghr1	F	TGGACACCCAGTGCTTGATG	58	Liver	91	AF403539	Hevrøy et al. (2015)
	R	TCCCTGAAGCCAATGGTGAT		Muscle	96		
ghr2	F	TCGGAACATTCCAGAACCTC	58	Liver	93	NM_001123594	Hevrøy et al. (2015)
	R	GGTCATCCAGACCTTCGTGT		Muscle	102		
igf1	F	TGACTTCGGCGGCAACA	62	Liver	90	M81904	Hevrøy et al. (2015)
	R	GCCATAGCCCGTTGGTTTACT		Muscle	91		
dio1	F	GGTGGTCTACATTGCCGAGG	62	Liver	94	XM_021603351	Wang et al. (2018)
	R	CATCCACTACGATGGGGCAC		Muscle	96		
thra	F	GCACAACATTCCCCACTTCT	60	Liver	91	AF146775	Raine and Hawryshyn (2009)
	R	AGTTCGTTGGGACACTCCAC		Muscle	90		
thrb	F	TCACCTGTGAAGGATGCAAG	62	Liver	-	AF146777	Raine and Hawryshyn (2009)
	R	GACAGCGATGCACTTCTTGA		Muscle	94		
gpx1b2	F	GCGGGGAGTGACATTTACCA	62	Liver	91	HE687023	Wang et al. (2018)
	R	GTAATCCCTGGTGGTCGTGC		Muscle	90		
gpx4a1	F	AAGCTGTGGACTCGTTCTTGT	62	Liver	98	HE687024	Wang et al. (2018)
	R	AGTTTACCGGGGTTTTCCCTC		Muscle	94		
sod1	F	TGGTCCTGTGAAGCTGATTG	62	Liver	98	AF469663	Teimouri et al. (2019)
	R	TTGTCAGCTCCTGCAGTCAC		Muscle	96		
acc	F	TGAGGGCGTTTTCACTATCC	60	Liver	90	tcbk0010c.b.21_5.1.om.4	Librán-Pérez et al. (2015)
	R	CTCGATCTCCCTCTCCACT		Muscle	90		
cpt1b1	F	GATGTTCCGTGAGGGTAGGA	62	Liver	99	AJ606076	Morash et al. (2010)
	R	TTGTCTTGCATGGCTCTGAC		Muscle	90		
elovl2	F	GATGCCTGCTCTTCCAGTTC	58	Liver	93	KM244737	Gregory et al. (2016)
	R	CATTGGTGGAGACAGTGTGG		Muscle	98		
fas	F	TGATCTGAAGGCCCGTGTCA	62	Liver	94	tcab0001c.e.06_5.1.s.om.8	Kamalam et al. (2013)
	R	GGGTGACGTTGCCGTGGTAT		Muscle	-		
ldlr	F	CAGCGAAGGACTGGAGAAAC	58	Liver	99	AF542091	Al-Habsi et al. (2018)
	R	TTCAGCCCACTCTTCTCGAT		Muscle	96		

Reference genes: *β-actin*, actin beta; *ef1α*, elongation factor 1 alpha; *gapdh*, glyceraldehyde-3-phosphate dehydrogenase; *hprt1*, hypoxanthine phosphoribosyltransferase 1. Target genes involved in growth: *ghr1*, growth hormone receptor 1; *ghr2*, growth hormone receptor 2; *igf1*, insulin-like growth factor I; iodine metabolism: *dio1*, iodothyronine deiodinase 1; *thra*, thyroid hormone rector alpha; *thrb*, thyroid hormone receptor beta; oxidative stress: *gpx1b2*, glutathione peroxidase 1 isoform b2; *gpx4a1*, glutathione peroxidase 4 isoform a1; *sod1*, superoxide dismutase 1; and lipid metabolism: *acc*, acetyl-CoA carboxylase; *cpt1b1,* carnitine palmitoyl transferase 1 isoform beta 1; *elovl2*, elongation of very long chain fatty acids protein 2; *fas*, fatty acid synthase; *ldlr*, low density lipoprotein receptor.

2.6. Chemical analysis of the fillet

2.6.1. Fatty acids profile

Total lipids were extracted from muscle according to Cruz et al. (2013) . Briefly, 300 mg samples were extracted with a ternary mixture of propan-2-ol, cyclohexane, and 0.9% (m/v) KCl, with triundecanoate as internal standard (Sigma, St Louis, USA), and antioxidants (ascorbic acid and butylated hydroxytoluene; Sigma, St Louis, USA). The extracts were saponified using KOH (0.5 mol.L^{-1} in methanol; Panreac, Castellar del Vallès, Spain) at 100 °C (10 min) and further methylated with boron trifluoride (14% in methanol; Sigma, St Louis, USA) at 100 °C (30 min).

Fatty acids were analysed using analytical conditions and equipment as reported by Cruz et al. (2013). Briefly, a Chrompack CP 9001 (Chrompack, Middelburg, Netherlands) chromatograph equipped with a split-splitless injector, a FID, a Chrompack CP 9050 auto-sampler and a 50 m × 0.25 mm i.d. CP-Sil 88 column (0.19 µm film; Chrompack-Varian) column. The program column temperature was 140 to 220 °C, and the injector and detector temperatures were 250 and 270 °C, respectively. Total fatty acids content was estimated on the basis of total fatty acid methyl ester area counts in comparison with the undecanoic methyl ester, by converting fatty acid methyl esters (FAMEs) to their respective fatty acid equivalents.

2.6.2. Total cholesterol content

Muscle cholesterol was determined with the gas-chromatography–mass spectrometry (GC-MS) method reported previously (Cunha et al., 2006). Briefly, an accurate sample portion (1 g) was extracted with 4 ml of n-hexane/ethyl acetate (90:10, v/v). After evaporation of the solvent, 1 ml of sodium methoxide solution, and 50 µl of betulin as internal standard, were added. The mixture was left at room temperature for 20 min before adding 1 ml of water and 2 ml of n-heptane. The aqueous phase was withdrawn and replaced by 1 ml of 1% citric acid solution. Finally, the organic fraction was dried under a nitrogen stream and derivatized at 70ºC for 20 min with 125 µl of N,O-bistrimethylsilyltrifluoroacetamide (with trimethylchlorosilane 1%; Sigma, St Louis, USA) and 125 µl of pyridine (Fluka™, Seelze, Germany). One µl of extract was analysed using an Agilent gas chromatograph 6890 (Little Falls, DE, USA) equipped with an electronically controlled split/splitless injection port and interfaced to a MSD-5973N mass selective detector and a DB-5MS fused silica capillary column (30 m × 0.25 mm I.D., 0.25 µm film thickness; J&W

Scientific, Folsom, CA, USA) coupled directly to the mass detector. The injection was performed at 270 °C in the split mode (ratio 15:1). The column temperature was 270 °C during the entire run, and the transfer line temperature was 280 °C. Quantification was carried out in selective ion monitoring (SIM) mode using 329 as the quantification ion and 129, 73, 368 and 458 as qualifier ions for cholesterol, while, for botulin, the 189 ion was used as quantifier, and 496 and 493 as qualifier ions. The calibration curve, LOD and LOQ were calculated as 2 µg.mg^{-1} and 6 µg.mg^{-1}, respectively.

2.6.3. Vitamin D_3 content

Vitamin D_3 was analysed according to Byrdwell et al. (2013). Briefly, 0.5 g samples, with 50 mg ascorbic acid, 50 µl butylated hydroxytoluene (10mg/mL) and 25 µl tocol (100 µg/l; internal standard), were saponified with KOH 60% and extracted with hexane (2 ml x 3 times). After evaporation of the solvent, 500 µl of phase mobile B was added. Separation and quantification of vitamin D_3 was performed using a high-performance liquid chromatography (HPLC) system (Waters 2695, Waters, Milford, MA, USA) coupled to a Micromass Quattro micro API™ triple quadrupole detector (Waters, Manchester, UK), equipped with MassLynx 4.1 software for data processing. HPLC conditions were set according to Byrdwell et al. (2013). Chromatographic separation was achieved using a Kinetex® Phenomenex® C18 column (2.6 µm, 150 mm x 4.60 mm (i.d.)) with a pre-column from Phenomenex (Torrance, CA, USA). The column was kept at 40 ºC, but the auto sampler qA maintained at ambient temperature (± 25 ºC). The injection volume was 10 µL. Mobile phase A (water with 0.1% formic acid) and mobile phase B (methanol with 0.1% formic acid) flow rates were 0.300 mL.min^{-1}. The solvent gradient program was as follows: (1) 0-5 min, 25% A; (2) 5.0-11.0 min, 21% A; (3) 5-11min. 10 % A; (4) 11-13 min 25% A. MS/MS acquisition was operated in positive-ion mode with multiple reaction monitoring (MRM). The optimized MS parameters were as follows: capillary voltage, 3.00 kV; source temperature, 150 ºC; desolvation temperature, 350 ºC; and desolvation gas and cone gas flow, 350 and 50 L.h^{-1}, respectively. The optimized MS/MS parameters for vitamin D_3 were 385>259 quantifier transition and 385>367 qualifier transition, and for tocol, 388>123 as quantifier transition and 388>163 as qualifier transition. The calibration curve, LOD and LOQ were calculated as 10 µg.kg^{-1} and 31 µg.kg^{-1}, respectively.

2.7. Statistical analysis

Statistical analysis was conducted using SPSS 25.0 (SPSS Inc., Chicago, IL, USA). All data were tested for normal distribution and homogeneity of variances with Kolmogorov-Smirnov and Levene's tests, respectively. One-way ANOVA was performed followed by Tukey's multiple comparison test, where individual means were compared. When normality and/or homogeneity of variances assumptions were not met, data were log-transformed. A non-parametric test (Kruskal-Wallis H-test) was applied when these assumptions were not achieved after transformation. Significant differences were considered for a *P*-value < 0.05. A Spearmen's rank correlation coefficient (r_s) test was applied to all variables. Significant correlations were considered at the bilateral levels of 0.05 (*) or 0.01 (**).

3. Results

3.1. Growth performance

After 12 weeks feeding, fish fed diets with 4% *S. latissima* had significantly lower final body weights (458 ± 72 g) compared to remaining treatments (484 ± 81 g). Likewise, hepatosomatic indices (HSI) were negatively correlated with increased sugar kelp, resulting in significantly lower values in fish fed 4% *S. latissima* (Fig. 1). Zootechnical data regarding this growth trial is further detailed in Granby et al. (2020, Submitted).

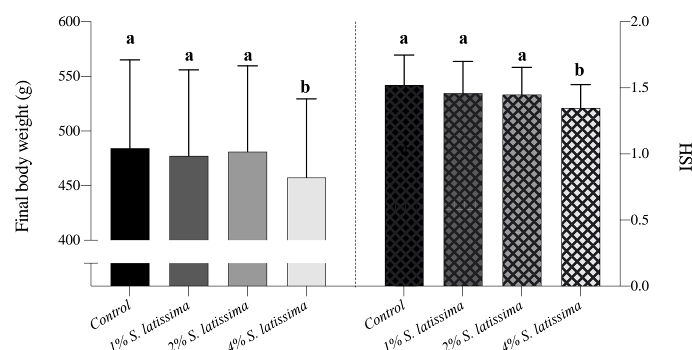

Fig. 1. Final body weight (g) and hepatosomatic index (HSI) of fish fed the control diet and experimental diets (1, 2 or 4% inclusion of *S. latissima*) over 12 weeks. Values presented as mean ± SD and different superscript letters denote significant differences among treatments (P < 0.05). Adapted from Granby et al. (2020, Submitted).

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3.2. Expression of genes related to growth, iodine metabolism, oxidative stress and lipid metabolism in liver and muscle

Expressions of a panel of 14 genes related to growth, iodine and lipid metabolism, and oxidative stress were analysed in the liver and muscle of rainbow trout. For the expressions of genes related to growth, namely *igf1* and growth hormone receptors (*ghr1* and *ghr2*) no significant differences were registered among dietary treatments (Figs. 2 and 3). However, in muscle *ghr* was upregulated with the inclusion of sugar kelp (though not significantly) and expression of both *ghr1* and *ghr2* were significantly negatively correlated with fish final body weight (Table 3).

In both liver and muscle, expression of genes involved in iodine metabolism (*dio1, thra and thrb)* was not significantly affected by dietary inclusion of *S. latissima*. In the liver, however, *dio1* was downregulated (though not significantly) by inclusion of sugar kelp in diets (Figs. 2 and 3).

Dietary inclusion of *S. latissima* resulted in a significant downregulation of *gpx1b2* in the liver of fish fed 2% of sugar kelp in comparison to fish fed the control diet. Expression of *gpx4a1* and *sod1* remained unaffected by dietary treatments in both liver or muscle (Figs. 2 and 3). Both *gpx1b2* and *gpx4a1* transcription levels were positively correlated with final body weight and HSI (Table 3).

Regarding lipid metabolism, expression of *acc* was not significantly affected by the dietary treatments, either in liver or muscle (data not shown). Although non-significant in the liver, *cpt1b1* showed a tendency for an upregulation with increasing amounts of *S. latissima*, whilst an opposing trend was observed in *elovl2* expression in both liver and muscle (Figs. 2 and 3). Expression of *cpt1b1* was significantly negatively correlated with HSI and final body weight (Table 3). In liver, a non-significant decrease in *ldlr* expression with increased incorporation of macroalgae was observed but, in muscle, a significant downregulation of *ldlr* was observed in fish fed diets with 1% sugar kelp compared to those consuming the control diet (Figs. 2 and 3). Moreover, this gene was significantly positively correlated with HSI in the liver and with HSI and final body weight in the muscle (Table 3). The *fas* expression in liver decreased with the inclusion of seaweed and was significantly reduced in fish consuming 4% kelp compared to fish fed with control diet (Fig. 2).

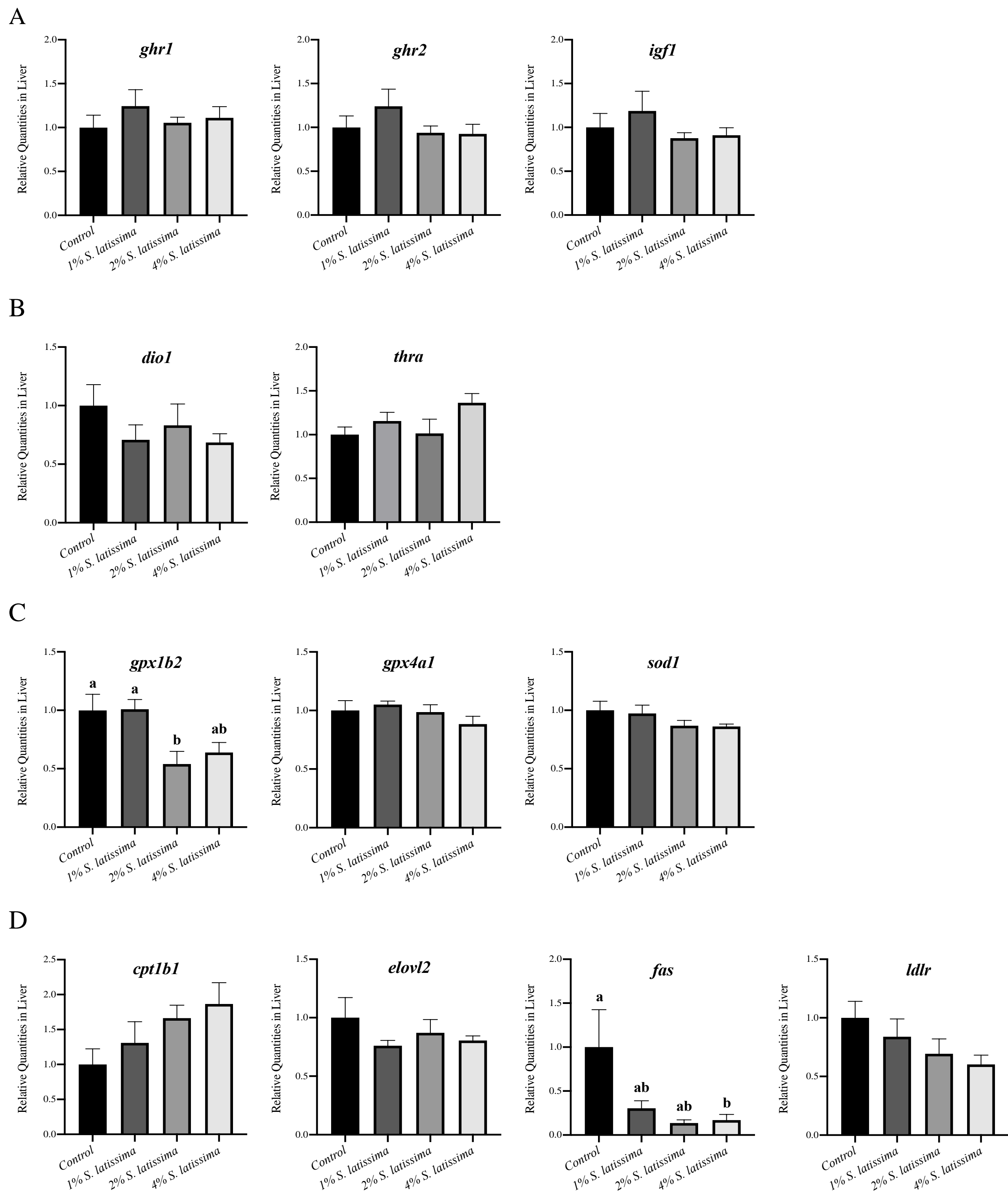

Fig. 2. Gene expression in the liver of rainbow trout fed the control diet and the experimental diets (1, 2 or 4% inclusion of *S. latissima*) over 12 weeks. A - Genes related to growth; B - Genes involved in iodine metabolism; C - Genes associated with oxidative stress; and D - Genes involved in lipid and cholesterol metabolism. Relative abundance in liver is presented as mean ± SEM (n=9 fish per treatment). Different letters denote significant differences among treatments (P = 0.004 for *gpx1b2* and P = 0.04 for *fas*).

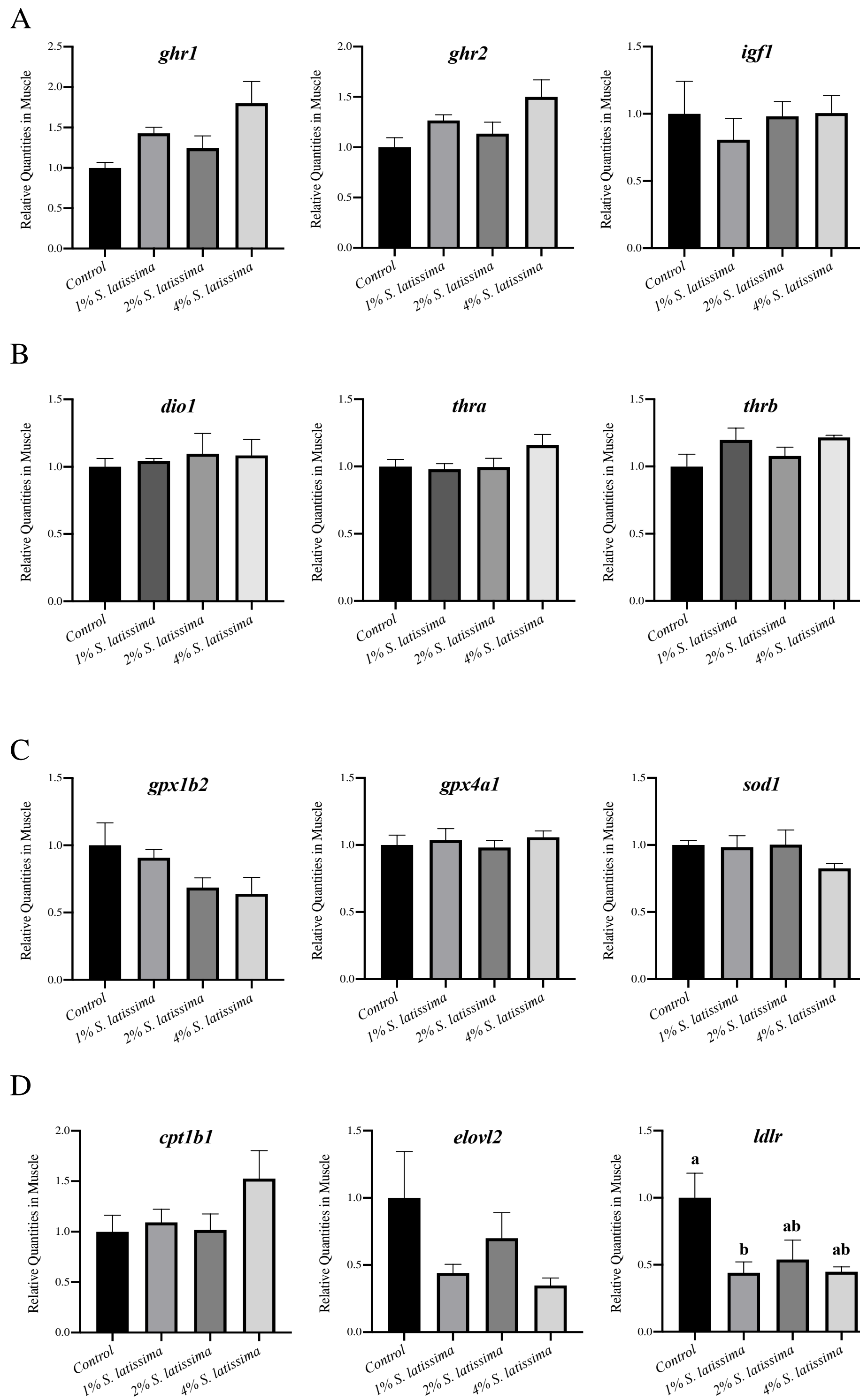

Fig. 3. Gene expression in the muscle of rainbow trout fed the control diet and the experimental diets (1, 2 or 4% inclusion of *S. latissima*) over 12 weeks. A - Genes related to growth; B - Genes involved in iodine metabolism; C - Genes associated with oxidative stress; and D - Genes involved in lipid and cholesterol metabolism. Relative abundance in muscle is presented as mean ± SEM (n=9 fish per treatment). Different letters denote significant differences among treatments (P = 0.02).

Table 3. Correlations with statistical significance between expression of selected genes (liver and muscle) and growth performance or fillet composition in rainbow trout fed the experimental diets.

	Final body weight	HSI	∑SFA	∑MUFA	∑PUFA	Cholesterol	Vitamin D_3
Liver							
dio1	NS	NS	NS	NS	NS	0.63*	NS
thra	NS	NS	NS	NS	NS	NS	NS
gpx1b2	0.60*	0.73*	0.69*	0.64*	0.61*	NS	0.71**
gpx4a1	0.66*	0.66*	NS	NS	NS	NS	NS
sod1	NS	NS	0.59*	NS	NS	NS	NS
cpt1b1	-0.89**	-0.92**	-0.65*	NS	NS	NS	-0.71**
fas	NS	NS	0.84**	0.76**	0.64*	NS	0.81**
ldlr	NS	0.58*	0.63*	NS	0.59*	NS	0.74**
Muscle							
thra	NS	NS	NS	NS	NS	NS	NS
thrb	-0.64*	NS	NS	NS	NS	NS	NS
ghr1	-0.66*	-0.75*	NS	NS	NS	NS	-0.83**
ghr2	-0.59*	-0.69*	NS	NS	NS	NS	-0.68**
cpt1b1	-0.63*	-0.74**	NS	NS	NS	NS	NS
elovl2	NS	0.64*	NS	NS	NS	NS	NS
ldlr	0.78**	0.76**	0.78**	0.75**	0.69*	NS	NS
Vitamin D_3	NS	0.68*	0.77**	0.64*	0.59*	NS	-

NS: not significant. Spearmen's rank correlation coefficient was performed. Correlation was considered significant at the bilateral levels of 0.05 (*) or 0.01 (**).

3.3. Fatty acids profile and content in cholesterol and vitamin D_3 of the fillet

Monosaturated fatty acids (MUFAs) were the primary fatty acids class observed in rainbow trout fillets, followed by polyunsaturated fatty acids (PUFAs) and, in a lesser amount, saturated fatty acids (SFAs). SFA content decreased significantly with the inclusion of *S. latissima*, being lower in fillets of fish fed 2 and 4 % *S. latissima* when compared to control. Both MUFAs and PUFAs decreased with the inclusion of sugar kelp and fish fed 2% *S. latissima* had significantly lower concentrations compared with controls, which was mainly due to reduced concentrations of oleic (18:1 n-9) and linoleic (C18:2 n-6) acids. No significant differences were observed with respect to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) contents (Table 4). Fillet SFA, MUFA, and PUFA contents were positively correlated with genes related to oxidative stress, *fas* and *ldlr* expression in liver, and with *ldlr* expression in muscle. SFA were also negatively correlated with the liver expression of *cpt1b1* and positively correlated with *sod1* (Table 3).

Table 4. Fillet total lipid content (g.100 g^{-1} wet weight) and fatty acid composition (g.100 g^{-1} wet weight).

	Control	1% *S. latissima*	2% *S. latissima*	4% *S. latissima*
Total Lipids	7.43 ± 0.4	7.12 ± 0.1	6.81 ± 0.4	6.92 ± 0.6
Fatty acids				
SFA				
C14:0	0.14 ± 0.003^{a}	0.13 ± 0.0003^{ab}	0.12 ± 0.006^{b}	0.13 ± 0.006^{b}
C16:0	0.74 ± 0.02^{a}	0.70 ± 0.004^{ab}	0.65 ± 0.03^{b}	0.67 ± 0.02^{b}
C18:0	0.21 ± 0.007^{a}	0.19 ± 0.003^{ab}	0.18 ± 0.01^{b}	0.18 ± 0.005^{b}
[1] ∑ SFA	1.18 ± 0.03^{a}	1.11 ± 0.008^{ab}	1.05 ± 0.05^{b}	1.07 ± 0.03^{b}
MUFA				
C16:1 n-7	0.22 ± 0.007^{a}	0.20 ± 0.003^{ab}	0.19 ± 0.01^{b}	0.20 ± 0.008^{ab}
C18:1 n-9	2.86 ± 0.08^{a}	2.68 ± 0.04^{ab}	2.55 ± 0.1^{b}	2.66 ± 0.09^{ab}
C20:1 n-9	0.14 ± 0.003^{a}	0.13 ± 0.003^{ab}	0.13 ± 0.009^{b}	0.13 ± 0.006^{ab}
[2] ∑ MUFA	3.33 ± 0.1^{a}	3.13 ± 0.04^{ab}	2.97 ± 0.2^{b}	3.10 ± 0.1^{ab}
PUFA				
C18:2 n-6	1.32 ± 0.04^{a}	1.24 ± 0.02^{ab}	1.18 ± 0.07^{b}	1.23 ± 0.04^{ab}
C18:3 n-3	0.32 ± 0.004^{a}	0.30 ± 0.004^{ab}	0.29 ± 0.01^{b}	0.30 ± 0.01^{ab}
C20:5 n-3 (EPA)	0.26 ± 0.01	0.26 ± 0.002	0.24 ± 0.01	0.25 ± 0.005
C22:6 n-3 (DHA)	0.73 ± 0.03	0.70 ± 0.02	0.70 ± 0.02	0.72 ± 0.02
[a] EPA+DHA	0.99 ± 0.04	0.96 ± 0.02	0.94 ± 0.03	0.96 ± 0.01
[3] ∑ PUFA	3.07 ± 0.1^{a}	2.93 ± 0.05^{ab}	2.82 ± 0.1^{b}	2.91 ± 0.05^{ab}

Values presented as mean ± SD (n=3) and different superscript letters denote significant differences among treatments ($P < 0.05$).

[1] ∑ SFA is the sum of saturated fatty acids and also includes also C12:0, C15:0, C17:0, C20:0, C:21, C22:0 and C24:0.

[2] ∑ MUFA is the sum of mono-unsaturated fatty acids and includes also C14:1, C17:1, C22:1 n-9 and C24:1 n-9.

[3] ∑ PUFA is the sum of polyunsaturated fatty acids and includes also C18:2 n-6t, C18:3 n-6, C18:4 n3, C20:2 n-6, C20:3 n-6, C20:4 n-6, C20:3 n-3, C20:4 n-3, C22:2 n-6, C22:3 n-3, C22:4 n-6, C22:5 n-6 and C22:5 n-3.

[a] EPA + DHA = eicosapentaenoic acid + docosahexaenoic acid.

S. latissima diets had no effects on total cholesterol levels, but vitamin D_3 concentrations significantly decreased in a dose-response manner in fish fillets fed kelp-rich diets. Fillets of fish fed 1% *S. latissima* had significantly lower concentrations of vitamin D_3 than those fed the control, whilst fish fed diets with 2 or 4% kelp showed a significant decrease in comparison with fish fed both control and 1% diets (Fig. 4). Fillet content of vitamin D_3 was positively correlated with HSI and expression of *fas, ldlr and gpx1b2*, but negatively correlated with *cpt1b1, ghr1 and ghr2* expression. A significant positive correlation was observed between vitamin D_3 content and SFA, MUFA and PUFA concentrations (Table 3), as well as whole-body fat ($r = 0.61$; $P = 0.03$). In contrast, fillet cholesterol content only showed a positive correlation with *dio1* expression in liver (Table 3).

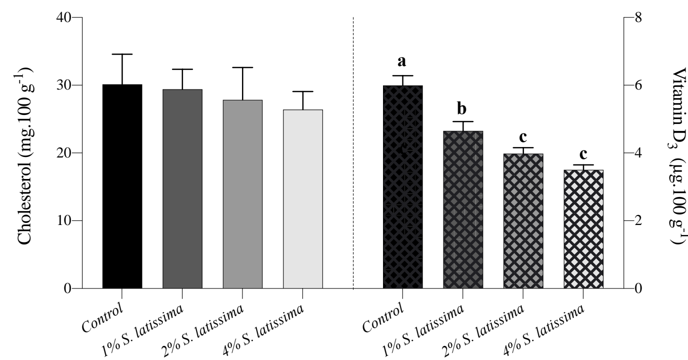

Fig. 4. Fillet composition in total cholesterol and vitamin D_3 of rainbow trout fed the control diet and the experimental diets (1, 2 or 4% inclusion of *S. latissima*) over 12 weeks. Values presented as mean ± SD (n=3) and different superscript letters denote significant differences among treatments ($P < 0.001$).

4. Discussion

The use of novel feeds with natural functional ingredients, like macroalgae *S. latissima*, has attracted much attention lately, due to a public health drive to improve diets by increasing essential nutrients in healthy products. In most EU Member States, dietary intakes of iodine are a major concern, with most food items not showing sufficient levels to enable consumers to reach the daily recommended intakes (Biban and Lichiardopol, 2017). Fish can be good vehicle of iodine and other nutrients for human consumption, mainly long-chain fatty acids and vitamins. Our results clearly show that the dietary inclusion of *S. latissima* at either 1 or 2% is feasible, but a 4% inclusion significantly reduced rainbow trout final body weight. As far we are aware of, this is the first study evaluating the inclusion of *S. latissima* in rainbow trout diets. Growth impairment at higher levels is probably associated with a limited capacity to digest the kelp. In a complementary study, Granby et al. (2020, Submitted) reported a significant decrease in protein digestibility in rainbow trout fed sugar kelp-rich diets, although lipid and energy digestibility did not vary among treatments. Likewise, Pereira et al. (2012) observed that *Sargassum* sp. was not well digested by rainbow trout, compared with other macroalgae (e.g. *Gracilaria* sp.), limiting its dietary inclusion in aquafeeds. The present study showed that it is possible to include *S. latissima* in diets for rainbow trout, but the inclusion level should be below 4%.

Body growth is highly dependent on protein deposition in muscle that, in turn, is regulated by endocrine factors (Mommsen, 2001). Igf1 is a key elicitor of growth and its synthesis is stimulated by Gh via Ghrs (Reindl and Sheridan, 2012). In this study, no significant differences in expression of *ghr*s or *igf1* were

observed, but there was a trend for upregulation in muscle of both *ghr1* and *ghr2* with dietary kelp. Expression of *ghrs* in muscle was also significantly and negatively correlated with fish final body weight, and fish fed diets with the highest seaweed level (4%) had significantly lower final body weight than fish fed the other experimental diets. Together, these data suggest that 4% sugar kelp can lead to subtle changes in the Gh/Igf-I axis at the molecular level, which lead to significant detrimental effects on rainbow trout growth.

Sugar kelp-rich diets, with high concentrations of iodine, had limited effects on genes involved in rainbow trout iodine metabolism. Iodine is a key component for Ths synthesis, but the supplementation of feeds with excess iodine did not lead to significant differences in the expression of *dio1* or *thr*s. Studies evaluating dietary iodine intake on fish metabolism are extremely scarce, but in mice, exposure to excess inorganic iodine led to a significant decrease in D_1 activity and serum T_3 (Xia et al., 2013). Excess iodine intake has also been associated with the inhibition of thyroid hormone receptor protein in liver without significant changes at the gene expression level (Zhao et al., 2010). Likewise, our results suggested a slight downregulation of *dio1* expression in liver with dietary kelp (although not significant), without any clear trend in *thra* transcription. Determination of circulating thyroid hormones concentrations might provide further insights into the physiological impact of high dietary organic iodine.

Dietary supplementation with seaweeds is associated with health benefits that include protection against oxidative stress, both in fish and humans (Wan et al., 2019). Present data demonstrated a significant reduction in *gpx1b2* mRNA levels in trout fed with 2% *S. latissima* diet. The *gpx1b2* encodes for glutathione peroxidase, which is a key enzyme in the regulation of the oxidative status and protection of cells against lipid peroxidation. Reduced *gpx1b2* expression in rainbow trout liver might indicate that *S. latissima* provides readily available antioxidants, leading to decreased demand for production of endogenous antioxidant enzymes. This concept is, in general, in accordance with previous results where rainbow trout fed increasing amounts of red seaweed, *G. pygmaea*, were reported to have reduced antioxidant Gpx and Sod activities (Sotoudeh and Mardani, 2018). Magnoni et al. (2017) highlighted the antioxidant properties of seaweeds during hypoxia trials with gilthead seabream, which suggested a protective role against oxidative stress, reducing the requirement for endogenous antioxidant enzymes.

In the present study, inclusion of *S. latissima* significantly affected lipid metabolism. Liver *fas* expression decreased with the inclusion of seaweed, being significantly reduced in fish fed 4% kelp compared with controls. The *fas* is involved in *de novo* fatty acid biosynthesis pathways. Previous studies in rat models reported inhibition of the lipogenic pathway, including downregulation of *Fas*, after ingestion of low levels of the brown seaweed *Undaria pinnatifida* (Yoshinaga et al., 2018). In contrast, Xia et al. (2013) reported an upregulation of hepatic *Fas* mRNA in mice challenged with high doses of iodine via water, ultimately resulting in hepatic steatosis. Different sources of iodine, and their related bioavailabilities, could in part explain these variations since, in the latter study, potassium iodate (KIO_3) was used as vehicle for iodine rather than a brown macroalgae. Brown seaweeds contain several bioactive compounds, for instance fucoxanthin, that can affect lipid metabolism or protect from other harmful impacts (Afonso et al., 2019). Ribeiro et al. (2017b) observed a decrease of whole-body fat and lipid retention in gilthead seabream fed diets supplemented with fucoxanthin-rich microalgae *Phaeodactylum tricornutum*. The downregulation presently observed in *fas* suggests decreased lipogenesis that, together with the (non-significant) trend for increased *cpt1b1* expression, could account for the significant lower HSI observed with 4% *S. latissima* feed. In fact, fish HSI was negatively correlated with *cpt1b1* expression. Cpt1 is the main regulatory enzyme in fatty acid β-oxidation, the catabolic process in fatty acid metabolism. A previous study evaluating the effect of dietary *Spirulina* sp. supplementation in red seabream (*Pagrus major*) highlighted an elevated activity of hepatic Cpt accompanied by a decrease in total lipids both in serum and liver (Nakagawa et al., 2000). Granby et al. (2020, Submitted) detected a significant reduction in lipid retention and protein digestibility in fish fed diets containing kelp, resulting in significantly lower final body weights. These data suggest that fish metabolized lipids to produce energy and to compensate for reduced protein bioavailability, although this compensatory mechanism was not sufficient to meet energy requirements with 4% sugar kelp.

In fish, lipid storage occurs mainly in liver, visceral fat, and muscle, with only the last being of interest for human consumption. Decreased deposition of SFAs, MUFAs and PUFAs was observed in fillet from rainbow trout fed sugar kelp and was positively correlated with *fas* expression. This suggests that the inclusion of this macroalgae affects lipid metabolism, resulting in lower fatty acids content in the fillet. Previous studies in trout showed an increase in PUFAs, namely EPA and DHA (% total fatty acids), in fish

fed diets with 3 and 6% inclusion of the brown algae *Macrocystis pyrifera* (Dantagnan et al., 2009). In general, this is in accordance with our data, which demonstrated that, despite a decrease in total PUFAs content with algae inclusion, EPA and DHA deposition remained unaffected by dietary treatments. Taken together, these results suggest a selective retention of EPA and DHA in fillets, as reported previously (Dantagnan et al., 2009; Sissener, 2018). Fish are the most important natural sources of omega-3 fatty acids, which are essential for human health. The consumption of n-3 polyunsaturated fatty acids from seafood has been linked with lower incidence of cardiovascular diseases (Raatz et al., 2013). The EPA and DHA muscle content of all fish in this study (0.94 to 0.99 g.100 g^{-1}) were well above (3.7 times more) the minimum recommended values per 100 g portion of 0.25-0.50 g (EFSA, 2010). Therefore, the dietary inclusion of sugar kelp reduced fillet deposition of some fatty acids (e.g. oleic and linoleic acids) but did not compromise the contents of essential omega-3 fatty acids.

The liver plays a pivotal role in cholesterol homeostasis, as it is involved in the cholesterol biosynthesis pathway, uptake from plasma via *ldlr*, and degradation through conversion into bile acids (Go and Mani, 2012). In our study, there was a trend for diminished hepatic *ldlr* expression with increased seaweed supplementation, though no significant differences were registered between dietary treatments. This suggests that circulating Ldl-cholesterol might not be negatively impacted by the experimental diets. A previous study with juvenile turbot fed diets supplemented with increasing amounts of *Sargassum horneri* reported a significant increase in serum Ldl cholesterol at the highest levels (7.5 and 10%) without any differences in the Ldl-C/Hdl-C ratio, suggesting that algae meal had no detrimental effects on fish cardiovascular system (Wang et al., 2019). However, in mice exposed to high iodine concentrations via water (KIO_3), Zhao et al. (2010) reported an attenuation of *Ldlr* mRNA expression via TRβ1, accompanied by hypercholesterolemic effects. In this study, a significant downregulation of *ldlr* in muscle was detected in fish fed diets with 1% sugar kelp in comparison to controls. In terms of fillet composition, the effects detected at the molecular level did not result in significant differences in cholesterol content. Other natural active substances in *S. latissima* might have helped to balance the negative effects exerted by this seaweed on *ldlr* transcript levels. Thus, dietary inclusion of iodine-rich *S. latissima* might impact *ldlr* expression in different tissues, although liver was not significantly affected. For example, prolonged exposure or higher

levels of this macroalgae could lead to detrimental effects in cholesterol metabolism of rainbow trout, deserving further consideration.

Fish fed kelp-rich diets exhibited markedly decreased fillet cholecalciferol (vitamin D_3) content. Vitamin D deficiency is a global health threat and common to all human populations (Naeem, 2010). Fish are the preferred source of cholecalciferol to achieve an adequate vitamin D status (Tripkovic et al., 2012) and, therefore, a decrease in vitamin D_3 fillet content is bad for human nutrition. For adults, the vitamin D dietary reference value is 15 µg.day^{-1} (EFSA, 2016) and the reduction in vitamin D_3 observed in this study (from 6.0 µg.100 g^{-1} in control to 3.5-4.7 µg.100 g^{-1} in diets with *S. latissima*) would significantly impact fillet quality. Nonetheless, fillet of fish fed the experimental diets still provided 23-40% of the recommended levels of vitamin D_3. Vitamin D_3 is a lipid soluble component and, in humans, its absorption has been shown to be affected by dietary fat (Dawson-Hughes et al., 2015). In our study, fillet vitamin D_3 deposition was significantly positively correlated with HSI, whole-body fat, and muscle SFA, MUFA and PUFA contents. Furthermore, a positive correlation between genes involved in lipid metabolism and vitamin D_3 content was observed. These results suggest that the combined downregulation of *fas* and upregulation of *cpt1b1* might contribute to a lipid-lowering effect of diets with sugar kelp, which reduced vitamin D_3 content in the fillet. The potential benefits of *S. latissima* as a functional ingredient in human diets to reduce obesity should be further explored. Further studies are also required to understand the precise molecular mechanisms through which diets supplemented with *S. latissima* modulate vitamin D_3 deposition in rainbow trout. Such knowledge could lead to the development of strategies to fortify trout fillet with such an important nutrient for human health.

5. Conclusion

Our results demonstrate that the dietary inclusion of sugar kelp *S. latissima* below 4% is feasible, without detrimental effects on rainbow trout final body weight. Reduced *gpx1b2* expression in fish fed 2% sugar kelp diet might suggest the need for less endogenous antioxidants, evidencing the potential of *S. latissima* as a protective agent against oxidative stress. The deposition of certain fatty acids (e.g. oleic and linoleic acids) in the fillet was reduced with kelp supplementation. However, EPA and DHA concentrations were not compromised and the fillet provided 3.7 times the recommended levels for human consumption.

Muscle *ldlr* expression was reduced with macroalgae inclusion, but fillet total cholesterol was not affected by dietary treatments. Diets with sugar kelp also had a lipid-lowering effect, with a significant downregulation of *fas* and tendency for upregulation of *cpt1b1*, which might have contributed to reduced vitamin D_3 content in the fillet. New approaches for improving vitamin D_3 content in trout fillets should be developed to provide products with health-promoting nutrients or human consumption.

Declaration of competing interest

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

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Authors contributions

K.G., B.K.L., L.M.P.V., J.D., M.L.N. and A.M. were responsible for the experimental design. B.K.L. and K.G. conducted the growth trial. M.F., K.G., B.K.L., S.C.C., C.M., J.O.F, I.C., L.F.C.C. were involved in chemical analysis and gene expression data. M.F. and L.M.P.V analysed data, performed the statistical analysis and wrote the manuscript draft. All authors provided comments to the manuscript redaction.

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