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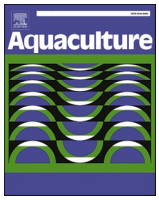

Dietary inclusion of the red seaweed *Asparagopsis taxiformis* boosts production, stimulates immune response and modulates gut microbiota in Atlantic salmon, *Salmo salar*

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ABSTRACT

Salmonids represent US$23 billion of global aquaculture value, yet Atlantic salmon farms lose approximately 10% of their production to diseases and parasites every year. New approaches to minimise such losses are urgently needed because current treatments (e.g. antibiotics) have environmental and human health impacts with increasing sea temperatures predicted to further exacerbate the impacts of disease. Immunostimulants that boost fish resistance to disease without negative environmental or human health impacts are currently being assessed. Seaweeds and their extracts are used as immunostimulants for land animals and are increasingly being investigated for use in finfish aquaculture, including for Atlantic salmon. Here we show that when the red seaweed *Asparagopsis taxiformis* and its extract were incorporated in Atlantic salmon feed, fish growth rates were enhanced up to 33%, feed intake was enhanced up to 13%, FCR were reduced, and innate immune responses were enhanced up to 58% compared to fish fed unsupplemented control diets over 4 weeks. Overall, fish fed the methanolic extract of *A. taxiformis* (at an inclusion of ~1% on a dry weight basis, D:D of feed) had the best combination of enhanced growth rate, feed intake and immune response. Fish fed the immunostimulant lipopolysaccharide (LPS) derived from *Escherichia coli* had the highest innate immune response in our trial, however LPS had no enhanced effect on growth or feed intake. Additionally, we provide evidence that the seaweed and LPS supplements modulated the expression of immune and stress-related genes in both the liver and head kidneys. More specifically, the fish fed the supplemented diets showed increased expression of the HSP70 gene in both their liver and head kidney after 2 weeks of treatment. At 4 weeks high HSP70 and lysozyme gene expression was observed in the fish fed the two seaweed methanolic extract diets. The seaweed diets also enhanced the diversity of bacterial communities within the hindgut of Atlantic salmon while the LPS treatment appeared to have the opposite effect. Whole *A. taxiformis* or its methanolic extracts could therefore be used as functional feed ingredients that boost the immune response and enhance the growth rate of Atlantic salmon without affecting feed efficiencies.

1. Introduction

Disease outbreaks, climate change and antimicrobial resistance are the top three challenges limiting the development of the aquaculture industry (Reverter et al., 2020; Stentiford et al., 2017). These challenges are interrelated and represent a wicked problem, as the intensification of farming practices and the warming of oceans are forecast to make disease outbreaks more severe and frequent, especially for thermally sensitive species such as salmonids (LaMere et al., 2020). Diseases and parasites can already cost fish farms up to 40% of their annual production (Stentiford et al., 2017). Furthermore, subclinical disease (i.e. diseases that do not require veterinary attention) incur additional opportunity costs by limiting fish growth and condition. Such subclinical diseases are poorly understood in fish aquaculture (Erkinharju et al., 2021), but in some species can represent as much as 7% of the overall production cost (Mohd Nor et al., 2019).

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The use of veterinary drugs such as antibiotics is not a sustainable solution due to its links with depressed immunity in treated fish, the environmental impacts of antibiotic use and the severe implications this poses for human and environmental health, i.e. the emergence of antimicrobial resistance (Henriksson et al., 2018). Although techniques such as selective breeding and gene editing have potential to improve the resistance of farmed fish to specific pathogens, they can be time consuming, costly and/or heavily regulated, which prevents their broad use on a global scale (Gjedrem, 2015; Gratacap et al., 2019; Zenger et al., 2019). Accessible and sustainable alternatives to prevent and treat extant and emerging disease risks for aquaculture are urgently required to reduce the environmental impact of the aquaculture industry in order to maintain the production of fish protein for a rapidly-growing world population (Stentiford et al., 2020).

A promising alternative to traditional disease treatments is the inclusion of functional ingredients or 'immunostimulants' in the diet of farmed fish, which can boost their immune systems and enhance their resilience to pathogens and disease (Sakai, 1999). Such dietary manipulations are generally attractive to industry because they are straightforward to implement and do not have negative impacts on the local environment or on human health (Thépot et al., 2021a). The key commercial consideration for inclusion of novel feed ingredients, including immunostimulants, is their potential impact on the production of farmed fish, for which any increase in growth or feed efficiency would confer direct economic benefits to farmers. The corollary is that any decreases in production will limit adoption of any novel ingredient. Furthermore, early adoption of natural feed ingredient that boosts fish health and productivity, and offers an environmentally and socially responsible alternative to veterinary drugs, would also provide a competitive advantage by minimising negative community and consumer perceptions (Campbell et al., 2015; Dessart et al., 2019). Finally, identifying novel marine-derived dietary additives which enhance feed conversion would lead to faster uptake of aquafeeds containing high inclusions of land protein if the detrimental effects on the food conversion ratios can be circumvented (Alhazzaa et al., 2019).

Immunostimulants include a wide range of additives such as probiotics, prebiotics, plants, fungi and organic acids (Dawood et al., 2018). Immunostimulants also include seaweed and their products, which we recently covered in a meta-analysis highlighting that whole seaweeds (dried and powdered) and their extracts have significant potential as immunostimulants for farmed finfish (Thépot et al., 2021a). A secondary finding was that some seaweed supplements also enhanced growth rates of fish; however, the relationship between boosting the immune response and the growth rate of the fish was weak (Thépot et al., 2021a). This review also highlighted that, despite emerging interest in seaweeds as potential immunostimulants, only 34 of the ~11,343 species of seaweeds have been investigated (Guiry and Guiry, 2021), with some studies working with whole seaweed tissue and others focusing only on seaweed extracts (Thépot et al., 2021a). Furthermore, only 48 of the 600 fish species produced in aquaculture have been investigated (FAO, 2020; Nelson et al., 2016), and the species more frequently studied are not necessarily those that represent most production in aquaculture globally (Thépot et al., 2021a). For example, Atlantic salmon (*Salmo salar*) represents 19% of global aquaculture value (USD 23 billion) yet only seven studies have assessed the efficacy of seaweed supplements on the immune status or growth of Atlantic salmon (Dalmo and Seljelid, 1995; Gabrielsen and Austreng, 1998; Kamunde et al., 2019; Moroney et al., 2017; Muñoz et al., 2019; Palstra et al., 2018; Wan et al., 2016). These studies testing the efficacy of seaweeds were inconclusive with respect to use as immunostimulants (Dalmo and Seljelid, 1995; Gabrielsen and Austreng, 1998) or growth promoters (Wan et al., 2016).

One seaweed species gaining significant attention, owing to its rich natural product chemistry with potent bioactive properties, is the red seaweed *Asparagopsis taxiformis* (Rhodophyta). This seaweed is native to warm waters of the Indo-Pacific, where commercial farming operations are presently being developed, but is also invasive and considered a pest in European waters (Zanolla et al., 2015), where it could potentially be wild-harvested with minimal environmental consequences. This species, when fed to cattle, alters ruminant gastrointestinal microbiota to such an extent that production of methane, a potent greenhouse gas, is almost reduced to zero (Kinley et al., 2016; Li et al., 2018; Machado et al., 2016; Roque et al., 2019). This result has been linked to the ability of *Asparagopsis taxiformis* to produce and concentrate bromoform (Paul et al., 2006). Importantly, there are no negative impacts on animal health or growth, rather animals supplemented with 0.1% (organic matter basis) *A. taxiformis* over a 90 day trial demonstrated up to 21% greater weight gain compared to the unsupplemented steers; a proposed benefit from utilisation of energy that would otherwise be wasted through methane emissions from the cow (Kinley et al., 2020). We recently showed that feeding the mottled rabbitfish *Siganus fuscescens* diets supplemented with this same species of red seaweed (*A. taxiformis*) elicited a four-fold increase in haemolytic activity, one of the main innate immune response parameters, compared to 15 other seaweed and microalgae supplements (Thépot et al., 2021b). However, it is unclear at this stage whether the dramatic effects of *A. taxiformis* on the immunochemistry is limited to one fish species, whether the active ingredients from *A. taxiformis* can be extracted from the seaweed and utilised directly, or whether there are any negative impacts on fish production traits that would influence commercial uptake of *A. taxiformis* as a novel feed supplement. A seaweed extract may be more useful than a whole (dried and powdered) additive if the bioactives are concentrated thereby displacing less of the formulated feed to achieve the same or better productivity and health outcomes in farmed fish.

Here we provide the first assessment of *A. taxiformis* as a dietary supplement for Atlantic salmon. We compared the innate immune responses of *S. salar* parr fed diets supplemented with whole (dried and powdered) *A. taxiformis*, or its extracts (exhaustive methanol extraction) at different inclusion rates, to those parr fed control diets currently used by the salmon aquaculture industry in Australia. We also compared parr fed diets supplemented with seaweed to those supplemented with a potent immunostimulant, lipopolysaccharides (LPS) from *Escherichia coli* (Paulsen et al., 2001). LPS has been reported to have a positive effect on Atlantic salmon fry weight (+10% compared to the control fish after 62 days; Guttvik et al., 2002). In addition to assessing the innate immune response, we measured and compared fish growth rates and feed efficiency across treatments, explored the expression of genes relating to immune and stress responses, and evaluated shifts in the bacterial communities that colonise the intestines of the experimental fish.

2. Material and methods

2.1. Fish rearing conditions

The 4-week feeding trial was carried out at the Bribie Island Research Centre (BIRC), Queensland, Australia (27°03′15.7″S 153°11′42.6″E). The Atlantic salmon fry, *Salmo salar*, were provided by Tassal Operations Pty Ltd. Fry (5 g) were shipped from the Rockwood hatchery in Tasmania to the BIRC. Here they were spread between two 1000 L fiberglass conical tanks where they remained for an acclimation period of 6 days and fed the control diet at 3% BW/day (Nutra Supreme-RC, Skretting Ltd., see below). The fish were then randomly allocated into 50 plastic tanks (55 L) with 18 fish per tank, such that each tank was an experimental 'replicate' and measurements from the 18 fish within each were either chosen randomly or averaged, depending upon the parameter (see below). To replicate aquaculture practise, fish were hand fed the experimental diets to satiation twice a day (10:00 a.m. and 3:00 p.m.). During the trials, to approximate conditions at their origin, water temperature was maintained at 15 °C by a heat pump (Oasis C16) and mean pH of 7.51 ± 0.06. The system was operated as a recirculating aquaculture system using dechlorinated town water and comprised two Waterco C50 bag filters in parallel (50 μm bags) followed by a Micron S602e sand-filter. The system was in a temperature and light controlled

room kept at 18 °C and on a 12 L:12D (08:00–20:00) light regime with a 30 min ramp up/down period.

2.2. Seaweed collection and preparation for dietary inclusion

A. taxiformis was collected from Moffat Beach in Queensland, Australia (26°47′21.7″S 153°08′36.0″E; Fig. 1). The seaweed was then cleaned using seawater to remove sand and epiphytes before being spun in a washing machine (Fisher & Paykel 5.5 kg Quick Smart) on spin cycle (1000 rpm) for 5 min to remove excess salt water. Following this, the seaweed was frozen at −80 °C before being processed in a freeze dryer (Thermo Savant model MODULYOD-230) for at least 3 days at approximately −44 °C and 206 mbar. Once dried, the seaweed was powdered and kept in a vacuum-sealed bag in the −80 °C until use (Fig. 1).

The seaweed extract was made using 150 g of freeze-dried *A. taxiformis*, which was extracted 4 times over 12 h (each time) in 500 mL of methanol in the dark at 25 °C. Briefly, the 150 g of freeze-dried *A. taxiformis* was added to 500 mL of methanol in a 1 L Schott bottle which was shaken by hand to ensure all dried powdered seaweed was in contact with the methanol. The bottle was left in the dark and after 12 h, once the seaweed biomass had settled, the supernatant methanolic extract was poured into a 2 L Schott bottle before another 500 mL of methanol was added to the original 1 L Schott bottle and homogenised as described above. This step was repeated two more times for exhaustive extraction, at which point the residual biomass appeared pale and the last 500 mL of methanol extract was lightly coloured (see Fig. 1).

The combined methanolic extract (2 L, 4 × 500 mL extractions) was filtered (Whatman® grade 2) prior to being slowly evaporated using a rotary evaporator (IKA® RV3 Eco) in a 30 °C bain-marie. Once the methanol was fully evaporated, the crude extract, representing by weight approximately 20% of the original seaweed biomass, was resuspended in 400 mL of deionised water and 100 mL of hexane (Fig. 1).

2.3. Chemical analysis of the freeze dried seaweed and its methanolic extract

The key natural products of both the original powdered seaweed and its methanolic extract were analysed using gas chromatography–mass spectrometry (GC–MS; Table S1, Fig. S1). The extract was reconstituted in methanol with ethyl benzoate as an internal standard, filtered and vialled for GC–MS analysis. For the whole seaweed treatment, freeze dried seaweed was extracted in methanol with ethyl benzoate as an internal standard, filtered and vialled for GC–MS analysis, which was performed on a Perkin Elmer Clarus SQ8S fitted with a DB-5 column (Perkin Elmer Elite-5MS, 30.0 m × 0.25 mm, 025 μm, hereafter referred to as GC). Injections (1.0 μL) were introduced with a 50:1 split ratio with a sample rate of 1.56250 pts./s. The GC was held at 40.0 °C for 1 min, ramped at 20.0 °C min^{-1} to 250.0 °C and held for 0 min followed by a 0.5 min equilibration time prior to the next injection. Helium was used as the carrier gas with a flow rate of 1 mL/min. Mass spectrometry was performed on a Perkin Elmer Clarus 580 across a weight range of 50–340 *m/z*. Analysis occurred from 3.0–12.0 min with a scan rate of 0.3 s. Compounds were identified by referencing mass spectral chromatographs to the National Institute of Standards and Technology library. The GC confidence intervals were then averaged across samples as well as within samples using different areas of the peak and subtraction of background ion profiles. Relative quantitation was achieved by comparison of peak area ratios (as determined using supplied TurboMass software) of compound to internal standard (equivalent to parts per million or compound (mg)/solvent (L)) which were then evaluated

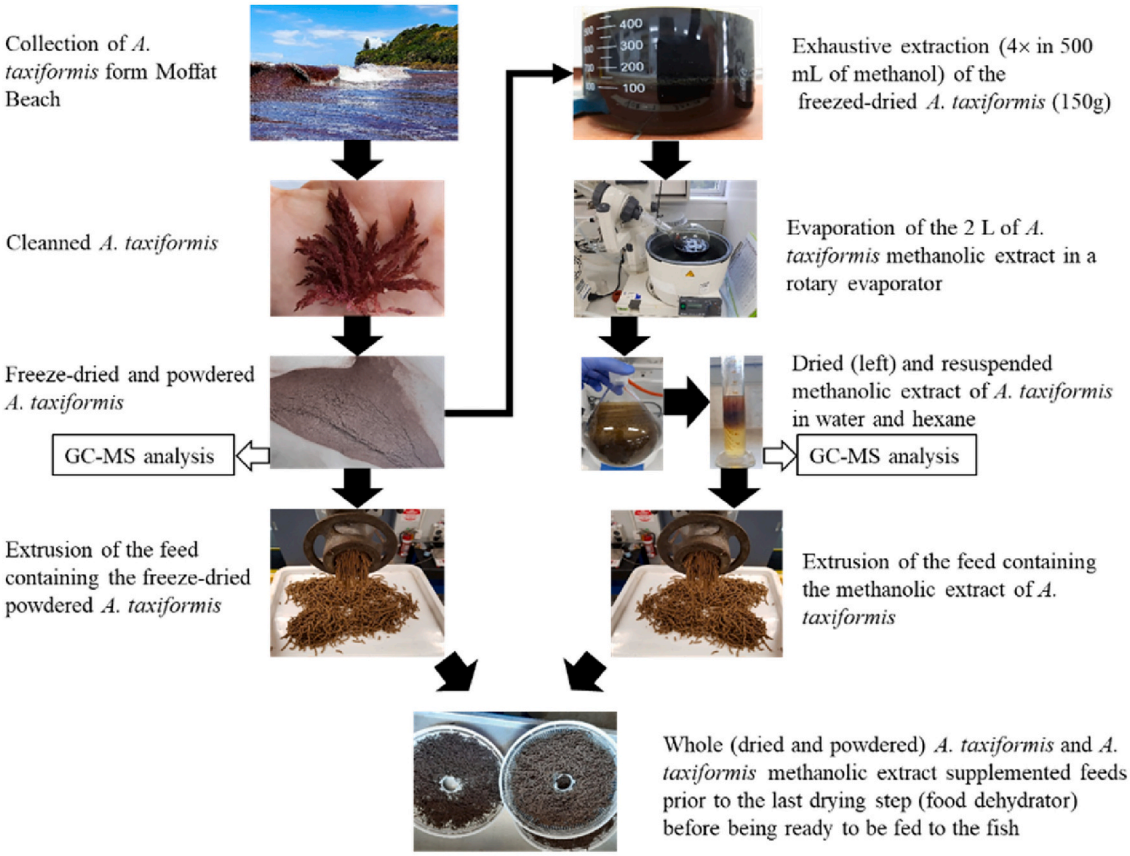

Fig. 1. Diagram showing the different steps involved in making the *A. taxiformis* supplemented feed from the collection of the seaweed to the drying stage of the extruded pellets.

to give compound (g)/seaweed material (g).

2.4. Experimental diets

S. salar parr were fed one of five experimental supplemented diets for 4 weeks: an unsupplemented control, whole seaweed diet (whole dried and powdered *A. taxiformis* at 3% inclusion, hereafter referred to as 'whole diet'), seaweed extract diet at 0.6% inclusion (exhaustive methanolic extract of *Asparagopsis taxiformis* representing the equivalent "extract" content of the 3% whole seaweed diet, hereafter referred to as 'extract diet'), a double dose of seaweed extract at 1.2% inclusion (twice the dose of the extract diet, representing the equivalent "extract" content contained in a hypothetical 6% whole seaweed diet, hereafter referred to as 'extract×2 diet') and an LPS diet (0.01% lipopolysaccharide from *Escherichia coli*, Sigma-Aldrich) as positive control. The control, unsupplemented diet was produced based on the commercial diet Nutra Supreme-RC (Skretting Ltd). The pellets were first powdered and then combined with deionised waster (30% of the pellet mass) in a blender (Hobart A120) at 104 rpm using a dough hook for approximately 10 min to produce a stiff dough. The dough was then extruded through a 2 mm die on trays and dried in a fan-driven food dehydrator (Ezidri$_{TM}$ Ultra FD1000) at 28 °C for 12 h. Once dried, the feed was packaged in airtight bags and subsequently stored at 4 °C until use.

The whole diet was made using the same procedure but received the powdered and sieved (300 μm) seaweed at 3% (D/D) prior to adding the water during the blending step. The two extract diets were made as described above but involved the addition of seaweed extract instead of powdered seaweed. Seaweed extract was added during the water addition step in the feed making process (e.g. 3% extract diet = methanol extracted seaweed biomass present in the 3% whole seaweed diet). The effective feed displacement for the extract and extract×2 diets were 0.6% and 1.2% respectively, which represented a 2.6% and 1.8% lower feed displacement respectively compared to the whole diet. A positive control, lipopolysaccharide (LPS from *Escherichia coli*) was added at 0.01% D:D into the feed (hereafter referred to as 'LPS') at the same step to provide our final experimental diet treatment.

2.5. Sample collection and analyses

2.5.1. Sample collection

After 2 weeks on the control and treatment diets, fish were fasted for 24 h prior to sampling. Once fasted, three fish per tank were randomly sampled and euthanized in 10 ppt Aqui-S® then weighed. Samples from different fish (e.g. blood or organ) were pooled in the same collection tube to give one sample per tank and ten replicate tanks and measurements per treatment. Euthanised fish had blood drawn using a heparinised (lithium heparin) 29G 1 mL insulin syringe for whole blood analyses (phagocytic activity and respiratory burst activity) and non-heparinised syringe for serum preparation (for lysozyme activity, haemolytic activity and glucose concentration). To obtain serum, the blood samples collected without heparin were left to clot for 1 h at 18 °C and 8 h at 4 °C before centrifugation at 700 G. The separated serum was aliquoted in new collection tubes and immediately transferred to a − 80 °C freezer. These fish also had their liver and head kidney excised and placed in RNAlater™ for gene expression experiments. The whole gut from three replicate fish from each tank was excised using a sterile scalpel blade and a 0.25 cm section of hindgut with digesta was taken starting 1 cm internally to the anal pore. The three hindgut samples were pooled together in one PowerBead® tube from the PowerSoil® DNA isolation kit containing 60 μL of C1 solution (Mo Bio, San Diego, CA, USA). All remaining fish in the replicate tanks were weighed and returned to their tanks for another 2 weeks of twice-daily feeds of their treatment diets. Following this, fish were fasted (24 h), euthanised and sampled as above so that we had fish sampled at 2 weeks and at 4 weeks.

2.5.2. Lysozyme activity

Serum lysozyme activity was determined using the turbidimetric assay, which gives a direct measure of lysozyme activity (Ellis, 1990). Briefly, lyophilized *Micrococcus lysodeikticus* (75 mg) was rehydrated and suspended in 100 mL of buffer (0.05 M Na2HPO4, pH 6.2) to achieve a 0.075% *w/v* concentration. Flat bottom microtiter plates were dosed with 140 μL of buffer and 10 μL of thawed serum sample. The plate was then shaken and absorbance was measured at 450 nm at 0.5 and 4.5 min. One unit of lysozyme (U/mL) was defined as a decrease of 0.001 in absorbance over that period.

2.5.3. Haemolytic activity

The haemolytic activity of the alternative complement pathway (ACH50) was determined as the method described by Oriol Sunyer and Tort (1995). Briefly, rabbit red blood cells (R-RBC) were washed thrice in Hanks buffered saline solution (HBSS) supplemented with 7 mM $MgCl_2$ and 10 mM ethylene glycol tetraacetic acid (HBSS-Mg-EGTA) with successive centrifugation (2000 rpm for 5 min at 4 °C). The R-RBC were rinsed three times with HBSS-Mg-EGTA for 1 min at 1000 rpm and made up to 3% volume in the same buffer. In 96-well plates, 20 μL of test serum was diluted with 30 μL of HBSS-Mg-EGTA and four-fold serial dilutions were made to achieve dilutions ranging from 0.15–10.00%. Subsequently, 20 μL of R-RBC suspension was added to each tube and incubated for 100 min at 25 °C with occasional shaking. Three replicate negative control were also made for the 0% and 100% lysis of R-RBC by adding 20 μL of the R-RBC suspension to 120 μL of HBSS-Mg-EGTA and distilled water respectively. After incubation, the plates were centrifuged at 2000 rpm for 2 min and 50 μL of supernatant of each dilution was then transferred to a new microtiter plate and read at 450 nm. The degree of haemolysis was calculated by dividing the corrected absorbance value by the 100% haemolysis control. The reciprocal of the serum dilution giving 50% haemolysis was used as the ACH50 titre (U/mL).

2.5.4. Respiratory burst activity

The production of reactive oxygen species by leukocytes was measured using nitrotetrazolium blue chloride (NBT, Sigma) and triggered using phorbol 12-myristate 13-acetate (PMA, Sigma) following the method from Secombes (1990) and subsequently modified by Stasiak and Baumann (1996). Briefly, 50 μL of blood samples were loaded in 'flat bottom' microtiter plates and incubated at 20 °C for 1 h to allow adhesion of cells. The supernatant was decanted and the wells were washed thrice with PBS. Fifty microliters of 0.2% NBT containing 200 ng/mL of PMA was loaded in the wells and incubated for 1 h at 20 °C. The cells were then fixed using 100% methanol for 2 min and washed thrice using 70% methanol. The plates were air-dried after which 60 μL of 2 N potassium hydroxide and 70 μL of dimethyl sulfoxide were added to all well to dissolve the formazan blue precipitate formed by the reactive oxygen species. Finally, the optical density of each well was measured at room temperature and recorded in an EnSpire multimode plate reader (PerkinElmer) at 540 nm.

2.5.5. Phagocytic index

The phagocytic index assay was based on the method from Anderson and Siwicki (1995). Fifty microliters of heparinised blood sample was place in the wells of microtiter plate followed by 50 μL of 1×10^7 1 μm fluorescent beads (Sigma) suspended in phosphate buffered saline (pH 7.2). The mixture was mixed thoroughly and incubated for 1 h at 20 °C. Five microliters was then taken out onto a glass slide to prepare a smear. The smear was air dried (10 min) and then fixed with 95% methanol. Once the methanol evaporated, the smear was stained with a Giemsa solution. The number of engulfed fluorescent latex beads in phagocytes was counted using an epifluorescence microscope (Nikon Eclipse T*i*—U with X-Cite series 120 Q from Lumen Dynamics). The phagocytic index (PI) were calculated as follows:

PI = total number of beads engulfed by phagocytes/total number of phagocyte −containing beads

2.5.6. Microbial DNA isolation and 16S rRNA gene sequencing and analysis

DNA was extracted from five randomly selected samples (pool of three fish) from each treatment due to cost limitations using the PowerSoil DNA isolation kit (Mo Bio, San Diego, CA, USA) following the manufacturer's instructions and thereafter stored at −20 °C.

Microbial diversity profiling of was completed at the Australian Genome Research Facility (AGRF), who amplified the hypervariable region V3–V4 of the 16S rRNA gene by the polymerase chain reaction (PCR) using the forward primer 341F (CCTAYGGGRBGCASCAG) and the reverse primer 806R (GGACTACNNGGGTATCTAAT). The sequencing was performed on a MiSeq platform (2 × 300 bp) and the resulting reads were analysed with Illumina bcl2fastq pipeline version 2.20.0.422. Trimmed sequences were processed and denoised using the DADA2 package (Callahan et al., 2016) and QIIME2 (v2018.8) software, with amplicon sequence variants (ASVs) tables constructed and aligned against the Silva 16S rRNA 99% reference database (release v132; Quast et al., 2012). Subsequent quality filtering included the removal of singletons, chimeric sequences, mitochondrial DNA, and unassigned or Eukaryotic amplicon sequence variants (ASVs). Raw sequences have been deposited in the National Center for Biotechnology Information (NCBI) sequence read archive (SRA) under the bioproject number PRJNA727066.

2.5.7. Gene expression of immune and stress related genes in Atlantic salmon

RNA was extracted from five randomly selected samples (pool of three fish) from each treatment due to cost limitations. All RNA extraction was performed using RNeasy mini kit (Qiagen, Australia), as per manufacturer's instructions. For the elimination of DNA, DNase I (Sigma-Aldrich, Australia) was used, as per manufacturer's instructions. Prior to reverse transcription, extracted RNA was quantified using Qubit® 2.0 fluorometer (Invitrogen), and for each reverse transcription reaction we used a total 1000 ng RNA in reaction. Reverse transcription was performed using iScript reverse transcription supermix (Bio-Rad, Australia), as per manufacturer's instructions. The cDNA concentration and quality from all samples were evaluated on a Qubit® 2.0 fluorometer (Invitrogen). cDNA was then stored at −20 °C until quantitative real-time PCR (qPCR) assays for gene expression.

cDNA, extracted from livers and head kidneys, was used to measure the level of Atlantic salmon IFN-γ, IL-1β, Lys, C3a, HSP gene expression with EF1 used as a reference gene using previously described primers (Table 1). Prior to qPCR assays, we have performed both gene and primer sequence analyses using blastn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm sequence specificity for each gene target. Conventional PCRs were done to check amplification from cDNA and to generate amplicons for each gene target for use in standard curves.

Table 1
Genes targeted in this study.

Gene	Primer sequence (5′–3)	Amplicon size (bp)	R^2	Efficiency (%)
$EF1_A$	CCCCTCCAGGACGTTTACAAA CACACGGCCCACAGGTACA	57	0.998	105.0
IL-1β	ATGCGTCACATTGCCAAC GGTCCTTGTCCTTGAACTCG	91	1.000	92.4
Lys	CTACAATACCCAGGCCACCAA GTCACACCAGTAGCGGCTGTT	85	0.999	100.3
IFNγ	CCGTACACCGATTGAGGACT GGGCTTGCCGTCTCTTCC	98	0.999	95.5
C3a	GAGGAAAGGTGAGCCAGATG TGTGTGTGTCGTCAGCTTCG	103	0.998	97.8
HSP70	CCCCTGTCCCTGGGTATTG CACCAGGCTGGTTGTCTGAGT	121	0.995	93.8

Amplifications were carried in a total of 30 μL reactions, consisting of 15 μL of 2× Amplitaq Gold Master mix (ThermoScientific), 1 μL of 10 μM each of forward and reverse primer, 10.5 μL PCR grade water and 2.5 μL template cDNA under the following cycling conditions: 95 °C for 10 min; 35 cycles of 95 °C for 15 s, 58 °C for 20 s, 72 °C for 30 s; and a final extension step of 72 °C for 7 min. The PCR products were checked by electrophoresis on 2% agarose gels stained with SybrSafe, and visualized under UV transilluminator. The resulting amplicons were purified using the Roche High Pure PCR Product Purification Kit (Roche, New South Wales, Australia) following the manufacturer's instructions.

Detection limit and efficiency for each gene qPCR assay was determined using a standard curve constructed using serial dilutions from 10^6 to 10^{-1} copies/μL of the purified target gene amplicons, tested in triplicate. PCR efficiency values (E) were calculated for each gene from the given slope after running standard curves and following the formula:

$$E = 100 \times \left(10^{\left(\frac{-1}{\text{Slope}}\right)}\right)$$

For each gene target, the E values were within the acceptable range (92.4%–112.8%), the r^2 values were 0.99–1.00, with the Cq values within the linear quantifiable range for the relevant standard curve.

After initial optimisation, the qPCR reactions were performed in a final volume of 20 μL, including 10 μL iTaq master mix (Biorad), 1 μL of 10 μM each of forward and reverse primer (equating to 0.75 μM primer concentration in reaction) (Table 1; Easy oligos, Sigma-Aldrich), 6 μL PCR grade water and 2 μL template cDNA. Cycling conditions consisted of 3 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 25 s at 60 °C and 30s extension at 72 °C, followed by melt curve analysis (65 °C to 95 °C in 0.5 °C increments). Samples were tested in duplicates, and negative controls (MilliQ water and no transcribed sample) were included in each assay. In each assay, a Cq value was recorded for each sample. Each gene amplicons were also characterised with a high-resolution melt (HRM).

Gene expression of IFN-γ, IL-1β, Lys, C3a and HSP was measured in both the liver and head kidney and compared to the house-keeping gene $EF1_A$. To quantify the absolute mRNA abundance for each gene, qPCR products were used to generate a standard curve using a 10-fold dilution series (initial concentration 10^6 number of copies). Subsequently, the absolute mRNA expression level for each gene was determined based on the respective standard curve using the following equation: Copy number $= 10^{\left(\frac{\text{Cq}-\text{intercept}}{\text{Slope}}\right)}$

The copy number was normalised using the total ng of RNA used for each target gene.

2.6. Calculations and statistics

The relative growth rate and feed intake were calculated over the 4 week period and also from the start to week 2 and form week 2 to week 4 (supplementary files). Feed intake, relative weight gained, and food conversion ratios (FCR) were calculated as follow:

$$\text{Feed intake (\%of biomass per day)} = \left(\frac{100}{\text{trial duration (days)}}\right) \times \left(\frac{\text{mass of feed given (g)}}{\text{average fish biomass (g)}}\right)$$

$$\text{Relative weight gain (\%)} = 100 \times \left(\frac{\text{final weight (g)–initial weight (g)}}{\text{initial weight (g)}}\right)$$

$$\text{FCR} = 100 \times \left(\frac{\text{amount fed (g)}}{\text{weight gain (g)}}\right)$$

Statistical analyses and graphical representation were performed in R version 3.6.3 (Core-Team, 2013). Differences in relative growth rate,

feeding rate and FCR between treatments were evaluated using one-way ANOVAs, with square root transformed data to meet the assumptions of homogeneity of variance and improve normality.

To explore potential variation within and between treatments at different sampling time (week 2 vs. week 4) and because we know very little about the effects of seaweed on the immunochemistry of fish and their immune and stress gene expression response the innate immune parameters and gene expression responses were explored using two-way repeated measures ANOVA (immune response vs. dietary treatment + sampling week) using the EMSaov package (Choe et al., 2017). When significant at the treatment level the one-way ANOVA and the two-way repeated measures ($p < 0.05$), the tests were followed by a Dunnett's test. The effect of the seaweed diet as a dietary group (whole, extract and extract×2 together, hereafter referred to as 'Seaweed') was also evaluated using a regression model for all measurements (growth and feed efficiencies, immunochemistry and gene expression) and followed by a Tukey's HSD post hoc test when significant to identify whether the dietary supplementation with any seaweed treatments was influencing the regression results.

Beta diversity of the fish hindgut microbiome was visualized using non-metric multidimensional scaling (nMDS) ordinations and Bray-Curtis community dissimilarity index and compared between treatments using the Adonis function of the Vegan package (Dixon, 2003). The hindgut microbial composition of the fish fed the four dietary treatments were compared to that of the control fish using pairwise comparisons of changes in the relative abundances of ASVs with Wald tests in the DESeq2 function (Love et al., 2014). The interactions between the measurements taken at 2 weeks: innate immune response, growth and feed intake, hindgut ASVs representing a minimum of 5% relative abundance and immune and stress gene expression, were further explored in nMDS using Bray-Curtis dissimilarity matrix.

3. Results

3.1. Effect of dietary seaweed supplements on growth and feed intake

The addition of whole *Asparagopsis* or its extracts led to significantly elevated growth rates of Atlantic salmon parr (Fig. 1A). The relative weight gain of fish fed diets supplemented with seaweeds (average relative weight gain for both whole seaweed and extract fed fish) over the 4-week trial (from start to 4 weeks) was 26% higher than those of the fish fed the control or LPS diets (84% and 87% relative weight gain respectively; regression model ANOVA of relative weight gain from start to 4 weeks vs. dietary treatments, $F = 10.33$, $p = 0.0002$, Tukey's HSD seaweed treatments vs. control $p = 0.001$ and seaweed treatments vs. LPS $p = 0.005$).

3.2. Effect of dietary seaweed supplements on feed intake

The fish fed the diets containing *A. taxiformis* extract and extract×2 (Fig. 1B), led to the highest feed intake out of the five treatments, representing an average 10% increase in feed intake for the fish fed the seaweed extract diets compared to those fed the control diet (Dunnett's test, $p = 0.001$ and $p = 0.015$ for the extract diet and extract×2 diet respectively vs. the control). The overall feed intake of the fish fed the whole or extract seaweed diets was 8% higher on average than those fed the control and LPS diet (regression model ANOVA of feed intake from start to 4 weeks vs. dietary treatments, $F = 6.15$, $p = 0.004$, Tukey's HSD 'Seaweed vs. Control' $p = 0.024$ and 'Seaweed vs. LPS' $p = 0.019$). The feed intake of the fish fed the whole diet was not significantly different from that of the control and LPS-fed fish (Dunnett's test, $p = 0.966$). A weak negative correlation was found between the feed intake and the relative growth rate of the Atlantic salmon fed all the supplemented diets (Fig. S2, adjusted $R^2 = 0.16$, $F = 20.01$, $p < 0.001$).

3.3. Effect of dietary seaweed supplements on food conversion ratio

No statistical differences in FCR were observed between the Atlantic salmon fed the different treatments (ANOVA, $F = 1.46$, $p = 0.231$) or treatment groups (regression model ANOVA, $F = 1.92$, $p = 0.154$) during the 4 week trial (Fig. 1C). Fish fed the control diet had more variance around the FCR (1.38 ± 0.21 SE; Fig. 1C) compared to those fed the whole diet (1.09 ± 0.04) and those fed the two extract diets (1.15 ± 0.05 and 1.16 ± 0.08 for the extract and extract ×2 diets respectively).

3.4. The innate immune response of Atlantic salmon fed seaweed supplements

There were no treatment effects on the haemolytic activity (Fig. 2A). However, both the effect of weeks and its interaction with the treatments were statistically significant (repeated measures ANOVA, treatments $F = 1.95$, $p = 0.118$, weeks $F = 101.80$, $p < 0.001$ and treatment:weeks $F = 4.57$, $p = 0.003$). At week 2, the fish fed the positive control LPS (410 ± 40.06 U/mL) had a higher ACH50 compared to the control fish (221 ± 28.73 U/mL, Dunnett's test, $p = 0.008$), which corresponded to an increase of 85% compared to the control but this was no longer the case at week 4 (Fig. 2A). The fish fed the whole diet had a mean ACH50 value 1.6 times higher (352.12 ± 40.93 U/mL) than that of the control fish but this difference was not statistically significant. After 4 weeks, the fish fed the LPS diet no longer had a significantly higher ACH50 compared to the control fish and it appeared that the best performing treatment was the extract×2 fed fish which had a mean ACH50 (304 ± 36.8 U/mL) 21% higher than that of the control fish (239.6 ± 17.3 U/mL), but this difference was not resolved statistically (Fig. 2A).

No significant different treatment effect existed for the respiratory burst response (Fig. 2B) of the fish fed the different diets, however, there was a significant week effect with a four-fold reduction in respiratory burst activity for all fish between week 2 and week 4 (repeated measures ANOVA, treatments $F = 0.60$, $p = 0.066$, weeks $F = 206.54$, $p < 0.001$, $p = 0.711$).

There were no significant effects of treatment in regards to the fish serum lysozyme activity but as for the respiratory burst activity, a significant week effect was observed with lower lysozyme activity at week 4 (average of 5.62 U/mL) compared to that of week 2 (average of 14.39 U/mL; repeated measures ANOVA, treatments $F = 1.60$, $p = 0.26$, weeks $F = 117.04$ $p < 0.001$). At 4 weeks, the fish fed the seaweed supplemented diets had, on average, 58% higher serum lysozyme activity than the control fish (regression model ANOVA of lysozyme activity vs. dietary treatments, $F = 3.44$, $p = 0.040$, Tukey's HSD 'Seaweed vs. Control' $p = 0.035$). Although at week 4 the fish fed the supplemented diets all had a higher lysozyme activity than the control fish, no dietary treatment stood out (ANOVA $F = 1.84$, $p = 0.138$; Fig. 2C).

The phagocytic index was not significantly influenced by the different diets at either 2 weeks (ANOVA, $F = 0.45$, $p = 0.774$) or at 4 weeks: (ANOVA, $F = 0.86$ $p = 0.498$; Fig. 2D).

The treatments had a strong effect on the serum glucose level of fish (repeated measures ANOVA, treatments $F = 5.09$, $p = 0.002$, weeks $F = 0.01$ $p = 0.930$) with after 2 weeks the fish fed the control diet had a 50% higher glucose level compared to those fed the seaweed (regression model ANOVA, $F = 6.09$, $p = 0.005$, Tukey's HSD 'Seaweed vs. Control' $p = 0.005$) and LPS diets (Tukey's HSD 'LPS vs. Control' $p = 0.033$; Fig. 3). More specifically, the fish fed the control diet had a significantly higher serum glucose level (11.35 ± 2.97 mmol L^{-1}; ANOVA, $F = 2.91$, $p = 0.035$) compared to the fish fed the whole diet (5.77 ± 0.68 mmol L^{-1}; Dunnett's test, $p = 0.029$) and extract diet (5.87 ± 0.28 mmol L^{-1}; Dunnett's test, $p = 0.031$). Although the control fish still had the highest serum glucose concentration after 4 weeks (7.32 ± 0.49 mmol L^{-1}) it was greatly reduced compared to the 2 week levels and the difference between diets was no longer significant (ANOVA, $F = 1.93$, $p = 0.122$; Fig. 3).

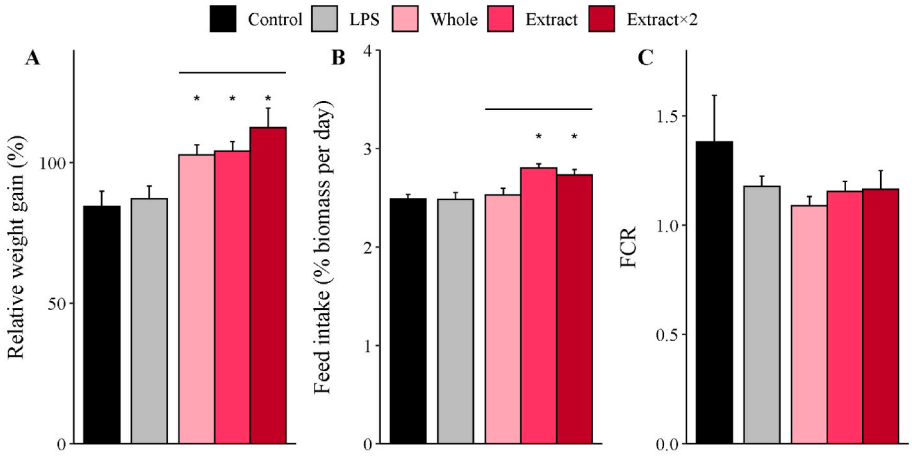

Fig. 2. A) Relative weight gain, B) feed intake and C) FCR of the fish fed the five treatments over the 4 week trial: control (black), LPS (grey), whole seaweed (pink), seaweed extract (light red) and seaweed extract×2 (dark red) after 4 weeks. Asterisks above individual treatments denote significant differences compared to the control (Dunnett's test, $p < 0.05$). The straight line over the 'seaweed' treatment group and LPS denotes significant differences compared to the control and 'seaweed' treatment group respectively (regression model ANOVA $p < 0.05$ followed by Tukey's HSD 'Seaweed vs. Control' or 'LPS vs. Seaweed' $p < 0.05$). Data shown are average values ± SE. The standard error of the mean was calculated from $n = 10$ tanks per treatment ($n = 10$–15 fish per tank). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

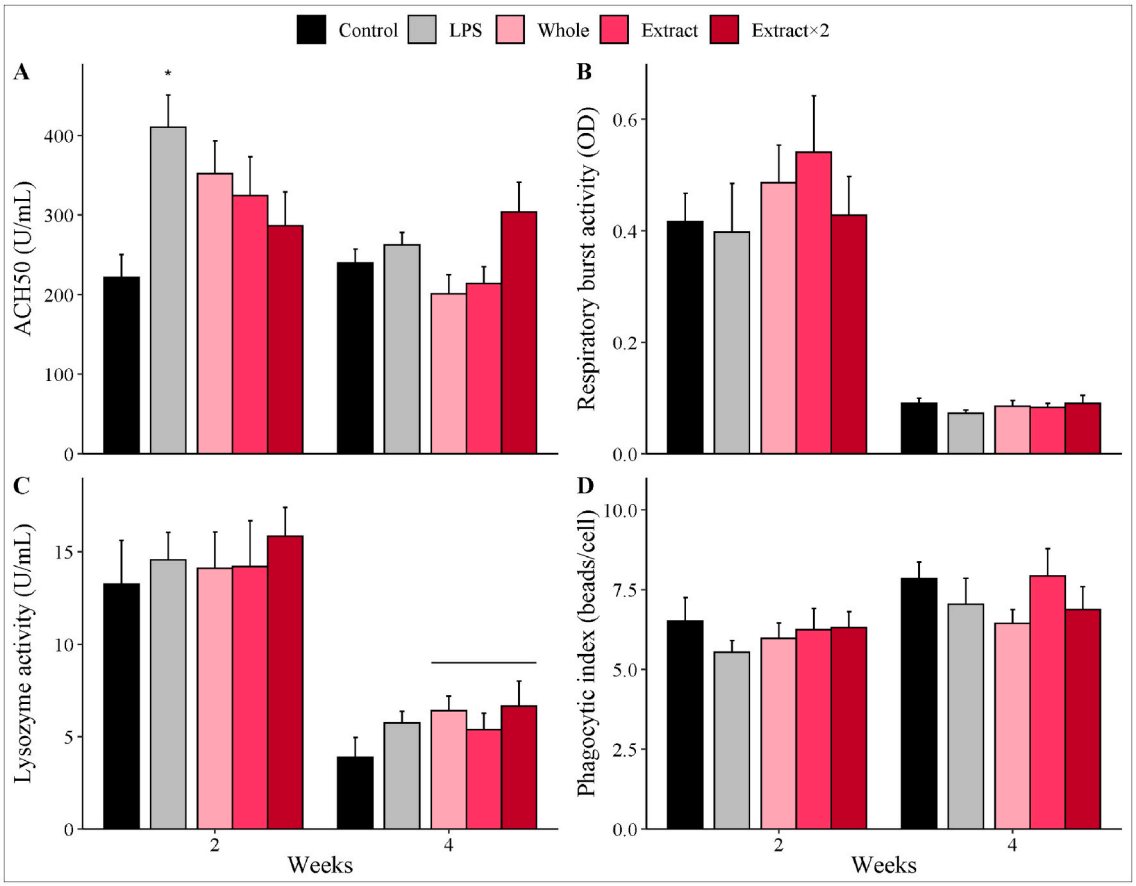

Fig. 3. Innate immune response, (A) haemolytic activity, (B) respiratory burst activity, (C) lysozyme activity and (D) phagocytic index of the fish fed the five treatments: control diet (black), LPS diet (grey), whole seaweed diet (pink), seaweed extract diet (light red), seaweed extract×2 diet (dark red) and sampled at 2 and 4 weeks. Asterisks above individual treatments denote significant differences compared to the control (Dunnett's test, $p < 0.05$). The straight line above the three seaweed treatments denotes significant differences compared to the control (regression model ANOVA $p < 0.05$ followed by Tukey's HSD 'Seaweed vs. Control' $p < 0.05$). Data shown are average values ± SE. The standard error of the mean was calculated from $n = 10$ tanks per treatment ($n = 3$ fish per tank). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.5. Immune and stress related gene expression of Atlantic salmon fed A. taxiformis

No significant treatment effect were observed for the fish head kidney HSP70 expression, however there was an interaction between the treatment and the sampling weeks (repeated measures ANOVA, treatments $F = 1.54$, $p = 0.229$, weeks $F = 1.44$ $p = 0.242$, treatments: weeks $F = 4.14$, $p = 0.013$). The significant interaction of treatments and weeks was caused by the higher head kidney HSP70 expression in the fish fed the LPS diet was 66% ± 30% (mean % change ± SE) higher than the control fish after 2 weeks (regression model ANOVA, $F = 3.76$, $p = 0.039$; Fig. 4C) but at 4 weeks the fish fed the LPS diet had a 73% ± 16% lower expression of HSP70 than the control fish (Dunnett's test, $p = 0.045$). The fish fed the whole diet for 2 weeks appeared to have higher

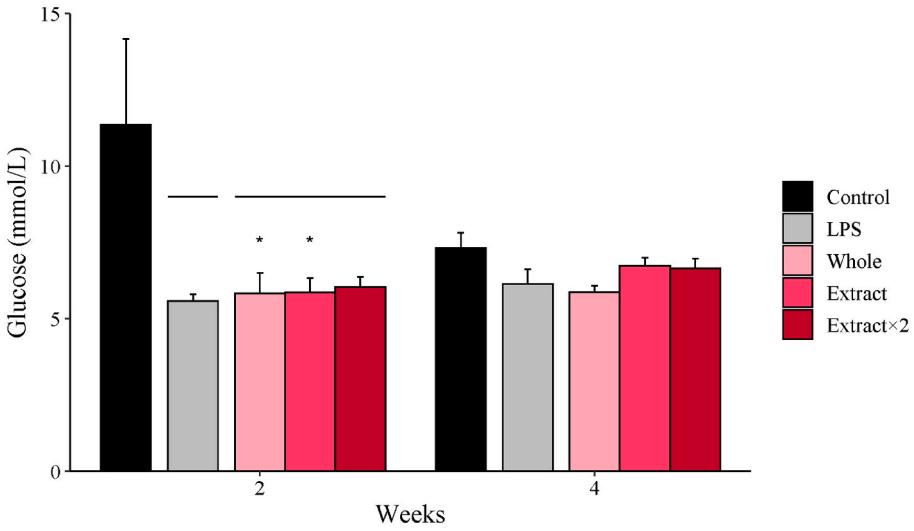

Fig. 4. Serum glucose level of the fish fed the five treatments: control (black), LPS (grey), whole seaweed (pink), seaweed extract (light red) and seaweed extract×2 (dark red) at 2 and 4 weeks. Asterisks above individual treatments denote significant differences compared to the control (Dunnett's test, $p < 0.05$). The straight line over the 'seaweed' treatment group and LPS denotes significant differences compared to the control (regression model ANOVA $p < 0.05$ followed by Tukey's HSD 'Seaweed vs. Control' or 'LPS vs. Control' $p < 0.05$). Data shown are average values ± SE. The standard error of the mean was calculated from $n = 10$ tanks per treatment ($n = 3$ fish per tank). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

expression of HSP70 and lysozyme in their head kidney and higher expression of C3a in their liver compared to the control fish but this was not resolved statistically (Fig. 4A–C). The fish fed the extract diet for 2 weeks appeared to have higher expression of HSP70 (+96% compared to the control) and lysozyme in their livers (+611% ± 485% compared to the control) compared to the other fish but these differences were not resolved statistically (Fig. 4D and Fig. S3).

At 4 weeks, the head kidney gene expression of the fish receiving the extract diet appeared to have the highest upregulation for the five monitored genes in that organ but it was also the most variable out of all treatments and these differences were not resolved statistically (Fig. 4B and Fig. S3). Although most of the gene expression results were not influenced by the dietary treatments or sampling week, the interleukin-1β and interferon-γ (IFN-γ) gene expression (liver, Fig. S3A, B, G and H) results both showed a strong week effect (repeated measures ANOVA, liver IFN-γ: treatments $F = 1.60$, $p = 0.212$, weeks $F = 6.35$ $p = 0.020$,

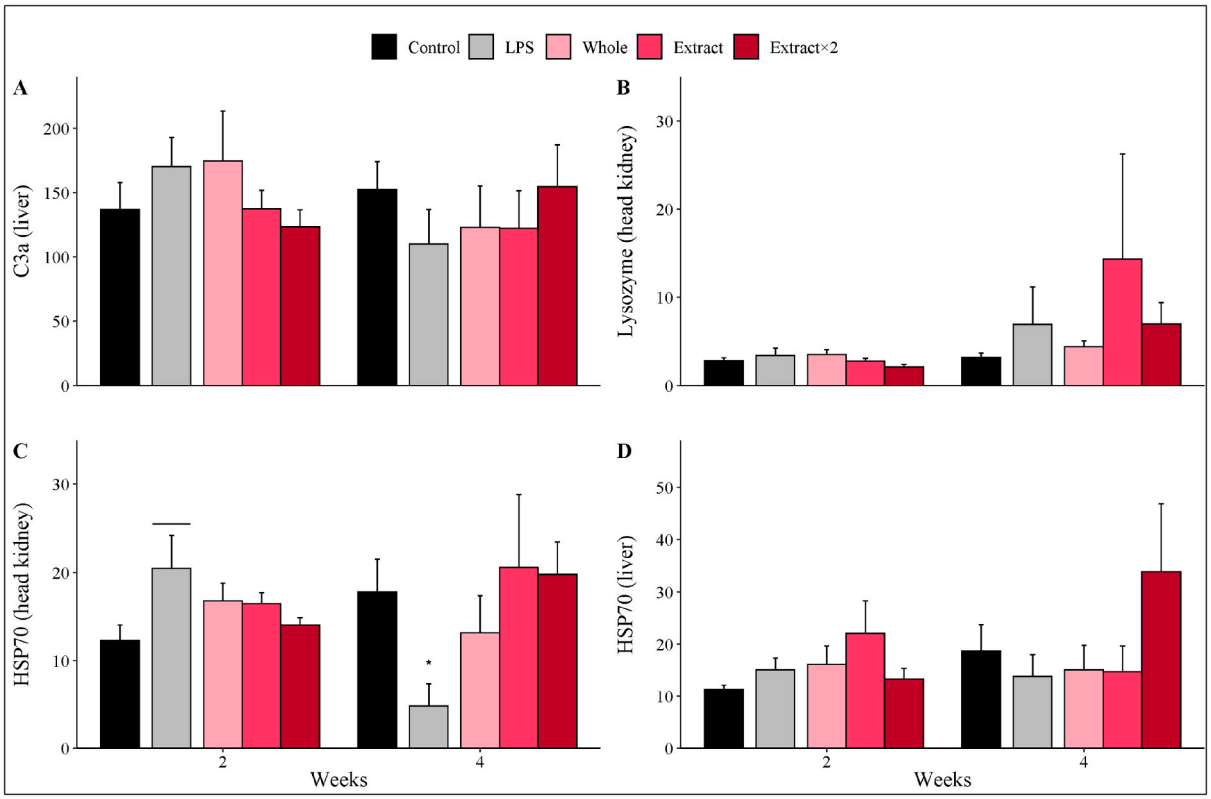

Fig. 5. Change in normalised relative expression of the 3 immune and stress-related genes in Atlantic salmon fed the control diet (black), or the diets supplemented with LPS (grey), whole seaweed (pink), seaweed extract (light red) or seaweed extract×2 (dark red) at 2 and 4 weeks in the liver and head kidney. Asterisks above individual treatment denote significant differences compared to the control ($p < 0.05$, Dunnett's test). The straight line over the LPS treatments (C) denotes significant differences compared to the control (regression model ANOVA $p < 0.05$ followed by Tukey's HSD 'Seaweed vs. Control' $p < 0.05$). Data shown are average values ± SE. The standard error of the mean was calculated from $n = 5$ tanks per treatment ($n = 3$ fish per tank). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and liver IFN-γ: treatments F = 0.93, $p = 0.464$, weeks F = 22.77 $p < 0.001$).

3.6. Effects of A. taxiformis supplementation on the hindgut microbiome of Atlantic salmon

In total, we recovered 162 ASVs and 146 ASVs after rarefaction from the hindgut of Atlantic salmon ($n = 25$) from this experiment. Out of the 162 ASVs, 17 ASVs were shared between all the fish sampled in this trial (Fig. 5A). The hindgut of the control fish and those fed the whole diet appeared to have most ASVs (92), while the hindgut of LPS and control-fed fish seemed to have the lowest ASVs (51 and 62 respectively).

Twenty-eight of the 162 ASVs represented at least 0.5% of the relative abundance of bacteria present in the hindgut of the fish across the different diets (Table S2). Overall no major differences were found for the hindgut microbial composition between the fish fed the control diet and the supplemented diets (PERMANOVA of 'Bray-Curtis dissimilarities matrix of the rarefied ASV abundance vs. dietary treatments', F = 0.67, $p = 0.943$, Fig. S4). Nonetheless, the alpha diversity was higher in fish fed diets supplemented with seaweed (ANOVA of Observed ASVs, F = 4.04, $p = 0.015$), especially in the hindgut of the fish fed the whole seaweed diet (Tukey HSD, 'whole vs. LPS', $p = 0.022$) while the diet containing LPS led to the lowest Observed ASVs in the fish's hindgut (Fig. 5B). There was a trend for increased abundance of *Proteobacteria* of the *Aeromodaceae* family in the hindgut of the fish fed the diets supplemented with seaweed, especially for the extract-fed fish (Table S2 and Fig. S5), although this was not significant. From this family (*Aeromodaceae*), eight ASVs (out of 28 ASVs with relative abundance >0.5%) were *Aeromonas* spp. and represented between 37% (control-fed fish) and 71% (whole seaweed-fed fish) of the total relative abundance of bacteria in the hindgut of the Atlantic salmon in this trial (Table S2 and Fig. S5).

Furthermore, the four supplemented diets each had between 29 and 54 ASVs that were differentially abundant between the hindgut of the fish fed those treatment diets compared to the fish fed the control diet (Table S3, $p < 0.05$). The hindgut of the fish fed the whole seaweed diet had the highest number of ASVs that were differentially abundant compared with that of the control fish (54 ASVs) while the LPS-fed fish had the lowest (29ASVs; Table S3). Only eleven of these ASVs were above 0.5% relative abundance and were significantly differentially abundant in the fish fed the supplemented diets compared to the control (Table S3). Out of these, two were different ASVs of the genus *Acinetobacter* sp. (ASV101 and ASV169), with ASV101 being less abundant in the hindgut of the fish fed the LPS diet (ASV101, absent), while ASV169 was more abundant in the hindgut of the fish fed the whole diet (1.2% relative abundance) and those fed the extract×2 diet (3.2% relative abundance) compared to the hindgut of the control fish (DESEQ, vs. control adjusted $p < 0.001$; Table S3). The genus *Streptococcus* sp. (ASV45 and ASV114) was lowest in the hindgut of the fish fed the three seaweed diets compared to the control fish with ASV45 being significantly lower in the hindgut of the fish fed the whole diet and the LPS diet while ASV114 was significantly lower in the hindgut of the fish fed the extract×2 diet (DESEQ, vs. control adjusted $p < 0.001$). ASV354, a *Pseudomonas* sp., was lower in the hindgut of the fish fed the seaweed diets (ranging from 0% to 0.03% relative abundance) compared to that of the fish fed the control (3.1% relative abundance) and that of the fish fed the LPS diet (1.1% relative abundance), however only the hindgut of the fish fed the extract×2 diet had a significantly lower abundance of ASV354 (DESEQ, vs. control adjusted $p < 0.001$; Table S3). ASV252 (*Escherichia/Shigella* sp.), ASV316 (*Arcicela* sp.), ASV381 (*Sphaerotilus* sp.), ASV385 (*Aquabacterium* sp.) and ASV21 (an unassigned genus of the family *Moraxellaceae*), were also significantly more abundant in the hindgut of the fish fed the control diet while absent in the hindgut of the fish fed the whole diet and lower in fish fed the two extract diets (Table S3). ASV16 (*Shewanella* sp.) was absent in the hindgut of the LPS-fed fish (DESEQ, vs control adjusted $p < 0.001$) yet was the most abundant ASV in the hindgut of the fish fed the whole seaweed diet (2.6% relative abundance) compared to the hindgut of the fish in the other treatments (Table S2 and S3).

3.7. Correlation between innate immunity, growth, microbiome and gene expression parameters

No significant differences were found between the different treatments when all the 2-week variables were included in a non-metric multidimensional scaling (nMDS) (PERMANOVA, F = 1.06, $p = 0.366$) and the fish from the different treatments did not cluster in any defined way in the nMDS (Fig. 6A). Although the growth and feed efficiency results of week 2 (Fig. S6) did not correlate with the other measurements, significant correlations were found between many of the different variables of the week 2 sampling event from the immunochemistry, gene expression and ASVs. The head kidney lysozyme gene expression closely aligned with ASV390 (*Acinetobacter* sp.), and to a lesser extent C3 gene expression but had opposite loading to the serum lysozyme (Fig. 6B, Table S4). The serum lysozyme positively correlated to some extent with ASV77, which was more abundant in the fish fed the LPS diet (Fig. 6B, Table S4). Two ASVs (ASV228 and ASV161) positively

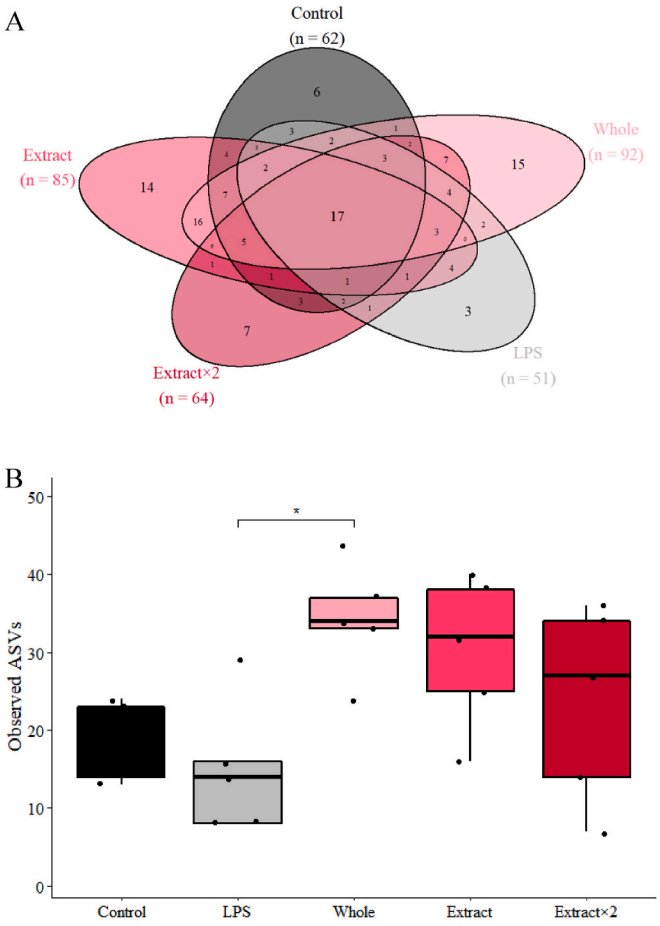

Fig. 6. (A) Shared and unique ASVs and (B) Observed ASVs from the hindgut of Atlantic salmon fed the control diet (black) or diets supplemented with LPS (grey), whole seaweed (pink), seaweed extract (light red) and seaweed extract×2 (dark red). The standard error of the mean was calculated from $n = 5$ tanks per treatment (comprised of $n = 3$ fish per tank). The bracket and asterisk denotes significant differences between observed ASVs from fish fed the LPS and whole seaweed diets (one-way ANOVA of 'observed ASVs vs. dietary treatments' followed by Tukey's HSD *post-hoc* test). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

correlated (loading on the nMDS1 axis) and were the two most abundant ASVs in the hindgut of the fish fed the seaweed diets. ASV355 and ASV95 were also more abundant in the hindgut of the fish fed the seaweed diets and positively correlated with the Shannon index (Fig. 6B). On the other hand, ASV192 and ASV83 were more abundant in the hindgut of the control and positively correlated (both negatively loaded on the nMDS1 axis) (Fig. 7B).

4. Discussion

This study comprehensively examined the dietary effects of the red seaweed *Asparagopsis taxiformis* on Atlantic salmon production, immunochemistry, gene expression and gut microbial community. We show for the first time that dietary supplementation of Atlantic salmon parr with whole *A. taxiformis* or its extract boosted fish growth and enhanced feed intake without compromising feed conversion. The seaweed also positively influenced the innate immune response as well as gene expression of immune and stress-related genes, and enhanced the diversity of their hindgut microbiomes through increases in potentially beneficial microbes and lower abundances of potential pathogens. The mechanisms driving the observed changes remain unclear but they are likely multifactorial and at times counterintuitive (e.g. gene expression of lysozyme negatively correlated to serum lysozyme level). Overall, these findings are important as they highlight opportunities to refine the functional ingredients of *Asparagopsis* to maximise the benefits of the immune response knowing that there was no trade off with feed conversion and a positive influence on growth over the four-week trial.

Supplementing diets with *A. taxiformis* significantly increased the growth and improved the feed intake of Atlantic salmon. This was particularly obvious in fish fed the seaweed extract×2 diet (double dose at 1.2% feed inclusion), for which fish were 11% heavier and consumed 17% more feed than the control fish after 4 weeks. In a previous study, we fed the extract ×2 diet to the mottled rabbitfish *Siganus fuscescens* and found that growth after 4 weeks increased by 17% more than their counterparts fed the control diet (Thépot et al., in press). Similar effects of the whole seaweed seaweed were observed for the growth of the orbiculate batfish *Platax orbicularis* by Reverter et al. (2016), who reported a significant increase in fish weight of similar magnitude (~14%) and an increase in fish appetite in a three-week trial. In our study the feed intake of the fish fed the seaweed diets was 15% higher than for those fed the control diet. Taken together with an effectively lower FCR in the seaweed treatments, we conclude that the *A. taxiformis* supplements likely act as both feeding and growth stimulants. Paradoxically, it is possible that *Asparagopsis* species, which produce potent secondary metabolites capable of deterring herbivores (Paul et al., 2006), could also produce compounds that stimulate feeding by Atlantic salmon. Increased feed intake associated with diets incorporating the brown seaweed *Laminaria* sp. has previously been reported for Atlantic salmon (Kamunde et al., 2019). It is generally assumed that an increase in feed intake would be the most important factor driving an increase in fish weight, however, we found no correlation between feed intake and relative weight gain, suggesting that there are some growth promoting effects of *A. taxiformis* on *S. salar* which require further investigation.

Microbial modulation is one potential factor in the increase in growth of Atlantic salmon fed *A. taxiformis* as the natural products have demonstrated antimicrobial activity against aquaculture pathogens (Manilal et al., 2009; Paul et al., 2006). The gram-positive bacteria *Streptococcus* spp. (ASV45 and 114) and the gram-negative *Pseudomonas* sp. (one ASV354) are two potential pathogens in lower abundance in the hindgut of the Atlantic salmon fed the seaweed diets compared to those receiving the control diet. Furthermore, another *Pseudomonas* sp. (ASV274) was more abundant in the hindgut of the fish fed the seaweed and LPS diets compared to those receiving the control diet. These changes to the gastrointestinal microbiome in the seaweed fed Atlantic salmon may produce positive production and immune outcomes. Although the mechanisms involved are poorly understood, the growth promoting effect of antibiotics are well known for livestock, with 4–8% improvements in antibiotic-fed animals (e.g. chicken; Butaye et al., 2003). Recently, inclusion of *A. taxiformis* was shown to drastically reduce the methane emissions of cattle through significant reductions in the abundance of methanogenic archaea (Machado et al., 2016). The previously lost energy in the form of methane was purportedly then

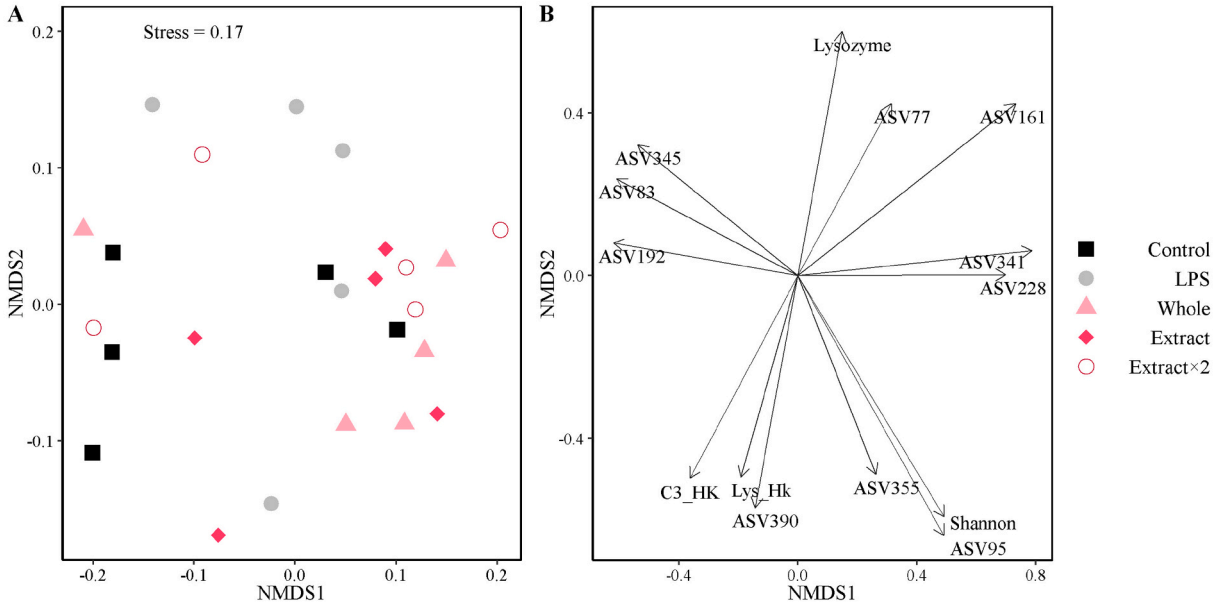

Fig. 7. Non-metric multidimentional scaling (nMDS) plot (Bray-Curtis dissimilarity matrix) of the four innate immune parameters (lysozyme activity, phagocytic activity, haemolytic activity and respiratory burst activity), the four growth/feed variables and health idicators (relative weight gain, FCR, feed intake, glucose), the five immune/stress related gene expressions for both the head kidney and liver and the top 11 most abundant ASVs (>80% relative abundance on average across all fish) at the 2 week sampling point plotted as (A) the individual fish fed the different dietary treatments and (B) the original variables loaded as vectors in nMDS space (with loading >0.7). The different colours represent the control diet (black), LPS diet (grey), whole seaweed diet (pink), seaweed extract diet (light red) and the seaweed extract×2 diet (dark red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

available for the ruminants, which grew 22–26% more than steers on a control diet (Kinley et al., 2020), a result similar in magnitude to what we report for Atlantic salmon. Since fish are not recognised as major methane emitters, it is not clear which – if any – of these antimicrobial interactions are at play for Atlantic salmon fed *A. taxiformis*.

An alternative microbially-mediated effect is the possibility that growth-promoting gut microbes are fostered by the inclusion of seaweed in the diet. We found *Shewanella* sp. (ASV16) more abundant in fish fed the seaweed diets compared to those fed the control diet, yet was also absent in the LPS-fed fish. *Shewanella* spp. include strains that have successfully been used as probiotics to improve the growth and immune response of fish (Guzmán-Villanueva et al., 2014; Lobo, et al., 2014). In addition, *Areomonas* spp., the most abundant genus in the salmon fed the seaweed-supplemented diets in this trial (Fig. S5B), includes both fish pathogens (Reith et al., 2008) and beneficial bacteria shown to have postivie effects on the immune response of fish (Brunt and Austin, 2005; Irianto and Austin, 2002). Both *Shewanella* spp. and *Aeromonas* spp. are "broad range metabolisers" (Harris et al., 2018; van der Kooij, 1991) and were in higher abundance in the gut of Atlantic salmon parr fed increased level of carbohydrates in another study (Egerton et al., 2020). We found no clear pairwise correlations between the abundance of any single ASV and fish growth or immune response. For example, those LPS-fed fish with the greatest improvement in immunity had lower abundance of both *Aeromonas* spp. and *Shewanella* spp. compared to fish fed seaweed. These results indicate that deeper exploration and empirical tests are required to elucidate the potential mechanisms, particularly for bacterial taxa that hold promise for development of probiotics (i.e. *Shewanella* sp. and *Aeromonas* sp.).

Given the lack of clarity on microbially mediated interactions, it appears that the mode of action for the positive immune and production effects of *A. taxiformis* is a direct effect on fish metabolism. This direct effect also appears to be relatively specific as supplementation of Atlantic salmon diets with *A. taxiformis* (whole or extract) had positive effects on some, but not all, innate immune parameters. After 4 weeks, the lysozyme activity of the fish feeding on the *A. taxiformis* supplemented diets was higher than that of the control fish or those fed the LPS diet, although lysozyme values across all treatments were lower in week 4 compared to week 2. These findings, and those from previous studies (Amphan et al., 2019; Elbesthi et al., 2020; Liu et al., 2012), highlight the variability in immunochemistry variables under different settings and the limitations of using immunochemistry as proxies for immune response compared to pathogen challenges (Thépot et al., 2021a). Our innate immune data for Atlantic salmon build on the body of work emerging on the inclusion of *Asparagopsis taxiformis* with different fish species, including the dramatic increases in the innate immune response for haemolytic activity of the mottled rabbitfish (*Siganus fuscescens*), which increased by 4-fold compared to the control and 14 other dietary supplements (Thépot et al., 2021b). Another similarity between the current salmon trial and the rabbitfish trial is a reduction in serum glucose levels, a secondary marker of stress in fish (Fazio et al., 2015). Taken together, the reduction in serum glucose level and the increase in innate immune responses in Atlantic salmon fed *A. taxiformis* provide evidence that this seaweed could bolster fish performance against pathogens. However, further research is necessary to adance our understanding of the relationship between the immunology responses seen in this study and gene expression including the selection of other immune and stress related genes in addition to those investigated here.

The use of molecular markers to understand the gene expression behind observed innate immune responses could be a powerful tool but is one that is not yet realised for the influence of *A. taxiformis* in the salmon as we did not observe any correlation between gene expression (e.g. of lysozyme gene) and in situ markers (e.g. serum lysozyme activity). This contrasts with findings from the addition of *A. taxiformis* to the feed of orbicular batfish, which led to a significant increase in the expression of immune-related genes (lysozyme and transforming growth factor) after 3 weeks with diets supplemented with the seaweed at 1.5% and 3% *W*/W (whole; Reverter et al., 2016). The authors postulated that the improvements in lysozyme gene expression would translate to improved immune response. In our study, the gene expression for lysozyme did not differ between the different diets, even though the serum lysozyme activity of the fish fed the *A. taxiformis* supplements was significantly higher than the control diet after 4 weeks. Whilst the investigation of immune-related genes in fish as a response to dietary immunostimulants provides a new angle to evaluate fish responses to novel ingredients, the increase in expression at the gene level did not translate in an increase in the protein that gene is coding for (e.g. lysozyme). In fact, the relationship between mRNA transcripts and protein abundance is often quite low with, for example, ~30–40% of the variance in protein abundance being explained by mRNA abundance in mammals (Vogel and Marcotte, 2012). Therefore, we recommend complementing gene expression assays with in situ measurements wherever possible. Furthermore, when testing a novel ingrediets with unknown effects on fish, transcriptomics could provide a more holistic evaluation on gene expression than nominating individual target genes. The lack of PCR product for some of the chosen gene/organ combinations (e.g. head kidney IFN-γ) is evidence that a more holistic approach is required when the concequences of specific dietary additives are unknown.

The present study highlights considerable variation in the reponse of the different immune-related and microbial variables to fish diets including *A. taxiformis* and its extracts. This variation included clear but inexplicable temporal variation in multiple variables across two time points in the 4 week experimental period. Clearly the time of exposure needs to be considered when using potent functional ingredients, as well as the dose of immunostimulants, as there can be immunosuppression due to overexposure to the supplement (Sakai, 1999). For example, immunostimulants can have a strong effect at one time point with abrupt changes later in the experimental period (Yoshida et al., 1995) and other immunostimulants cause detrimental effects at high doses (Robertsen, 1994), including LPS which led to a significant drop in lysozyme activity in catfish after 6 weeks (Bich Hang et al., 2016). Regardless of well documented variation in immune responses, we found that the most consistent results of *A. taxiformis* inclusion were the positive impacts on growth and feed efficiencies. Here, it is possible that the halogenated natural products contained in the seaweed extract directly influenced the metabolism of the fish. Seaweed and plant bioactives have "growth factor" properties (Chakraborty et al., 2014; Virgili and Marino, 2008) as well as antioxidant and nutrient protection properties (e.g. reduced amino acid degradation from decaboxylation; Kosina et al., 2004). Single compounds isolated from plants can increase the white-muscle weight of rainbow trout, *Onchorhynchus mykiss*, presumably due to an anabolic effect (Fernández-Navarro et al., 2008). The diverse bioactive natural products in the extracts of *A. taxiformis* likely include compounds that produced the clear growth effects and compounds that produced the less consistent immune responses. Potentially, these effects may be due to the same compound. Subsequent research should identify and purify *A. taxiformis* bioactives to evaluate the direct and indirect effects of these compounds on fish production. Additionally, on-farm trials of the *A. taxiformis* feed additive should be conducted on a larger scale and for a longer period to demonstrate the commercial potential for *S. salar* and other species of farmed fish.

5. Conclusions

Dietary supplementation of Atlantic salmon parr with *Asparagopsis taxiformis* enhanced growth rates, feed intake and boosted aspects of their innate immune response without any negative impact on the feed conversion ratio. This last point is crucial to the uptake of novel ingredients, with a goal to displace as little of the feed as possible and to maximise the benefit of the feed additive, which in the present study tended to improve feed conversion compared to the control diet. The *A. taxiformis* feed supplements also enhanced the diversity of fish gut

microbiomes, increased the abundance of potentially beneficial bacteria and decreased the abundance of potentially pathenogenic ones highlighting other potential benefits of seaweed supplements in fish production. While we acknowledge the considerable variation in the findings between and among the variables examined, the isolation and purification of *A. taxiformis* bioactive compounds could potentially improve the outcome and help our mechanistic understanding of the immune and production responses. The ultimate goal of refining the seaweed extracts is to minimise the feed displacement and improve feed conversion ratio, particularly over the full production cycle where small differences in inclusion rate can have compounded effects. The lack of clear differences in fish response between forms of seaweed supplements (whole seaweed and methanolic extracts) enables a flexible approach for industry to incorporate *A. taxiformis* as a feed supplement for Atlantic salmon to improve growth rates and fish welfare. Consideration of which product to commercialise can also be given to consumer versus business angles, e.g. seaweed extracts from natural sources versus synthetic ingredients. The ideal feed supplement or combination of supplements is one that can both promote immune response together with improved production performance, which we have demonstrated is possible when using *A. taxiformis* as a supplement. Future efforts may find synergistic value in the combination of *A. taxiformis* with a commercial immunostimulant (Thépot et al., 2021a).

Authors' contributions

V.T., A.C., M.R., M.J., C.J., B.E. and N.P. co-designed the experiment. V.T. collected the data, performed laboratory assays, statistical analyses, generated figures for the manuscript and wrote the initial draft. V.T. and M.J. performed laboratory work relating to the gene expression. N.P. provided additional help with statistical analysis. A.C. provided guidance with the gastrointestinal microbiome analsyses. All authors contributed to writing the publication and all gave final approval for publication.

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Declaration of Competing Interest

The authors acknowledge that the results from this study are part of two patent applications (publication number PCT/AU2020/051354 and PCT/AU2019/051427).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2021.737286.

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