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Seaweed dietary supplements enhance the innate immune response of the mottled rabbitfish, *Siganus fuscescens*

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ABSTRACT

Disease is one of the major bottlenecks for aquaculture development, costing the industry in excess of US $6 billion each year. The increase in pressure to phase out some traditional approaches to disease control (e.g. antibiotics) is pushing farmers to search for alternatives to treat and prevent disease outbreaks, which do not have detrimental consequences (e.g. antibiotic resistance). We tested the effects of eleven seaweed species and four established fish immunostimulants on the innate immune response (cellular and humoral immunity) of the rabbitfish *Siganus fuscescens*. All supplements including different seaweeds from the three groups (Chlorophyta, Phaeophyta and Rhodophyta) were included in the fish pellet at 3% (by weight) and had variably positive effects across the four innate immune parameters we measured compared to control fish. Diets supplemented with the red seaweed *Asparagopsis taxiformis* and the brown seaweed *Dictyota intermedia* led to the largest boosts in humoral and cellular innate immune defences, including particularly significant increases in haemolytic activity. Diets supplemented with *Ulva fasciata* also led to promising positive effects on the fish innate immune responses. We conclude that dietary seaweed supplements can boost the immune response of *S. fuscescens* and thus the top three species highlighted in this study should be further investigated for this emerging aquaculture species and other fish species.

1. Introduction

Aquaculture now produces more than 50% of the seafood consumed globally, but disease outbreaks pose persistent threats to its further development and cost this global industry more than US $6 billion every year [1]. Traditional responses to disease outbreaks have often involved the use of high volumes of veterinary drugs delivered as prophylactics or to treat particular pathogens [2]. However the use of such drugs in aquaculture settings can cause environmental damage and create public health concerns by infiltrating the human food chain, either directly via the consumption of treated cultured fish or the consumption of wild-caught fish or shellfish within the vicinity of the treated farm [3,4]. Furthermore, as has occurred in other animal husbandry sectors, the heavy use of antibiotics in aquaculture is inevitably leading to an increase in antibiotic-resistant bacteria in marine environments and human food products [5]. This not only impairs the effectiveness of antibiotics in aquaculture but also increases the likelihood of passage of antibiotic-resistant pathogenic or non-pathogenic bacteria to aquatic [6] and terrestrial animals including humans [7].

There is now strong societal and consumer pressure to find alternatives to antibiotic treatments that prevent or reduce the impacts of disease in aquaculture without impacting fish health, productivity or quality [8]. These alternatives include prebiotics, probiotics and immunostimulants (e.g. phytochemicals). Probiotics are live bacteria that are ingested and become part of the gastrointestinal (GI) microbiomes of the fed host. As well as colonising the gut, probiotics may also elicit an immune response from the host [9]. Prebiotics, or non-digestible fibres, are complex polysaccharides that stimulate the growth of beneficial bacteria within the host GI tract [10]. Furthermore, intestinal epithelial cells can recognise prebiotics as foreign bodies (pathogen associated molecular patterns; PAMPs), thus eliciting an immune response [11]. Both probiotics and prebiotics can improve the gut- and overall health of animals, thereby increasing their resistance to disease. Dietary 'immunostimulants' include any ingredient (synthetic, natural product or living organism) that boosts the immune system of an organism. Terrestrial and aquatic plants (including algae), and their chemical derivatives called phytochemicals, have been widely used as alternative medicine for humans for thousands of years to help treat or

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prevent common diseases [12]. The potential use of plants in aquaculture as dietary immunostimulant both commercially and at the research level as an alternative to antibiotics was amplified by legislation introduced in the European Union to ban the use of subtherapeutic antibiotics in animal faming [13]. The effect of plant based dietary supplement is not limited to the boosting of the fish innate system but can also promote fish growth, reduce stress and improve the fish resistance to pathogens [14,15]. More recently, within this large group of plant immunostimulants, seaweeds are receiving increased attention in animal studies due to their diverse taxonomy (>10,000 species), their biosynthesis of a broad range of unique bioactive compounds and the complex polysaccharides they contain, which can function as strong prebiotics [16,17].

Ironically, the testing of seaweed-based immunostimulants has been predominantly performed on animals that would not naturally eat or even encounter seaweed [e.g. sheep, cattle, pig, chicken and human; [18–25]]. Despite this, the tested dietary seaweed supplements have been shown to have positive immunostimulatory effects in land animals. We understand less about the immunostimulatory effects of seaweed on fish but a recent review by Thépot et al. [17] showed that seaweeds do have overall positive effects on fish immune responses. This review also highlighted that what we do know about the immunostimulatory potential of seaweeds for farmed fish comes mostly from carnivorous species which again, are not known to consume seaweeds naturally.

It is possible that seaweed dietary supplements would have a less obvious effect on the immune response of marine herbivores who are accustomed to its inclusion in their normal diets. On the other hand, because seaweed is a major part of the natural diet of marine herbivorous fish, its presence in aquafeed might yield substantial benefits to farmed marine herbivorous fish, which despite being underrepresented in this particular research area, are the most commonly farmed species globally [26]. Indeed, only two studies have investigated the effect of seaweed (two species of *Ulva* sp. and one of *Gracilaria* sp.) in a marine herbivorous fish, the rabbitfish *Siganus canaliculatus*, with both identifying potential for immune stimulation [27,28]. Rabbitfish (*Siganus* spp.) production mainly comes from wild fisheries with 130,000 tonnes landed in 2018 of which 107,000 tonnes were caught in Indonesia and the Philippines [29]. Although the farming of *Siganus* spp. is in its infancy (with only 250 tonnes produced annually worldwide [30]), this species is an attractive sustainable aquaculture candidate because of its low trophic-level and ability to produce long chain polyunsaturated fatty acids *de novo* (omega-3s; [31]). Because only three seaweed species have been explored (out of >10,000 species) in this marine herbivore and because different seaweed species will produce vastly different natural products, there is a need to test more seaweeds as functional ingredients for this fish [27,28]. For these reasons, we aimed to investigate the immune-boosting potential of 11 different seaweed species from within the Chlorophyta, Phaeophyta and Rhodophyta, when provided as dietary supplements to the mottled rabbitfish, *Siganus fuscescens*, on both humoral and cellular innate immune parameters. For comparison, we also used four existing immunostimulant supplements as positive controls, comprising two commercial immunostimulants (Hilyses® and sodium alginate) as well as two documented algal immunostimulants (astaxanthin and spirulina).

2. Material and methods

The study was conducted at the Bribie Island Research Centre (BIRC) on Bribie Island, Queensland, Australia (27°03′15.9″S 153°11′42.9″E) under the University of the Sunshine Coast Animal Ethics Approval ANS1751. The fish, *S. fuscescens*, used in the experiment were collected under the general fisheries permit (permit number 195305) issued by the Queensland Department of Agriculture and Fisheries (Fisheries Act 1994). Note that *S. canaliculatus* and *S. fuscescens* are regarded by some [32,33] as colour morphs of the same species, but for simplicity we have retained the two names.

2.1. Seaweed and experimental diets

The origin, treatment and processing methodology for all eleven seaweed species used in this study are summarised in Table 1. For the preparation of seaweed-supplemented diets, fresh seaweeds were rinsed with saltwater (34.5 ppt) to remove sand and biological contaminants. They were then spun in a washing machine (Fisher & Paykel 5.5 kg Quick Smart) on spin cycle (1,000 rpm) for 5 min to remove excess water, frozen at −80 °C, and then lyophilized in a freeze dryer (Thermo Savant model MODULYOD-230) for 3 days at approximately −44 °C and 206 mbar. Once dried, each seaweed species was vacuum sealed in individual bags with silica desiccant and stored at −20 °C until used for the experimental diets. Initial identification of the different seaweed species was done using morphological characters and were subsequently confirmed, where possible, using DNA analytic methods by Dr. Zuccarello at the Victoria University of Wellington following methodology described in Zuccarello and Paul [34].

As comparisons to the seaweed treatments, four 'positive controls' (existing immunostimulatory products currently used in the aquaculture industry and research) were also included in the feeding trials. These products were Hilyses® (MarSyt Inc), a hydrolysed yeast culture derived from the sugarcane fermentation process (and a source of β-glucans), sodium alginate, the anionic polysaccharide extracted from brown seaweeds, the cyanobacteria *Arthrospira platensis* (high strength organic spirulina, Swiss Wellness Pty Ltd, hereafter referred to as "spirulina") and the microalga *Haematococcus pluvialis*, which is rich in astaxanthin (Pacific Biotechnologies Pty Ltd).

All the experimental diets were combined with the commercial aquaculture feed product (hereafter 'aquafeed') 'Native' (Ridley Aquafeeds Ltd). The pellets (1.5 kg for each experimental diet) were powdered then added to a blender (Hobart A120) together with deionised water (30% weight/weight) and the seaweed or positive control supplement which had been powdered and sieved through a 300 μm mesh. The ingredients were combined for approximately 10 min at low speed (agitator rpm of 104) using a dough hook to produce a stiff dough. The dough was extruded through a 4 mm die onto trays which were then placed in a fan-forced oven overnight at 50 °C. Once dried, the feed was packaged in airtight bags and stored at 4 °C until required and for the duration of the trial. The control diet was produced using the same procedure, but without the inclusion of any supplement.

2.2. Experimental design

The rabbitfish, *S. fuscescens*, were captured using a drag net (15 m long by 2.1 m deep with a 2.5 cm mesh size) at Moffat Beach, Queensland Australia (26°47′21.7″S 153°08′36.0″E) from rocky reefs adjacent to the beach and transferred to the BIRC in an oxygenated 500 L tank. Once at BIRC, they received a hydrogen peroxide bath (200 mg/L for 30 min) to rid them of potential external pathogens and parasites. After treatment, the fish were transferred to three 1000 L fibreglass tanks where they were acclimatised and fed the control diet for two weeks. Following acclimation, each fish (N = 144) was classified into a size class, (small, medium and large; n = 48), for which the initial fish weight (mean ± SE) was 85.83 ± 7.85 g, 112.60 ± 8.17 g and 150.59 ± 14.59 g respectively. Fish were allocated into one of 48 plastic tanks (55 L) to a density of 3 fish per tank such that one fish from each size group was in each tank and each tank contained one small, one medium and one large fish. The *Ulva* dietary treatment comprised only 2 replicate tanks after the loss of one tank due to water and air supply issues.

Diets were hand fed at 3% body weight twice a day (10:00 a.m. and 3:00 p.m.). During the trials, water temperature was maintained at 27 °C and pH in a range of 7.9–8.1. The system was operated as flow-through using seawater pumped from approximately 300 m off the beach adjacent to the station. The influent seawater was physically filtered to <10–15 μm, sterilised with ozone, and then treated with ultraviolet filtration and activated carbon to remove any residual products of the

Table 1
Physical and chemical attributes, pre-inclusion processing method, collection site, and proximate composition of the different supplements used in this study.

Functional ingredient	Morphology	Chemical attribute	Processing	Collection site	Lipid	Protein	Carbohydrate	Ash	Moisture
Aquafeed supplements									
Sodium alginate	The sodium salt of alginic acid which is extracted from the cell wall of brown algae	β-1,4-D-ManA, or M blocks, α-1,4-L-GulA, or G blocks	Dried (unreported method)	Commercial supplier	0%	0%	75%	16%	9%
Hilyses®	Hydrolysed yeast (*Saccharomyces cerevisiae*)	β-glucans and mannan oligosaccharides	Dried (unreported method)	Commercial animal feed supplier	1%	30%	48%	15%	5%
Spirulina (*Arthrospira platensis*)	Marine filamentous blue-green algae	Phenolic and flavonoid compounds	Unknown	Pharmacy	9%	52%	22%	13%	5%
Haematococcus pluvialis	Unicellular, spherical cysts	Astaxanthin, phenolic compounds	Refractance window dried	Commercial algae producer (19°28′39.9″S 147°28′24.3″E)	37%	10%	35%	8%	11%
Seaweed									
Chlorophyta									
Caulerpa taxifolia	Soft, fleshy	Caulerpin, flavonoids, terpenoids, alkaloids and phenols	Freeze dried	Pt. Cartwright, QLD, Australia (26°40′53.4″S 153°08′19.9″E)	5%	12%	48%	31%	4%
Halimeda sp.	Hard, calcifying	Phenolic compounds, diterpenes, Halimeda tetraacetate and halimedatrial	Freeze dried	Moffat Beach, QLD, Australia (26°47′21.8″S 153°08′35.0″E)	5%	11%	34%	45%	4%
Ulva fasciata	Single cell thick blade	Ulvans, phenols and flavonoids	Freeze dried	Cultured by the USC Seaweed Research Group(27°03′14.1″S 153°11′39.8″E)	2%	7%	56%	34%	2%
Rhodophyta									
Asparagopsis taxiformis	Soft, fleshy	Haloketones, haloalkanes, halomethanes (bromoform) and haloacids	Freeze dried	Moffat Beach, QLD, Australia (26°47′21.8″S 153°08′35.0″E)	3%	16%	23%	56%	2%
Hydropuntia perplexa	Soft fleshy, branching	Agar, chlorophyll *A*, carotenoids, phycobilins, halogenated compounds and polyphenol compounds	Freeze dried	Coolum Beach, QLD, Australia (26°32′22.9″S 153°05′51.7″E)	1%	8%	39%	49%	2%
Kappaphycus alvarezii	Rigid, branching	Carrageenan, flavonoid and polyphenol compounds	Sun dried	Gunu village, Yasawa Islands, Fiji (17°04′38.3″S 177°14′22.0″E)	1%	0%	27%	67%	5%
Laurencia obtusa	Fleshy, turgid	C_{15}-acetogenins, sesquiterpenes and diterpenes	Freeze dried	Moffat Beach, QLD, Australia (26°47′21.8″S 153°08′35.0″E)	6%	9%	43%	42%	1%
Sarconema filiforme	Soft fleshy, branching	Sulphated polysaccharides, secondary metabolite	Freeze dried	Cultured by the USC Seaweed Research Group (27°03′14.1″S 153°11′39.8″E)	3%	14%	28%	53%	2%
Phaeophyta									
Dictyota intermedia	Fine, dichotomously branching	Fucoidan, terpenoid compounds (dictyterpenoids)	Freeze dried	Coolum Beach, QLD, Australia (26°32′22.9″S 153°05′51.7″E)	8%	6%	45%	36%	5%
Lobophora nigrescens	Tough, leathery, thallus	Fucoidan, sulfoquinovosyldiacylglycerol, phenolic compounds	Freeze dried	Moffat Beach, QLD, Australia (26°47′21.8″S 153°08′35.0″E)	2%	6%	61%	30%	1%
Sargassum spinuligerum	Fleshy, turgid	Fucoidan, tannins, saponins, sterols, polyphenols, steroids and terpenoid compounds	Freeze dried	Pt. Cartwright (26°40′53.4″S 153°08′19.9″E) and Coolum Beach (26°32′22.9″S 153°05′51.7″E), QLD, Australia	3%	5%	49%	37%	6%

sterilisation procedure. The treated seawater was pumped to a header tank, which fed directly into a pipe system delivering treated seawater to this experiment. The system was in a temperature and background light controlled room kept at 24–26 °C and on a 24 L:0D light regime.

2.3. Sample collection and preparation

Experimental fish received their allocated diets for 14 days, after which they were starved for 24 h. Then the fish from one replicate tank per treatment were harvested each day over a three day period (as

described above). Blood samples were collected from the caudal vein of all the fish in each replicate tank using a 1 mL syringe and a 29G needle. The samples were immediately transferred to two Eppendorf tubes: one with and one without heparin. The tube with heparin was stored at 4 °C until analysed (<24 h). The tube without heparin was allowed to clot for 1 h at room temperature then 8 h at 4 °C and subsequently centrifuged at 1500 g for 5 min at 4 °C. The separated serum was then collected, aliquoted into 1.5 mL Eppendorf tubes and stored at −80 °C until analysed. The heparinised blood samples were analysed the same day for cell counts. Each fish was then weighed and its liver dissected out to calculate the hepatosomatic index as part of the baseline health measurements (HSI; see equation below).

2.4. Baseline health and glucose measurement

To confirm that the dietary supplements did not produce detrimental health side effect baseline health and glucose measurements were performed. The tubes containing the heparinised blood were inverted gently and diluted 1:500 in phosphate buffered saline (137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, pH 7.4) containing 1% of formalin (37%). The diluted samples were then gently inverted and 10 μL was transferred to a Neubauer improved cell counting chamber to enumerate erythrocytes and leukocytes using a light microscope (×100; Nikon Eclipse E200).

The number of cells per μL of blood for each replicate fish was calculated as the average of three counts from the same sample tube to minimise the potential for error associated with mixing and loading the samples on the Neubauer improved cell counting chamber, as follows:

Cell/μL = (number of cells × dilution factor (500))/volume of haemocytometer (0.1 μL).

The plasma concentration of glucose was measured using a blood glucose meter (Accu-check, Performa) with Accu-check test strips code 222 and a 2 μL serum sample.

The hepatosomatic index was determined using the following equation:

Hepatosomatic index (HSI) = 100 × (weight of the liver (g)/weight of the fish (g))

2.5. Immunological assays – humoral innate immune response

2.5.1. Haemolytic activity of the alternative complement pathway

The haemolytic activity of the alternative complement pathway (ACH50) was determined as the method described by Sunyer and Tort [35]. 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 EGTA (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% to 10.00%. However, some samples were over the desired range for accurate measurement so they required further dilution. In this instance the four-fold serial dilution went from 0.005% to 5%. Subsequently, 20 μL of R-RBC suspension was added to each tube and incubated for 100 min at 27 °C with occasional shaking. Three replicate negative controls 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. Then 50 μL of supernatant of each dilution was transferred to a new microtiter plate and read at 540 nm. The degree of haemolysis was calculated by dividing the corrected absorbance 540 nm value by the 100% haemolysis control. The volume of serum that gave 50% haemolysis was used for calculating the ACH50 using the following formula:

ACH50 (units/ml) = 1/K

Where K is the amount of serum giving 50% haemolysis.

2.5.2. Serum lysozyme activity

Serum lysozyme activity was determined using the turbidimetric assay, which gives a direct measure of lysozyme activity [36]. Briefly, lyophilized *Micrococcus lysodeikticus* (75 mg) was rehydrated and suspended in 100 mL of buffer (0.05 M Na_2HPO_4, 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 freshly thawed serum sample. The plates were then shaken and absorbance was measured at 450 nm at 0.5 min and 4.5 min. One unit of lysozyme was defined as a decrease of 0.001 in absorbance over that period.

2.6. Immunological assays – cellular innate immune response

2.6.1. Respiratory burst activity

The production of reactive oxygen species by leukocytes was measured using nitrotetrazolium blue chloride (NBT, Sigma) following the method from Secombes [37] and subsequently modified by Stasiack and Bauman [38]. Briefly, 50 μL of blood samples were loaded in 'flat bottom' microtiter plates and incubated at 27 °C for 1 h to allow adhesion of cells. The supernatant was decanted and the wells were washed thrice with PBS. Fifty μL of 0.2% NBT was loaded in the wells and incubated for 1 h at 27 °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 wells 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 620 nm. It is noted that the results from 11 fish out of 141 (mostly those from the "small" category) were excluded due to the rapid coagulation of the blood. This led to residual blood being present in the reaction wells of the microtiter plates until the addition of potassium hydroxide and DMSO and thus erroneously increased the absorbance during the reading of the plate at 620 nm.

2.6.2. Phagocytic activity

The phagocytic activity assay was based on the method from Anderson and Siwiski [39]. The phagocytic activity and the phagocytic index were determined using 1 μm fluorescent beads (Sigma). A heparinised blood sample (50 μL) was placed 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 room temperature. A 5 μL sub-sample was then taken out onto a glass slide to prepare a smear. The smear was air dried and then fixed with 95% methanol. Once the methanol evaporated, the smear was stained with a Giemsa stain. The number of phagocytes, phagocytising cells and the number of engulfed fluorescent latex beads was counted using an epifluorescence microscope (Nikon Eclipse Ti-U with X-Cite series 120 Q from Lumen Dynamics). The phagocytic activity (PA) and phagocytic index (PI) were calculated as follows:

PA = number of phagocytising beads/total number of phagocytes.

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

2.7. Statistics

All statistical analyses and figure production were performed using the statistical platform R version 3.6.3 [40]. Results are reported as

mean ± S.E (n = 3 for each treatment). All data were subjected to a log10 transformation to improve normality and meet the assumption of homogeneity of variance prior to analysis. Due to the loss one of the replicate tank for the *Ulva* treatment, the average of the remaining two replicates was used for the different parameters of interest to produce a balanced design. The cellular and humoral innate immune responses were analysed using a one-way ANOVA. In case of significance among treatments, multiple comparisons using a Dunnett's test (95% confidence interval) were performed using the DescTools package. All the results of the statistical analyses are presented in Tables S1 (ANOVA) and S2 (Dunnett's test).

3. Results

We observed no differences in feed palatability between the different treatments, with all the pelleted feed being eaten within 5 min from when first offered. The fish appeared healthy during the trial with no dietary effects on fish death or morbidity. Additionally, no dominance behaviour (e.g. large versus small fish) were observed in any tank during the trial. Finally, although evaluation of growth was not the aim of this trial, no fish lost weight and growth rates were comparable among treatments, with an average weight gain of 17.5 ± 0.7 g per fish after two weeks.

3.1. Humoral immune response

Although there were significant differences between the 15 treatments (ANOVA, F = 3.10, $P = 0.003$) only two treatments resulted in significantly higher haemolytic activity than the control: these were fish fed *Asparagopsis*-supplemented feed, which had significantly higher haemolytic activity (821.6 ± 158.4 units/mL, Dunnett's test Control vs *Asparagopsis*: $P < 0.001$) and those fed the *Dictyota* diet (392.4 ± 60.4 units/mL, Dunnett's test Control vs *Dictyota*: $P = 0.041$). Although not significantly different from the control, the fish fed the diet supplemented with the green seaweed *Ulva* (Dunnett's test Control vs *Ulva*: $P = 0.0504$) also had an increased mean haemolytic activity (double that of the control fish; Fig. 1A).

The lysozyme response was similar across treatments, with no significant differences between treatments (ANOVA, F = 1.73, $P = 0.095$). Overall, the lysozyme results (Fig. 1B) were more variable within each treatment compared to the haemolytic activity results (Fig. 1A) and this variability may have clouded any treatment effects. Albeit not statistically significant, the mean lysozyme activity values for the fish fed two of the green seaweeds (*Caulerpa* and *Ulva*), two of the brown seaweeds (*Dictyota* and *Sargassum*) and two of the red seaweeds (*Kappaphycus* and *Sarconema*) were at least 1.5–2 times higher than that of the control fish (Fig. 1B).

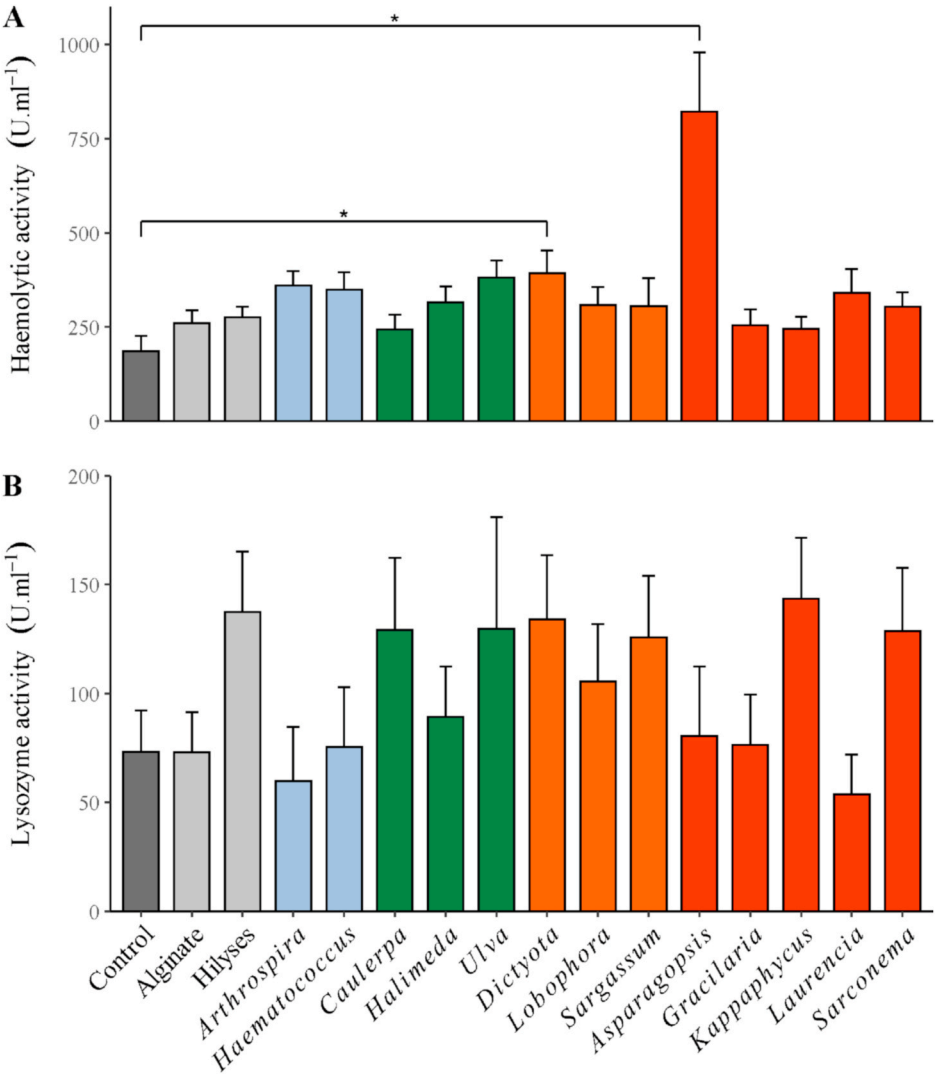

Fig. 1. Humoral innate immune parameters in *S. fuscescens* fed the control diet (dark grey) and the different treatments from the five treatment categories including the commercial immunostimulants (light grey), documented immunostimulants (light blue), green seaweeds (green), brown seaweeds (brown) and the red seaweeds (red) for (A) the haemolytic activity of the alternative complement pathway and (B) serum lysozyme activity. A bracket with a star denotes a significant difference (Dunnett's test, $P < 0.05$) between the control and the indicated treatment. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.2. Cellular immune response

There were no significant differences between the respiratory burst activity of leukocytes in fish fed the different experimental diets (ANOVA, F = 1.04, $P = 0.446$; Fig. 2A).

Similarly, no statistically significant differences were observed between phagocytic activity in fish fed the different diets (ANOVA, F = 1.49, $P = 0.168$). Although not statistically significant, those that received diets supplemented with the red seaweeds *Asparagopsis* and *Laurencia* had a respective proportion of phagocytes with engulfed beads of 43% for *Asparagopsis* and 40% respectively, which appeared higher than that of the control fish (29%; Fig. 2B).

The results for the phagocytic index (Fig. 2C) resembled those for phagocytic activity (Fig. 2B) with no significant differences detected (ANOVA: F = 0.61, $P = 0.843$) between the phagocytic index of fish fed different diets. Although not statistically significant, the fish fed *Asparagopsis* (6.9 ± 0.3 beads phagocyte^{-1}) and those fed the brown seaweed *Lobophora* (6.3 ± 0.5 beads phagocyte^{-1}) appeared to have higher phagocytic index compared to the fish fed the control fish (4.7 ± 0.3 beads phagocyte^{-1}).

Interestingly, none of the commercial immunostimulant compounds led to significantly higher immune response than fish fed control diets,

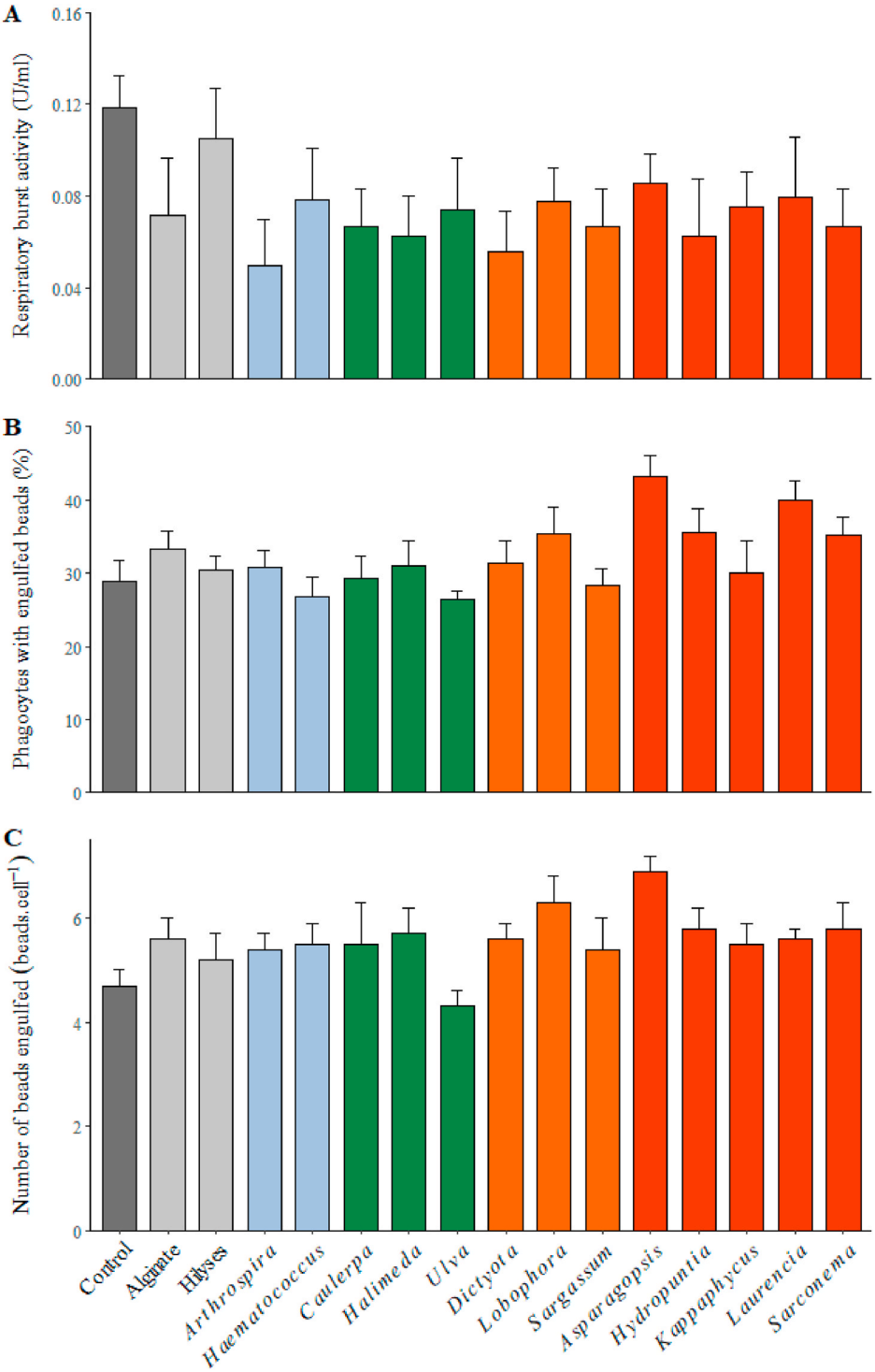

Fig. 2. Cellular innate immune response in the fish fed the control diet (dark grey) and the different treatments from the five treatment categories including the commercial immunostimulants (light grey), documented immunostimulants (light blue), green seaweeds (green), brown seaweeds (brown) and the red seaweeds (red) for (A) respiratory burst activity of leukocytes, (B) phagocytic activity of phagocytes, and (C) phagocytic index of phagocytes. A bracket with a star denotes a significant difference (Dunnett's test, $P < 0.05$) between the control and the indicated treatment. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

for any of the immune response parameters we measured (Figs. 1 and 2).

3.3. Cell counts and serum glucose concentration

When we compared the general health of experimental fish, there were no significant differences between the fish in the different treatments and the control fish regarding erythrocyte density, leukocyte density, haematocrit index, hepatosomatic index and mean corpuscular volume (Table 2). There were also no statistical differences in blood glucose concentration between treatments (ANOVA: $F = 1.81$, $P = 0.077$). Despite the lack of statistical significance, the highest blood glucose concentration was reported in the control diet (14.29 mmol L^{-1}) which was 1.78 times higher than the lowest blood glucose concentration of the fish fed the *Caulerpa*-supplemented diet.

4. Discussion

We identified a couple of seaweed species that caused significant and dramatic increases in the innate immune response of the mottled rabbitfish *S. fuscescens*. In particular, the inclusion of the red seaweed *Asparagopsis taxiformis* in the diet of *S. fuscescens* led to a 4-fold increase in haemolytic activity compared to the control fish. Fish fed diets with *Dictyota intermedia*, had similar, albeit less dramatic increases in this innate immune parameter. Importantly, these seaweed supplements stimulated the haemolytic activity in rabbitfish *S. fuscescens* without hindering the overall health of the fish, providing potentially valuable insights for industry development for this candidate aquaculture species. This trial highlighted the potential benefits of using seaweed, and especially *Asparagopsis taxiformis* and *Dictyota intermedia*, as functional ingredient for fish in the context of aquaculture.

A. taxiformis and *A. armata* are considered promising species as animal feed supplements because of their production and accumulation of biologically active halogenated metabolites (including bromoform), which have a range of applications including antibacterial, antiviral, antifungal and anti-inflammatory [41,42]. *A. taxiformis* is mainly known for its methane reduction potential in ruminants [43] but it has also previously been fed at 3% dietary inclusion to the orbicular batfish, *Platax orbicularis*, which led to a significant increase in immune-related gene expression (lysozyme G and TGF-β1) after three weeks of exposure but as for our trial they found no differences in lysozyme after two weeks [44]. In another study [45], *A. taxiformis* was fed to gilthead seabream, *Sparus aurata*, and European seabass, *Dicentrarchus labrax*, at 10% dietary inclusion using whole seaweed (dried powder) and 10% dietary inclusion as an ethanolic extract. That study found strong *in vitro* bactericidal effects of *A. taxiformis* against important aquaculture bacterial pathogens (e.g. *Aeromonas salmonicida* and *Vibrio alginolyticus*), but the *in vivo* trial led to a reduction in white blood cell counts in both fish species evaluated, which was thought to be caused by the high dietary inclusion of the seaweed. In another study, Castanho, et al. [46] used a 0.5% solution of *A. armata* extract (Ysaline 100, YSA) to treat live feeds and found that it resulted in lower survival (8%) in gilthead seabream larvae compared with the control group (16%). Despite this negative effect, which the authors also attributed to the dose being too high, the algal extract resulted in better growth of the fish larvae and lower *Vibrionaceae* counts in the rearing water and in the gut of the fish larvae.

The brown seaweed *Dictyota intermedia* also led to a significant increase (double) of haemolytic activity in rabbitfish. Unlike *A. taxiformis*, this is the first time this seaweed has been explored as a functional ingredient in fish. This genus was explored once before but *in vitro* and as an extract [47]. In that study *D. dichotoma* had positive effects on the respiratory burst of turbot phagocytes [47], which was not an effect we observed when feeding *D. intermedia* to rabbitfish in our trial. The genus *Dictyota* appears to be an under-explored resource as fish immunostimulant as similarly to *A. taxiformis* it is a known rich source of bioactive secondary metabolites with significant biological activities [e.g. antiviral, antioxidant and antitumor; [48]]. *Sargassum* spp. is the brown seaweed genus most commonly used as a fish dietary immunostimulant, however in our trial it did not induce any significant immunostimulatory effects. The absence of profound immunostimulatory effects of *S. spinuligerum* in *S. fuscescens* in this trial may be the result of multiple factors including the length of the trial (14 days) which might have been too short to produce a significant immune response. Because other immunostimulant ingredients (including several used in this study) have been found to generate positive immune responses within days in fish [49], it is also possible that *S. spinuligerum* had limited immunostimulatory effects when fed to *S. fuscescens* in this specific experimental context. As for the other seaweeds tested in this trial, the three brown seaweeds had varying effects on the different innate immune parameters measured. This was also the case for the brown seaweeds tested by Wang et al. [50], who found that supplementing with *Sargassum horneri* (2.5%, 5%, 7.5% and 10%) the diet of juvenile turbot, *Scophthalmus maximus*, negatively impacted their lysozyme activity compared to the control diet but improved their haemolytic activity. However, the fish fed the *S. honeri* diets showed a 10% increase in survival when exposed to the pathogen *Edwardsiella tarda* compared to the control fish, which also highlights the importance of measuring multiple innate immune parameters when they are to be used as proxy for pathogen resistance [50].

The variability among immune parameters and the different effects of different seaweed species on various immune parameters had previously been highlighted [17]. For this reason the review by Thépot et al. [17] recommended a holistic approach and the measurement of multiple

Table 2
Health indicators of the rabbitfish fed the control and the different treatment diets. Values shown are mean ± SE.

Treatment	Erythrocytes (10^6 cell.µ L^{-1})	Leukocytes (10^3 cell.µ L^{-1})	Haematocrit index (%)	Mean corpuscular volume (fl)	Hepatosomatic index (%)	Glucose (mmol. L^{-1})
Control	1.70 ± 0.08	66.3 ± 7.15	28.56 ± 2.23	169.83 ± 13.16	2.26 ± 0.37	14.29 ± 1.92
Alginate	1.92 ± 0.12	70.37 ± 7.30	30.11 ± 1.78	160.03 ± 11.45	2.25 ± 0.27	9.61 ± 0.94
Hilyses®	1.96 ± 0.13	71.85 ± 4.94	31.22 ± 1.62	162.50 ± 8.19	2.23 ± 0.20	8.12 ± 1.07
Haematococcus	2.00 ± 0.13	80 ± 10.37	33.78 ± 2.28	170.31 ± 8.26	3.03 ± 0.15	9.78 ± 1.05
Spirulina	1.91 ± 0.11	55.74 ± 6.35	30.00 ± 1.95	158.17 ± 8.36	2.35 ± 0.34	12.09 ± 1.25
Caulerpa	1.80 ± 0.11	64.44 ± 6.22	28.67 ± 1.89	162.34 ± 12.67	2.58 ± 0.27	8.04 ± 1.21
Ulva	1.65 ± 0.10	49.72 ± 5.73	34.00 ± 1.75	209.76 ± 14.87	2.22 ± 0.45	10.92 ± 1.49
Halimeda	1.97 ± 0.15	59.44 ± 5.32	33.11 ± 1.06	178.65 ± 18.16	2.51 ± 0.27	11.68 ± 1.03
Lobophora	1.90 ± 0.09	73.15 ± 8.76	30.33 ± 1.76	162.85 ± 11.58	2.55 ± 0.24	12.60 ± 2.14
Dictyota	2.00 ± 0.07	66.3 ± 7.58	34.44 ± 1.37	173.28 ± 7.99	2.54 ± 0.28	12.17 ± 1.46
Sargassum	1.88 ± 0.10	69.07 ± 10.60	31.33 ± 0.73	170.90 ± 10.00	2.94 ± 0.25	10.00 ± 1.15
Laurencia	2.04 ± 0.11	60.37 ± 8.30	34.00 ± 1.59	169.09 ± 9.44	2.66 ± 0.32	12.09 ± 0.74
Sarconema	2.21 ± 0.13	74.26 ± 6.01	33.11 ± 1.95	151.38 ± 7.22	2.89 ± 0.33	11.30 ± 1.21
Kappaphycus	2.09 ± 0.13	71.67 ± 7.52	30.67 ± 1.00	152.77 ± 12.28	2.34 ± 0.34	8.49 ± 0.58
Gracilaria	1.81 ± 0.09	63.52 ± 8.65	30.00 ± 1.94	168.70 ± 13.61	2.84 ± 0.54	11.08 ± 1.73
Asparagopsis	1.80 ± 0.07	62.96 ± 7.35	30.33 ± 1.42	168.32 ± 5.18	2.42 ± 0.16	10.41 ± 0.31

immune parameters, particularly at initial stage like for our screening trial where being able to detect a potential beneficial effect or trend of a particular seaweed is key to then further explore in detail that particular seaweed (e.g. *A. taxiformis*). The results from our study support this recommendation as several seaweed species had dramatic impacts on only one out of five immune parameters and suggests that a lack of effect in studies that looked at only 1 or 2 parameters, does not necessarily indicate that the potential immunostimulant will have no effect on other parameters [51,52].

We aimed to assess the immunostimulatory potential of multiple seaweed species simultaneously, because until now, only three species of seaweeds have ever been tested in this context on a marine herbivorous fish. The screening approach taken in this study limited the statistical power, which led to some interesting trends in our data that were statistically un-resolvable. Despite this, several of those trends are potentially very interesting from an industry perspective and therefore worth highlighting here. For example, fish fed the diet supplemented with the green seaweed *Ulva fasciata* had haemolytic activity levels twice that of the control fish ($P = 0.050$, Table S2). Future trials using *S. fuscescens* could also use captive bred fish to further minimise the potential variance in responses between fish due to genetic differences or because of exposures to different foods or environmental conditions in the wild.

In our recent review, we found that seaweeds provided in combination with known immunostimulant products often elicited the biggest immune responses in fish [17]. Although not explicitly tested here, it is possible that the inclusion of seaweeds, in combination with existing products, may yield the greatest response. However, certain seaweeds, such as *Asparagopsis* spp., may be more effective when provided independently and may, if commercial scale production of this seaweed is established, provide a more economically sustainable alternative to fish farmers. Future feeding trials with *Asparagopsis* spp. should investigate the long term positive and/or negative effects of the form (dried whole and as an extract) and the dose of the seaweed in the diet of fish on their growth and feed efficiency. The link between the immune system of fish and their intestinal microbiome has been highlighted previously but has so far been scarcely investigated in herbivorous fish [17]. Studies combining molecular tools to investigate the effect of dietary seaweed or other immunostimulants on the fish intestinal microbiome with the measurement of innate immune responses could enrich our current understanding of dietary supplements on fish physiology and gastrointestinal health.

5. Conclusion

This study is the first to screen more than two seaweed species as immunostimulant candidates simultaneously, and measure how multiple immune parameters changed as a result in a marine herbivorous fish. Whilst all seaweeds and supplements tended to have a positive influence on the fish immune responses, *A. taxiformis* appears to be a potent immunostimulant, boosting haemolytic activity in fish that received it by four times compared to control fish. Inclusion of the brown seaweed *Dictyota* in experimental diets also led to significantly higher haemolytic activity, and although not statistically resolvable, the green seaweed *Ulva* consistently increased immune responses in fish that consumed it. Seaweeds have exciting potential as immunostimulants to boost the immune responses of fish without compromising their health or condition, providing an alternative for aquaculture farmers to reduce their reliance on antibiotics and other chemotherapeutics. Seaweeds, particularly *Asparagopsis taxiformis*, could thus help provide a solution to disease, which remains one of the major challenges for aquaculture development globally.

CRediT authorship contribution statement

Valentin Thépot: Conceptualization, Methodology, Data curation, Formal analysis, Visualization, Writing – original draft. **Alexandra H. Campbell:** Conceptualization, Methodology, Formal analysis, Visualization, Writing – review & editing, Supervision, Resources. **Nicholas A. Paul:** Conceptualization, Methodology, Formal analysis, Visualization, Writing – review & editing, Supervision, Resources. **Michael A. Rimmer:** Conceptualization, Methodology, Formal analysis, Writing – review & editing, Supervision, Resources.

Declaration of competing interest

The authors acknowledge that the results from this study are part of a patent application (publication number WO/2020/124167).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2021.03.018.

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