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Influence of immunostimulant polysaccharides, nucleic acids, and *Bacillus* strains on the innate immune and acute stress response in turbots (*Scophthalmus maximus*) fed soy bean- and wheat-based diets

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Abstract Immunostimulants are widely applied in aquaculture practice and may have beneficial effects on the immune system and physical functions allowing higher tolerance to stress. In the current study, the impact of four (i–iv) dietary active ingredients on the immune and stress response of turbot was examined in two experiments (I and II). A basal low fish meal (FM; 32%) diet was formulated and supplemented with (i) yeast β-glucan and mannan oligosaccharide (GM), (ii) alginic acid (AC), (iii) yeast nucleotides and RNA (NR), or (iv) *Bacillus* strains (BS). The basal diet (C-LF) and a high FM (59%) control (C-HF) were maintained. All six diets were fed to juvenile turbots for 84 days in experiment I and for additional 28 days prior to experiment II. Immunological and hematological parameters were determined in experiment I. In experiment II, physical stress response to a typical short-term (<1 day) aquaculture handling procedure (combination of capture, netting/transfer, and crowding) was investigated. For this, turbot blood was sampled before and at 0.5, 1, 4, and 24 h post stress. Plasma lysozyme activity, neutrophil reactive oxygen species (ROS) production, and total plasma protein levels did not significantly differ between treatment groups; however, plasma cholesterol increased significantly in fish fed GM, AC, NR, and C-HF compared to C-LF (I). A significant increase in plasma glucose and triglyceride was observed in GM and NR treatments, while glucose levels were significantly higher in C-HF compared to C-LF. Moreover, the immunostimulant-supplemented diets exhibited significantly lower cortisol levels compared to controls C-LF (at 0.5 h) and C-HF (at 1 h) post stress, respectively (II). According to our findings, FM substitution did not modulate the innate immune response but was associated with reduced levels of cholesterol. Dietary immunostimulants were not effective enough to boost the immune response, but we believe they might be helpful to trigger metabolic advantages during stressful handling events on fish farms.

Keywords Turbot *Scophthalmus maximus* · Fish meal reduction · Immunostimulants · Lysozyme activity · Reactive oxygen species · Cortisol

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Introduction

Fish health and welfare in aquaculture is a key issue in the provision of high quality, consumer-orientated, and sustainable fish products (Naylor et al. 2000; Focardi et al. 2005; Huntingford et al. 2006; Ashley 2007; Kiron 2012). Handling, feeding, transport, vaccination, water quality, and high stocking densities in aquaculture are

potential stressors affecting the physiology and health condition of fish (Barton 2002; Ashley 2007; Segner et al. 2012). Severe or long-lasting stress is found to be detrimental to animal health and welfare, affecting the physiology and performance in fish such as energy metabolism, oxygen uptake, immune function, growth, disease resistance, and survival (Mugnier et al. 1998; Gregory and Wood 1999; Barton 2002; Laiz-Carrión et al. 2003; Verburg-Van Kemenade et al. 2009). The organism has to deal with an energy-demanding process by synthesizing stress-related proteins and other compounds to fuel cellular processes (Tort 2011; Segner et al. 2012).

Assuring an optimal nutritional status and efficient immune system in farmed fish can help to reduce susceptibility to stress and diseases. At present, the use of preventative measures is central to disease control in aquaculture production. Vaccinations are known to be effective prophylactic methods but, as earlier mentioned, can be stressful for fish and remain costly and labor-intensive for the producers (Lillehaug 1989; Horne 1997; Gudding et al. 1999; Thorarinsson and Powell 2006). The application of immunostimulants to counter stress and diseases has been increasingly promoted in aquaculture (Anderson 1992; Sakai 1999; Merrifield et al. 2010). Injection of immunostimulating compounds seemed to be an effective method; however, bath immunization and oral administration showed to be less invasive and stressful for fish; in particular, the dietary uptake is a simple and time-saving way (Anderson 1992; Sakai et al. 2001; Peddie et al. 2002; Selvaraj et al. 2005).

Immunostimulants can be synthetic compounds or biological extracts derived from bacteria, fungi, or plants commonly in the form of polysaccharides. Alginic acids, β-glucans, mannan oligosaccharides (MOSs), nucleotides, and probiotic bacteria have essential physiological and biochemical functions in mediating energy metabolism, cell signaling, encoding and deciphering genetic information, or modifying intestinal microbial communities (Carver and Walker 1995; Dalmo and Bøgwald 2008; Kesarcodi-Watson et al. 2008; Holdt and Kraan 2011; Jung-Schroers et al. 2015). They are reported to strengthen the immune system, protecting fish against physiological stress and susceptibility to infections (Bagni et al. 2005; Staykov et al. 2007; Yoo et al. 2007; Ai et al. 2011; Peng et al. 2013). The fish's innate immune system is the first line of defense against disease vectors and is considered a fundamental mechanism in fighting all kinds of pathogens (Magnadóttir 2006; Whyte 2007).

Dietary inclusion of β-glucans, MOSs, algal derivates, nucleic acids, and probiotics has been shown to modulate immune defense, stress response, and survival in various fish species (Balcázar et al. 2006; Ringø et al. 2010, 2012). Immunostimulants can promote the production of antibacterial peptides, such as lysozyme, and the phagocytic activity of macrophages. Elevation in serum lysozyme activity has been found in various fish species, such as sea bass, *Dicentrarchus labrax* (Bagni et al. 2005); Nile tilapia, *Oreochromis niloticus* (El-Boshy et al. 2010); sturgeon, *Huso huso* (Heidarieh et al. 2011); and rainbow trout, *Oncorhynchus mykiss* (Staykov et al. 2007), which were fed on β-glucan-, MOS-, or alginic acid-supplemented diets. Dietary uptake of β-glucan in combination with a feed stimulant (BAISM) was able to improve the lysozyme activity in juvenile olive flounder, *Paralichthys olivaceus* (Yoo et al. 2007). Phagocyte respiratory burst was enhanced with dietary ribonucleic acid in rohu (*Labeo rohita*; Choudhury et al. 2005) and β-glucan in sea bass (Bonaldo et al. 2007). Dietary supplementation of β-glucan and MOS enhanced respiratory burst and survival of juvenile turbot, *Scophthalmus maximus* (Li et al. 2008). However, Ogier de Baulny et al. (1996) observed no effect of β-glucan on the lysozyme activity in turbot.

Information on the effect of immunostimulants on stress response in fish is scarce. Alginic acid-enriched (Gioacchini et al. 2008) and nucleic acid-enriched (Leonardi et al. 2003; Tahmasebi-Kohyani et al. 2012; Palermo et al. 2013) diets can suppress stress response (expressed as plasma cortisol levels) in rainbow trout and sole (*Solea solea*) exposed to different stressors (vaccination, virus disease, handling, and crowding). Recent attempts to minimize the fish meal (FM) content in formulated finfish feeds due to economic and environmental concerns may increase susceptibility to stress and infections potentially as a result of the reduction of high-quality animal proteins and lipids in diets (Burrells et al. 1999; Urán et al. 2008; Bonaldo et al. 2014; Khosravi et al. 2015).

Diets for carnivorous fish species, such as turbot (*S. maximus*), are yet to be optimized. In particular, the adverse effects of dietary plant substitutes and stressful husbandry conditions must be overcome. The interaction of immunostimulants and plant proteins acting as FM substitutes as regards immune and stress response remains, however, poorly studied. The influence of

immunostimulant supplementation in FM-reduced diets on plasma cortisol levels after an acute stress challenge remains unstudied for turbot. Therefore, the current study aims to evaluate the effect of commercially applied and piloted immunostimulants with the following active ingredients: (i) yeast β-glucan and mannan oligosaccharide (GM), (ii) alginic acid from brown algal extracts (AC), (iii) purified yeast nucleotides and ribosomal RNA (NR), and (iv) probiotic bacteria strains *Bacillus subtilis* and *Bacillus licheniformis* (BS), on the immune and stress responses in juvenile turbot fed low fish meal content diets.

Materials and methods

Fish species and experimental setup

The feeding experiment was conducted at the aquaculture facilities Zentrum für Aquakulturforschung (ZAF) at the Alfred Wegener Institute Helmholtz Center for Polar and Marine Research (AWI) in Bremerhaven, Germany. Juvenile turbots were obtained from Maximus A/S (Bedsted Thy, Denmark). Fish were examined for infectious diseases before and at the end of the experiment to monitor health conditions of experimental animals. The experiment was performed under the guidelines of the local authority (Department of Food Safety, Veterinary Affairs and Plant Protection) in Bremen with the permission to carry out animal experiments (522-27-11/02-00(112)).

The rearing system consisted of 36 tanks (0.8 m^2 bottom surface, 500 l total water volume). Tanks were connected to a recirculating aquaculture system (RAS; total water volume 40 m^3) and equipped with a drum filter, protein skimmer, moving bed biofilter, and disinfection unit (ozone generator; Sander Aquatec GmbH, Uetze-Eltze, Germany). The photoperiod was maintained at a 12 h light/12 h dark cycle throughout. Physical water parameters were monitored constantly (temperature 17.3 ± 0.5 °C, salinity 28.6 ± 1.4 g l^{-1}, dissolved oxygen 9.3 ± 0.5 mg l^{-1}; SC 1000 Multiparameter Universal Controller, Hach Lange GmbH, Düsseldorf, Germany). Chemical water parameters ammonia, nitrite, and nitrate were determined in a 3-day interval before feeding (NH_4-N 0.01 ± 0.02 mg l^{-1}, NO_2-N 0.04 ± 0.03 mg l^{-1}, NO_3-N 80.6 ± 16.7 mg l^{-1}; photometer DR 2800; Hach Lange GmbH, Germany).

Experimental diets

Six diets were formulated with regard to an isonitrogenous (565 ± 7 g CP kg^{-1}) and isocaloric (22 ± 0.5 g MJ kg^{-1} DM) content (Table 1). Four experimental diets were supplemented with active ingredients of commercially applied or piloted feed additives: (1) a yeast (*Saccharomyces cerevisiae*) product consisting of 20% beta-1,3/1,6 glucan and 17% mannan oligosaccharide (ProEnMune, ProEn Protein and Energie GmbH, Soltau, Germany) (GM), (2) an alginic acid product of brown algal extracts containing 99% *Laminaria digitata* and 1% *Ascophyllum nodosum* (Ergosan®, Intervet/Schering-Plough Aquaculture, Saffron Walden, UK) (AC), (3) a product of purified yeast nucleotides (cytidine-5V-monophosphate (CMP), disodium uridine-5V-monophosphate (UMP), adenosine-5V-monophosphate (AMP), disodium inosine-5V-monophosphate (IMP), disodium guanidine-5V-monophosphate (GMP)) and ribosomal RNA (Vannagen®, Chemoforma Ltd., Augst, Switzerland) (NR), and (4) a probiotic product of bacteria strains *B. subtilis* and *B. licheniformis* (Probiotic-plus.ru, Russia) (BS). Two control diets, a FM-based diet (C-HF; 585 g FM protein kg^{-1} feed) and a FM-reduced diet (C-LF; 320 g FM kg^{-1} feed), were additionally investigated. The protein content in C-LF and the supplemented diets (GM, AC, NR, and BS) was partly replaced with soy protein concentrate (SPC) and wheat gluten (WG) including 56% protein from plants. All diets were extruded to floating pellets of 5 mm in diameter, manufactured by the Institute of Food Technology and Bioprocess Engineering (BILB-ttz Bremerhaven, Germany).

Experiment I: feeding trials

During the acclimatization period, fish were fed with a commercial dry feed with 55% crude protein and 16% crude fat (R Europa 15, 2 mm diameter; Skretting ARC, Stavanger, Norway). For the experiment, 900 turbot individuals were weighed (initial mean body weight 95.8 g ± 17.7 g) and measured in total body length (initial mean length 18.0 cm ± 1.1 cm) and randomly stocked in the 36 experimental tanks (25 individuals $tank^{-1}$; 3.0 kg m^{-2} stocking density). Fish were starved 24 h prior to weighing. The feeding trial was designed to contain six fish groups consisting of six replicates each. The turbots were hand-fed until apparent satiation twice

Table 1 Ingredients (in g kg^{-1} dry matter (DM)), proximate composition (g kg^{-1} DM/MJ kg^{-1} DM), and amino acid composition (g 16 g^{-1} N) of the experimental diets

	Diets					
	C-HF	C-LF	GM	AC	NR	BS
Ingredients (g kg^{-1})						
Fish meal[a]	585	320	320	320	320	320
Soy protein concentrate[a]	125	250	250	250	250	250
Corn gluten[b]	30	40	40	40	40	40
Wheat gluten[c]	20	147	146.7	146.8	146.9	147
Wheat starch[c]	184	160	154.3	155.2	158.1	159.4
GM	0	0	6	0	0	0
AC	0	0	0	5	0	0
NR	0	0	0	0	2	0
BS	0	0	0	0	0	0.6
Fish oil[d]	45	72	72	72	72	72
Vitamin/mineral mixture[e]	10	10	10	10	10	10
Titanium dioxide[f]	1	1	1	1	1	1
Nutrient composition[g] (g kg^{-1})						
Moisture	72	70	66	65	74	64
Crude protein	553	567	571	569	562	571
Crude fat	117	112	112	112	118	113
Crude ash	108	76	75	77	71	77
Calcium	18	11	11	11	11	11
Phosphorus	15	10	10	10	10	10
Gross energy (MJ kg^{-1})[h]	21	22	22	21	22	22
Essential amino acids (g 16 g^{-1} N)						
Arginine	4.5	4.3	4.4	4.4	4.3	4.4
Cystine	1.2	1.5	1.5	1.6	1.5	1.5
Histidine	3.0	2.7	1.9	2.6	2.4	2.7
Isoleucine	3.3	3.2	3.4	3.4	3.2	3.5
Leucine	5.9	6.2	6.2	6.1	6.0	6.2
Lysine	5.9	4.8	4.8	4.8	4.5	4.6
Methionine	2.1	1.9	1.9	1.9	1.9	1.8
Phenylalanine	3.4	3.8	2.6	3.8	3.7	3.8
Threonine	3.2	3.0	3.0	3.0	3.0	2.9
Valine	3.7	3.5	3.7	3.7	3.5	3.8
Non-essential amino acids (g 16 g^{-1} N)						
Alanine	4.5	4.0	3.9	3.8	3.7	3.9
Aspartic acid	6.7	6.2	6.3	6.4	6.2	6.2
Glutamic acid	7.9	10.4	10.7	10.4	10.1	10.6
Glycine	4.3	3.8	3.8	3.7	3.5	3.8
Proline	4.1	5.9	6.1	5.6	5.5	5.8
Serine	3.3	3.8	3.8	3.7	3.7	3.6
Tyrosine	2.5	2.6	2.7	2.6	2.7	2.5

Additive concentrations were recommended by the manufacturers and literature (Burrells et al. 2001a, b; Merrifield et al. 2011)

C-HF high fish meal control, *C-LF* low fish meal control, *GM* β-glucan/MOS, *AC* alginic acid, *NR* nucleotides/RNA, *BS Bacillus* spp.

[a] Köster Marine Proteins GmbH, Hamburg, Germany

[b] Cargill Deutschland GmbH, Krefeld, Germany

[c] Kröner Stärke, Ibbenbüren, Germany

[d] Vereinigte Fischmehlwerke Cuxhaven GmbH & Co KG, Cuxhaven, Germany

[e] Spezialfutter Neuruppin GmbH & Co. KG, Neuruppin, Germany

[f] Kronos Titan GmbH & Co.OHG, Nordenham, Germany

[g] Weender analysis (Dumas): moisture (VDLUFA Bd. III 3.1), crude protein (VDLUFA Bd. III 4.1.2), crude fat (VDLUFA Bd. III 5.1.1), and ash (VDLUFA Bd. III 8.1); ICP-mass spectrometry: calcium and phosphor (PM DE01_018)

[h] Bomb calorimeter (6100, Parr Instrument GmbH, Frankfurt a. M., Germany)

a day (10:00 a.m. and 2:00 p.m.) over an 84-day period. The effect of fish meal substitution and feed additives on feed conversion and growth performance was reported in a previous communication (Fuchs et al. 2015).

At the end of the experiment, 108 individual fish (3 fish tank^{-1}) were selected randomly and placed into a 500 mg l^{-1} solution of tricaine methanesulfonate (MS 222; Sigma-Aldrich Co. LLC., Munich, Germany) until death (Neiffer and Stamper 2009). Subsequently, blood was drawn from the caudal vein into disposable syringes pre-filled with a lithium-heparin bead (Sarstedt AG & Co. KG, Nümbrecht, Germany), centrifuged at 2.000*g* for 15 min to collect the supernatant plasma. Plasma was stored at −80 °C for biochemical analysis. Furthermore, head kidneys of another fish per tank (6 fish treatment^{-1}, 36 fish in total) were isolated and placed in centrifuge tubes (50 ml; Sarstedt AG & Co. KG, Germany) filled with 15 ml of wash medium (RPMI medium mixed with 10,000 IU sodium heparin; Sigma-Aldrich Co. LLC., Germany). Head kidney samples were processed immediately to measure the production of the reactive oxygen species (ROS).

Experiment II: handling simulation (capture, netting/transfer, crowding)

Prior to experiment II, the remaining turbots from all six tanks of the six treatment groups were restocked in three replicate tanks (35 fish tank^{-1}, 630 in total). Turbots were kept in 500-l tanks, and feeding (twice a day) was continued for additional 28 days with the beforementioned experimental diets. In this experiment, the influence of a typical short-term (<1 day) aquaculture handling procedure (combination of capture, netting/transfer, and crowding) on physical stress response in turbot was determined. After 28 days, the mean initial stocking density of fish was 16.1 kg m^{-2} (C-HF), 13.5 kg m^{-2} (C-LF), 13.3 kg m^{-2} (GM), 13.3 kg m^{-2} (AC), 12.6 kg m^{-2} (NR), and 13.3 kg m^{-2} (BS), respectively. Fish were starved 24 h prior to sampling as feeding has shown to influence plasma cortisol levels (Arends et al. 1999). As a pre-treatment control, three fish were simultaneously taken from each tank (nine fish per treatment), immediately anesthetized with a lethal dose of MS 222 and bled using lithium-heparinized syringes (Sarstedt, Germany). Subsequently, all other fish were subjected to handling treatment, which consisted of netting from the rearing tanks, and transferred into aerated 90-l tanks (0.3 m^{-2} bottom surface, 72 cm length × 42 cm width × 30 cm depth) at a stocking density of 39.4 kg m^{-2} (C-HF), 32.9 kg m^{-2} (C-LF), 32.4 kg m^{-2} (GM), 32.4 kg m^{-2} (AC), 30.8 kg m^{-2} (NR), and 32.5 kg m^{-2} (BS). Fish were kept there in crowded conditions for 5 min before relocating them into the rearing tanks. At 0.5 h, 1 h, 4 h (11:30 a.m., 12:00 a.m., 3:00 p.m.), and 24 h after the end of the handling simulation, three fish per tank (nine fish per treatment and time point) were simultaneously captured, while ensuring minimal disturbance to other tank occupants, placed into anesthetic, and bled as described above. Only three treatment groups (C-HF, GM, and AC) were subjected to handling stress on the same day to allow equal timing for blood sampling (11:30 a.m., 12:00 a.m., 3:00 p.m.) during this narrow time frame. All samples (pre-treatment control and post stress) of the three treatments were taken directly before stress and within 24 h. The following day, the same procedure was applied to the other three groups (C-LF, NR, and BS). Blood samples were stored on ice and centrifuged at 2.000*g* for 15 min at 4 °C. The supernatant plasma was frozen for storage at −80 °C to determine the cortisol and glucose concentrations in fish at resting condition and after handling.

Immunological analysis

Generation of ROS by head kidney leucocytes (HKLs) was measured by a nitro blue tetrazolium salt (NBT) reduction assay (Pick et al. 1981; Verburg-van Kemenade et al. 1996) as described before by Skouras and Steinhagen (2003). Leucocytes were collected by pressing and washing the HK tissue through a 100-μm nylon mesh with three times 5 ml of wash medium (RPMI medium with 10,000 IU l^{-1} sodium heparin; Sigma-Aldrich Co. LLC., Germany). The isolated cells in medium were centrifuged at 580*g* for 10 min at 4 °C. After decanting the supernatant, cell pellets were resuspended with 15 ml of wash medium and centrifuged a second time. Subsequently, pellets were resuspended with culture medium (RPMI medium supplemented with 10% fetal bovine serum; Sigma-Aldrich Co. LLC., Germany). The viable cell concentration was determined by trypan blue staining and cell counting using a hemocytometer. Cells were adjusted to 10^6 cells ml^{-1} in the culture medium. To measure ROS generation, cell suspensions were incubated in 96-well flat-bottom microtiter plates (10^6 cells per well in a final volume of 175 μl medium) in triplicate with and without

a stimulator. Therefore, the culture medium, the indicator NBT (1 g l^{-1}; Sigma-Aldrich), the stimulator phorbol myristate acetate (PMA, 0.15 mg l^{-1}; Sigma-Aldrich), and cell suspension were added per well to induce ROS production. Additionally, cells were incubated without a stimulator to determine spontaneous ROS generation of cells. The solutions were discarded after 2 h of incubation at 22 °C. Cells were fixed for 10 min with 125 μl of 100% methanol (Carl Roth GmbH & Co. KG, Karlsruhe, Germany), and subsequently, wells were washed twice with 125 μl of 70% methanol. Plates were stored in the dark to allow air-drying of the fixed cells for 24 h at room temperature. On the following day, the reduced NBT (formazan) was solubilized in 125 μl of 2 M KOH and 150 μl DMSO (Carl Roth) per well (Rook et al. 1985). The optical densities were recorded immediately at 650 nm in a microplate reader (TriStar LB 941; Berthold Technologies, Germany) at 22 ± 0.5 °C.

Plasma biochemical analysis

Plasma lysozyme (LZY) activity was analyzed by a turbidimetric assay according to Parry et al. (1965), adapted for the measurement in microtiter plates (Skouras et al. 2003). A 0.2 g l^{-1} suspension of *Micrococcus lysodeikticus* was prepared with 0.05 M sodium phosphate buffer (pH 7.4) (chemicals: Sigma-Aldrich Co. LLC., Munich, Germany). The bacterial cell suspension was added to a 96-well microtiter plate and mixed with 25 μl plasma to receive a total volume of 200 μl per well. The optical density (OD) was measured after 0.5 and 4.5 min at 530 nm in a microplate reader (TriStar LB 941; Berthold Technologies GmbH & Co. KG, Bad Wildbach, Germany) at a temperature of 23 ± 1 °C. The lysozyme activity was calculated according to the decrease in absorbance, defining 1 unit of lysozyme activity as the amount of sample causing a decrease in absorbance of 0.001 OD min^{-1}. As an external standard, hen egg-white lysozyme (Sigma-Aldrich Co. LLC., Germany) was used (Hutchinson and Manning 1996). Analysis of total protein (TP), cholesterol (CHO), glucose (GC), and triglyceride (TG) was performed using an automatic biochemical analyzer (Pentra 400, Horiba Medical, Kyoto, Japan).

Plasma cortisol levels were measured by solid-phase enzyme-linked immunosorbent assay (ELISA; RE52611, IBL International, Hamburg), which was already used for turbot (Reiser et al. 2010). Before ELISA, plasma samples were treated with heat denaturation; 200 μl of plasma was denatured at 80 °C for 1 h, subsequently vortexed for 20 s, diluted with phosphate-buffered saline (PBS), vortexed for 20 s, and centrifuged at 13,000*g* for 20 min for separation of the supernatant. Duplicate aliquots (50 μl) of diluted plasma were then used in the assay. The accuracy of heat treatment was evaluated by cold spiking with cortisol (40 ng ml^{-1}) to determine the recovery (recovery = 94%).

Statistics

Data are presented as a mean ± standard deviation (SD) for each treatment. The SigmaPlot 11 for Windows (Systat Software, Inc., San Jose, CA, USA) software package was used for statistical evaluations. All data were tested for normal distribution by the Shapiro-Wilk test. If normality and homogeneity of variances were confirmed, multiple comparisons for the immune and hematological data were done by one-way analysis of variance (ANOVA). A non-parametric Kruskal-Wallis test was used when the normality assumption was not met. Data from acute stress measurements were analyzed by two-way ANOVA with two independent variables, treatment (diet) and time (hours), and cortisol or glucose concentration as dependent variables. Tukey's post hoc (HSD) or Dunn tests were carried out to identify significantly different groups. Differences between sets of comparisons were considered significant at a probability of error at $p < 0.05$.

Results

Overall survivorship during the experimental period was high with a total of 0 and 0.7% mortalities in treatments C-HF, C-LF, AC, and BS and treatments GM and NR, respectively ($p < 0.05$; one-way ANOVA). No signs of infections were observed during the experiments. The final weight (299.5 ± 92.0 g) of fish fed the high FM diet (C-HF) was significantly higher ($p < 0.01$) compared to that of low FM groups (NR = 246.3 ± 71.1 g, GM = 251.9 ± 69.6 g, BS = 254.0 ± 72.0 g, C-LF = 254.2 ± 77.9 g, AC = 257.5 ± 70.4) (Fuchs et al. 2015). Feed additives had no significant influence ($p > 0.05$) on growth performance.

Experiment I: plasma biochemistry and immunology

The mean lysozyme activity (units ml^{-1} plasma) did not differ significantly ($p > 0.05$) between treatments (Table 2). However, all fish treated with dietary immunostimulants or high FM (C-HF) showed a tendency towards increased lysozyme activities between 1006 ± 133 (BS) and 1108 ± 106 (AC), compared to an activity of 971 ± 118 in fish fed the low FM control diet (C-LF) (Table 2).

The values of the baseline reactive oxygen species production (OD), 0.02 ± 0.01, indicated no significant differences ($p > 0.05$) among all treatments (Table 2). Head kidney phagocytes from turbots could be stimulated by phorbol ester (PMA) and cells responded with a high NBT reduction. Stimulated ROS activities were measured between 0.53 ± 0.19 (NR) and 0.78 ± 0.38 (C-HF)/0.78 ± 0.53 (BS), while phagocytes in fish fed the low FM control diet (C-LF) showed a mean activity of 0.66 ± 0.34 (Table 2). However, upon cell stimulation, the mean production of ROS was not significantly different ($p > 0.05$) between all fish groups.

Mean concentrations of total plasma protein (mg ml^{-1}) ranged between 29.0 ± 2.7 (C-LF) and 31.7 ± 3.3 (AC) and did not significantly differ between dietary treatments (Table 2). However, plasma cholesterol (mg dl^{-1}) was significantly higher, between 43.7 ± 3.1 (AC) and 58.6 ± 7.4 (C-HF), in fish fed diets supplemented with GM, AC, NR, and the high FM control diet (C-HF) compared to the low FM control diet, 36.8 ± 2.8 (C-LF) (Table 2). Significant increases in plasma glucose (mg dl^{-1}), 44.4 ± 3.8 and 43.7 ± 4.5, and triglyceride (mol dl^{-1}), 105.5 ± 15.3 and 90.6 ± 19.2, were recorded under GM and NR supplementation compared to C-LF, 34.8 ± 2.4 and 38.3 ± 10.2, while glucose levels were significantly higher in C-HF, 45.4 ± 7.3.

Experiment II: stress indicators

Plasma cortisol concentrations (ng ml^{-1}) in unstressed turbots ranged between 4.9 ± 3.6 (NR) and 16.5 ± 14.9 (C-HF) (mean 10.0 ± 5.4) and can be defined as baseline levels (Fig. 1). At experimental outset, cortisol levels were not significantly different ($p > 0.05$) among dietary treatments. Cortisol levels in fish rose rapidly after handling, peaked by 0.5 and 1 h, and returned to basal concentrations in fish plasma 4 h after handling (Fig. 1). Fish fed the low fish meal control diet C-LF showed significantly higher levels (60.1 ± 10.0; $p < 0.05$) at 0.5 h post handling compared to all other fish groups (Fig. 1). In contrast, fish of the high fish meal control group C-HF exhibited significantly higher levels (59.4 ± 7.8; $p < 0.05$) at 1 h post handling. All other groups treated with supplemented diets showed the highest plasma cortisol concentrations between 23.7 ± 8.1 (BS) and 32.6 ± 13.5 (NR) 0.5 and 1 h after

Table 2 Immunological and physiological parameters measured in the blood plasma or head kidney of turbot juveniles fed the six experimental diets

Parameters	Diets					
	C-HF	C-LF	GM	AC	NR	BS
Immunological parameters						
LSZ (units ml^{-1} plasma)	1034 ± 93	971 ± 118	1009 ± 117	1108 ± 106	1080 ± 132	1006 ± 133
ROS (OD)	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
PMA stimulated ROS (OD)	0.78 ± 0.38	0.66 ± 0.34	0.74 ± 0.39	0.71 ± 0.17	0.53 ± 0.19	0.78 ± 0.53
Physiological parameters						
TP (mg ml^{-1})	30.8 ± 2.8	29 ± 2.7	31.1 ± 2.4	31.7 ± 3.3	29.7 ± 1.9	31.1 ± 2.1
CHO (mg dl^{-1})	58.6 ± 7.4^{a}	36.8 ± 2.8^{b}	49.4 ± 4.4^{a}	43.7 ± 3.1^{a}	55.0 ± 6.5^{a}	41.3 ± 3.2^{ab}
GLC (mg dl^{-1})	45.4 ± 7.3^{a}	34.8 ± 2.4^{b}	44.4 ± 3.8^{a}	39.9 ± 4.2^{ab}	43.7 ± 5.4^{a}	37.4 ± 3.7^{ab}
TG (mol dl^{-1})	69.9 ± 34.1^{ab}	38.3 ± 10.2^{b}	100.5 ± 15.3^{a}	80.0 ± 46.3^{ab}	90.6 ± 19.2^{a}	61.2 ± 18.3^{ab}

Data are presented as mean ± SD ($n = 6$). Different superscript letters within a line denote significant differences ($p < 0.05$)

LSZ lysozyme, *ROS* reactive oxygen species, *OD* optical density, *TP* total protein, *CHO* total cholesterol, *GLC* glucose, *TG* triglycerides

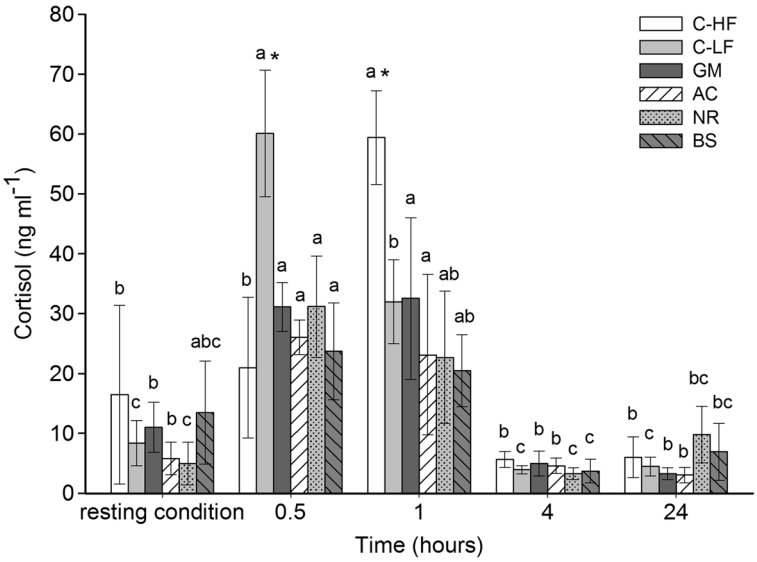

Fig. 1 Plasma cortisol concentrations in nanograms per milliliter after stress treatment collected from turbots of the six dietary treatments. Data are presented as mean ± SD ($n = 6$). *Different letters* denote significant differences within the same treatment, and *asterisk* and *section sign* denote significant differences between treatments at the same time point ($p < 0.05$)

the handling procedure (Fig. 1). At 4 and 24 h post handling, cortisol levels were not significantly different ($p > 0.05$) in turbot plasma among all fish groups. Plasma glucose concentrations (mg dl^{-1}) ranged between 31.8 ± 2.3 (AC) and 38.4 ± 4.8 (C-HF) (mean 34.9 ± 1.4) under initial resting condition (Fig. 2). Glucose concentrations were significantly increased up to 50.0 ± 5.0 in fish group C-HF following handling stimuli at 1 h post stress (Fig. 2). Only a trend towards elevated glucose concentrations was visible in the fish of the other groups at 0.5, 1, and 4 h post stress, although changes were not significant.

Discussion

The use of immunostimulating substances in fish and shellfish feeds has been widely accepted in the aquaculture industry despite their exact modes of action inside the organism remaining far from fully understood. Any prophylactic measures that have nutritional value and can support stress tolerance and defense mechanisms against pathogens are likely to be of commercial interest for producers of feed and fish. The current results provides new insight into the immunostimulant activity among common diet supplements and their effect on

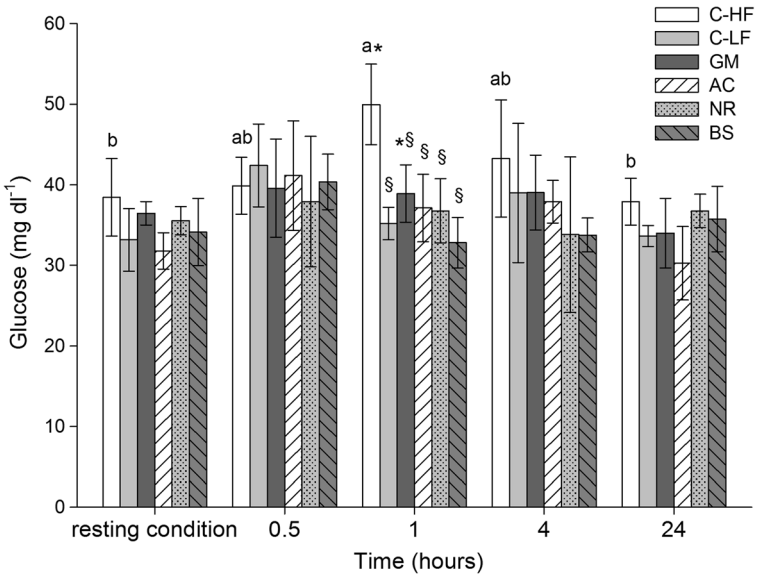

Fig. 2 Plasma glucose concentrations in nanograms per milliliter after stress treatment collected from turbots of the six dietary treatments. Data are presented as mean ± SD ($n = 6$). *Different letters* denote significant differences within the same treatment, and *asterisk* and *section sign* denote significant differences between treatments at the same time point ($p < 0.05$)

stress response in juvenile turbot maintained on reduced fish meal (FM) diets.

A FM reduction to 320 g kg^{-1} diet (44% protein derived from FM) did not affect innate immune activity in turbot, indicating that dietary SPC and WG do not compromise the potential of lysozyme and leucocyte ROS production to fight pathogens. Similar trends were observed in immune activities of Atlantic salmon (*Salmo salar*), gilthead sea bream (*Sparus aurata*), and turbot feeding on plant protein (PP)-rich diets (Bransden et al. 2001; Sitjà-Bobadilla et al. 2005; Kokou et al. 2012; Zheng et al. 2013). Stimulated respiratory burst of head kidney leucocytes did not show any changes in sea bream fed diets reduced to 350 and 110 g kg^{-1} FM, respectively (Sitjà-Bobadilla et al. 2005; Kokou et al. 2012). In general, plasma lysozyme activities between 971 and 1108 U ml^{-1} reveal relatively high values compared to activities between 8.8 and 9.4 U ml^{-1} (Zheng et al. 2013), between 500 and 900 U ml^{-1} (Sun et al. 2016), and between 900 and 1500 U ml^{-1} (Ogier de Baulny et al. 1996) in turbots, indicating an intact function of innate immune response in fish. No sign of inflammation was observed in the current study, unlike previous studies where high inclusion of PP in diets also increased immune responses in fish, an effect interpreted as either immunostimulating or inflammatory effects (Gabrielsen and Austreng 1998; Krogdahl et al. 2000).

Previous investigations proved capabilities of seaweed extracts and β-glucan products to enhance a respiratory burst activity of phagocytes in turbot, gilthead sea bream, Atlantic cod (*Gadus morhua*), and Atlantic salmon, after in vitro stimulation (Castro et al. 1999, 2004; Bridle et al. 2005; Caipang et al. 2011), although positive findings were not confirmed in vivo, feeding salmon β-glucan for 1 week. A short-term administration of dietary β-glucan and alginic acid (15 to 30 days) and that of oligonucleotides (42 to 56 days) improved some innate immune responses in fish (*D. labrax*, *Morone chrysops* × *Morone saxatilis* hybrid) (Li et al. 2004; Bagni et al. 2005). However, increased immune activities did not last for 112 weeks after long-term feeding of hybrid sea bass. Similarly, dietary supplementation with immunostimulating substances did not have any significant effects on lysozyme and ROS activities in turbot after an apparent long-term feeding of 84 days in this study, although a clear tendency towards increased activities is observable. In accordance with this finding, nucleotides in low fish meal diets (400 g kg^{-1} FM) did not affect activities of serum superoxide dismutase and catalase in juvenile turbot, although improved the antioxidative capacity after 60 days (Meng et al. 2016). Only tendencies of higher serum lysozyme activity and neutrophil oxidative radical anion production were found in red drum (*Sciaenops ocellatus*) with increasing nucleotide levels, 0.5 and 1%, in diets of 580 g kg^{-1} FM (Cheng et al. 2011). Similarly, dietary fructooligosaccharide (FOS) supplementation did not significantly improve turbot's innate humoral parameters, like lysozyme, and hematology when fed at 50% PP diets for 63 days at 15 and 20 °C water temperature (Guerreiro et al. 2014). On the other hand, dietary supplementation with yeast (*S. cerevisiae*) did improve the immune response in RAS-reared turbot (151 g mean initial weight) after 72 days (Li et al. 2008). Recent investigations on the effect of nucleotides and *B. subtilis* supplementation, respectively, in FM-reduced diets revealed positive effects on some immune parameters, either lysozyme or respiratory burst activity, in turbot and yellow croaker (*Larimichthys crocea*) (Ai et al. 2011; Peng et al. 2013). However, turbot individuals of another age/size class (9.2 vs. 96 g mean initial weights in the present study) were used and fed diets with increasing soy bean meal content for 60 days. Stressful farming conditions, e.g., vaccination, can impair physiological conditions and immune response. As an example, a β-glucan administration showed no effect in turbot, but combined with a vaccine, adjuvants increased immune activities compared to single vaccination (Ogier de Baulny et al. 1996; Figueras et al. 1998). Burrells et al. (2001b) confirmed this finding for vaccinated salmon whose immunity and survival were supported by a combined inclusion of 0.03% nucleotides that significantly enhanced the efficacy of vaccination. Obviously, the value of certain feed supplements varies not only between fish species and between methods of application, but is also highly dependent on the nutritional and physiological status of fish. Therefore, the influence of immunostimulants on fish has to be interpreted carefully, because many factors can change physical reactions to stimulants. Researchers assume that active compounds of dietary immunostimulants, like β-glucans, act as a ligand and may bind to specific β-glucan receptors on macrophages and neutrophils in fish influencing the functional status of phagocytes (Engstad and Robertsen 1993; Ainsworth 1994). At present, however, an uptake of particulate compounds, e.g., yeast β-glucans, in intestinal cells and the blood

system of fish to stimulate immune cells and components has not been proved yet (Dalmo and Bøgwald 2008).

A balance of essential amino acids (EAAs) in diets with high PP content, meeting the requirements of turbot, is essential for optimal growth and likely immune response in fish. Previous studies have shown high substitution with SPC and WG reduces growth performance in juvenile turbot (Bonaldo et al. 2011; Fuchs et al. 2015). However, these alternative ingredients did not affect turbot's immune capacity in the current study. Therefore, turbots tolerate relatively high contents of dietary SPC and WG associated with a balanced EAA profile to maintain normal immune function. It can, however, be assumed that gross energy demand for maintenance increases with dietary PP inclusion as indicated by Dietz et al. (2012) and, hence, affects the capacity to regulate immune responses.

In contrast of the unchanged immune functions, turbot's physiological status declined with FM replacement in terms of reduced plasma cholesterol levels. However, dietary supplementation of NR, GM, and AC showed a positive physiological change of juvenile turbot, as indicated by elevated cholesterol and triglyceride (NR, GM) concentrations. Plasma protein levels were not found to be modified with reduced FM content. Similar findings were reported for turbot and sea bream fed diets high in corn, wheat, or soy proteins (Regost et al. 1999; Sitjà-Bobadilla et al. 2005; Bonaldo et al. 2014).

At present, little has been published on the influence of dietary PP inclusion on stress response in fish (Bonaldo et al. 2014). High blood cortisol levels, above 50 ng ml^{-1}, are accepted as indicators of stress in fish (Barton and Iwama 1991; Wendelaar Bonga 1997). Base cortisol levels (4.9–16.5 ng ml^{-1}) of most turbots in the current study were similar to reported values for turbots at resting condition (Waring et al. 1996; Mugnier et al. 1998). Individual turbots in all replicate tanks of the six treatments exhibit higher base cortisol levels (>8 ng ml^{-1}) resulting in high variability in tanks (coefficient of variation: C-HF = 11, 36, and 75%; C-LF = 71, 87, and 118%; GM = 65, 78, and 84%; AC = 40, 70, and 71%; NR = 33, 61, and 61%, BS = 73, 117, and 125%). Discrepancies in basal cortisol levels between different studies may be the result of differential methods of killing, capture, or differences in overall holding conditions. In other studies, turbots were either killed using ice water (Reiser et al. 2010) and a blow to the head (Van Ham et al. 2003) or kept alive and cannulated (Waring et al. 1996; Mugnier et al. 1998). Holding conditions, e.g., temperature, stocking rate, or feeding, can modify cortisol resting values in fish and incidences of a stress response (Van Ham et al. 2003; Davis 2004). It is also possible that a visual contact with the sampler elicited a slight cortisol elevation in some fish, perhaps in anticipation of feeding in the morning. Among dietary treatments, stocking density was highest in C-HF which might have caused higher base cortisol levels in fish and higher variances between tanks.

A primary stress reaction induced by capture, netting, and crowding was clearly evidenced by significantly elevated cortisol values at 0.5 and 1 h. These were comparable to post-stress cortisol levels in turbot and gilthead sea bream (Van Ham et al. 2003; Ganga et al. 2011; Tahmasebi-Kohyani et al. 2012). But, cortisol levels in turbots remained quite low, in particular for the immunostimulant- treated fish. Only *unsupplemented* fish fed the control diets reached cortisol levels (59 and 60 ng ml^{-1}) above the 50 ng ml^{-1} threshold. During the repeated sampling at 0.5, 1, 4, and 24 h post stress, care was taken that all turbots from a particular tank were collected quickly to avoid further handling disturbance that may raise cortisol levels of the remaining tank occupants. According to Van Ham et al. (2003) and Mugnier et al. (1998), turbots are relatively insensitive to repeated handling disturbances. The peak stress responses, though with differences between the low fish meal groups C-LF and GM, AC, NR, and BS, were already recorded at 0.5 h post stress when no further disturbances had occurred. Cortisol levels returned to resting levels within 4 h, indicating an acute stress reaction after a short-term stress incidence. Post-exercise recovery was faster than the 24 h reported following capture and 9 min net confinement for turbot (Waring et al. 1996). However, concentrations of blood cortisol can vary between fish species, time of sampling depending on fish's circadian rhythm, and type and severity of stress (Pickering and Pottinger 1989; Barton and Iwama 1991). To our knowledge, no studies evaluating the influence of daytime on cortisol and glucose levels in turbot have been published. Compared to circadian rhythms of fish species, our data detected no similar pattern of stress response affected by these rhythms (Cerdá-Reverter et al. 1998; Montoya et al. 2010; Oliveira et al. 2013). In general, fluctuations of cortisol values due to a circadian rhythm show to be marginal during the course of the day when fish were not fed. Ferrari et al. (2015) also observed differences in

an individual behavior of fish held in groups identified as divergent coping styles that affect the intensity of stress response and release of corticosteroids.

Blood glucose level, a typical secondary stress response (Barton and Iwama 1991), increased parallel to those of cortisol in fish of the high FM control diet. In contrast, tendencies of increased blood glucose levels in low FM groups after 0.5, 1, and 4 h of handling exposure were apparent but did not reflect the pattern of cortisol release as a physiological stress response. Differences in magnitudes of the secondary stress response may be due to a higher stocking density of the high FM group due to higher individual weight at the end of the previous feeding trial. However, previous studies have detected that turbot did not exhibit a strong plasma glucose response when compared to other finfish, which may represent a fundamental difference in turbot stress physiology (Waring et al. 1992, 1996; Van Ham et al. 2003). Apparently, plasma cortisol is a more sensitive indicator of handling stress for turbot than plasma glucose changes.

All additives proved beneficial in suppressing stress response modifying the metabolism in turbot. Similarly, positive effects were noted in mannan oligosaccharide (MOS)-, Ergosan-, and nucleotide-treated European sea bass (*D. labrax*), rainbow trout, and sole (*S. solea*) subjected to stressors such as vaccination, bacterial or viral infection, and handling/crowding, respectively (Leonardi et al. 2003; Gioacchini et al. 2008; Tahmasebi-Kohyani et al. 2012; Torrecillas et al. 2012; Palermo et al. 2013).

Repeated stressors in routine husbandry procedures can affect strong physiological stress responses in turbot associated with impaired homeostasis and immunosuppression and changes in the energy metabolism (Mommsen et al. 1999; Costas et al. 2013). Particularly, stress response is associated with energetic costs to cope with stress, while consequently, less energy is available for other biological functions, including defense mechanisms (immune system) and physiological processes (e.g., growth) (Wendelaar Bonga 1997). Therefore, preventive methods and technologies have to be developed to ensure fish welfare and health to maintain profitable fish production.

In conclusion, juvenile turbot diets containing 320 g kg^{-1} FM and mixed PP (SPC and WG; 560 kg^{-1} diet) for juvenile turbots did not significantly affect the important innate immune mechanisms (lysozyme activity and reactive oxygen production of neutrophils). Dietary supplementation of immunostimulants (β-glucan/MOS, alginic acid, nucleotides/RNA, *Bacillus* strains) failed to enhance immunity; however, all effectively diminished an increase of blood cortisol levels in turbot subjected to handling stress. Further studies are required to determine an optimal inclusion level of PP that enables immunostimulants to activate immune responses in fish.

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

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