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The effect of supplementation with polysaccharides, nucleotides, acidifiers and *Bacillus* strains in fish meal and soy bean based diets on growth performance in juvenile turbot (*Scophthalmus maximus*)

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ABSTRACT

The investigation and application of a wide range of dietary supplements, such as probiotics, prebiotic and other additives, are increasingly popular in aquaculture research and practice. To date few studies have attempted to quantify the value of commercially available additives in improving growth performance of juvenile turbot (*Scophthalmus maximus*) and in compensating potential growth reduction resulting from high levels of plant protein (PP) in carnivorous fish diets.

Two experiments were conducted to investigate the effect of different active ingredients in diet additives on turbot. I) Five diets supplemented with (1) yeast b-glucan and mannan oligosaccharides (GM), (2) alginic acid from brown algal extracts (AC), (3) yeast nucleotides and RNA (NR), (4) potassium diformate (PDF) and (5) bacteria strains *Bacillus subtilis and B. licheniformis* (BS), containing fish meal (FM) as the only protein source, were fed to turbots (initial weight 48.8 g ± 5.2 g) over 112 days. II) Four diets supplemented with (1) GM, (2) AC, (3) NR and (4) BS, containing soy protein concentrate (SPC) and wheat gluten (WG) as a partial replacement of FM, were fed to turbots (initial weight 95.8 g ± 17.7 g) over 84 days. A non-supplemented FM diet (exp. I) and an FM- and PP-based diet (exp. II), respectively, were used as control diets.

Diet additives did not promote additional weight gain, specific growth rate (SGR), daily feed intake (DFI) and feed conversion ratio (FCR) in turbot fed FM- or PP-based diets ($p > 0.05$) when compared to isocaloric control diets in both experiments. Growth of turbots fed the high FM content control diet (II) was significantly higher than all other treatments ($p < 0.01$). Body proximate composition, condition factor (K) and liver index (HSI) remained unaffected by additive supplementation in fish fed either FM or PP diets ($p > 0.05$).

Results indicate that reported benefits for specific diet additives cannot be assumed to function or applied across species boundaries and age classes. In addition, dietary additive application may not be economically valid for larger animals and/or animals not exposed to specific culture-related stressors. The benefits of popular additives to high value species such as *S. maximus* remains to be tested under specific immune or physical stress situations and at crucial larval and early juvenile stages.

1. Introduction

Efforts to intensify aquaculture of valuable finfish, such as turbot (*Scopthalmus maximus*) can lead to increased stress, limited growth performance and poor welfare (Dalsgaard et al., 2013; Tal et al., 2009). Managing and avoiding outbreaks of infectious diseases are a challenge, particularly, since EU regulations banned the use of antibiotics as growth and health promoters in livestock production (EU 2003; EC No 1831/2003). Animal health and nutritional conditions are of particular importance to fish farmers to ensure a high quality and sustainable product for the consumer. A proper diet is essential to improve fish health and reduce susceptibility of fish to diseases. Turbot diets are recommended to have 500 up to 650 g kg^{-1} protein (dry matter) with fish meal (FM) as the main protein source (Cho et al., 2005; Lee et al., 2003). For the growing production of turbot, 5.5 kt to 12.7 kt (2002 to 2012), in Europe, a rising demand for fish meal is evident (FAO, 2014).

However, rising demand and limited supply (due to the sustainable use of fish stocks and therefore reduced fishery production, El Niño

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events, etc.) of high quality fishmeal have increased prices and forced the feed industry to partly substitute FM with alternative protein sources, mainly protein-rich plant ingredients (Rana et al., 2009; Tacon and Metian, 2008; Watanabe, 2002). Soy protein concentrate (SPC) and wheat gluten are popular alternative protein sources in aquafeeds due to their favorable profile of essential amino acids (EAAs), competitive price and availability (Gatlin et al., 2007; Hardy, 2010; Storebakken et al., 2000). Substitution with plant protein (PP) can, however, reduce growth performance, feed utilization and fish health due to diminished palatability, EAA deficiency, reduced energy content, antinutritional factors and lower nutrient digestibility compared to fish meal (Bakke-McKellep and Refstie, 2008; Bonaldo et al., 2011; Francis et al., 2001; Krogdahl et al., 2010). An alternative approach to reduce adverse factors of PPs may be the inclusion of diet additives that can improve growth performance in fish and, possibly, compensate performance loss in low FM diets.

A range of diet additives, including probiotics, prebiotics, acidifiers and plant or animal derived extracts, are commercially available for aquatic animals. Previous studies have evaluated several of these additives on their effect on growth performance, immune response and disease resistance, as well as intestinal microbial communities for various fish species (Balcázar et al., 2006; Kesarcodi-Watson et al., 2008; Merrifield et al., 2010; Ringø et al., 2010). The polysaccharides beta-1,3/1,6-glucans (BG) and mannan oligosaccharides (MOS), isolated from cell walls of yeasts, plants, algae, fungi or bacteria, are widely accepted as diet ingredients with positive effects on growth and health (Bohn and BeMiller, 1995; Meena et al., 2013; Zeković et al., 2005). Treatments with BG and MOS proved to promote growth performance in fish (Ai et al., 2007; Andrews et al., 2009; Kühlwein et al., 2014; Li et al., 2008; Misra et al., 2006; Staykov et al., 2007; Torrecillas et al., 2012; Yoo et al., 2007).

Among the many other substances and extracts investigated as diet additives, macroalgae and macroalgal extracts are rich in polysaccharides (e.g. alginic acid, laminarin, fucoidan) and contain bioactive substances (e.g. vitamins or polyphenols) that are known to affect animal health (Buchholz et al., 2012; Fleurence, 1999; Gupta and Abu-Ghannam, 2011; Holdt and Kraan, 2011; MacArtain et al., 2007). Macroalgae extracts containing alginic acids are reported to enhance growth performance in a variety of fish species (Ahmadifar et al., 2009; Heidarieh et al., 2011, 2012; Sheikhzadeh et al., 2012). Dietary uptake of exogenous nucleotides, isolated from yeast, may optimize cell proliferation in order to promote rapid growth, as the synthesis of nucleotides is a metabolically costly process (Sanderson and He, 1994). Particularly under stressful conditions additional nucleotides can be needed, for instance, for further signal transduction or immune cell proliferation (Carver and Walker, 1995; Li and Gatlin, 2006). The application of nucleotides has demonstrated a positive influence on growth performance when added to formulated fish diets (Burrells et al., 2001b; Lin et al., 2009; Tahmasebi-Kohyani et al., 2012).

Furthermore, acidifiers consisting of organic acids and their salts, used for instance as preservatives, are considered as promising growth promoters in animals (Lückstädt, 2008). Dietary supplementation of citric acid and potassium diformate showed improved growth and feed utilization in some fish species (Abu Elala and Ragaa, 2014; Baruah et al., 2007; Hossain et al., 2007). In addition, probiotics or beneficial bacteria are known to control pathogens through a variety of mechanisms and affect intestinal microbial communities (Kesarcodi-Watson et al., 2008). In some fish species the dietary inclusion of *Bacillus subtilis* and *B. licheniformis* strains had a positive effect on growth performance (Bairagi et al., 2004; He et al., 2011; Kumar et al., 2006; Raida et al., 2003).

Despite the progress made with various fish species, the effect of the above-mentioned feed additives on growth performance and feed utilization of commercially important turbot remains limited (Li et al., 2008; Peng et al., 2013; Yun et al., 2011). Few studies have investigated the effectiveness of diet additives on performance and health in fish comparing FM- and PP-based diets (Dimitroglou et al., 2010; Peng et al., 2013; Salze et al., 2010; Yun et al., 2011). Applied research, such as the current study, is needed to fill the knowledge gaps regarding feed additives' potential to support the increased use of plant proteins in diets for carnivorous fish species. The current study aims to determine the effect of selected feed additives on growth performance of turbot fed FM- and PP-based diets.

2. Materials and methods

Two trials were carried out to test the capacity of commercially available feed additives with the active ingredients, (1) yeast b-glucan and mannan oligosaccharides (GM), (2) alginic acid from brown algal extracts (AC), (3) purified yeast nucleotides and ribosomal RNA (NR), (4) acidifier potassium diformate (PDF; only used in trial I) and (5) probiotic bacteria strains *Bacillus subtilis and B. licheniformis* (BS), to improve growth performance (I) and/or to compensate performance loss in juvenile turbots (*S. maximus*) resulting from dietary fish meal reduction (II). The growth performance and feed utilization of fish fed (I) a high quality diet with 77% fish meal content and (II) a fish meal reduced diet with 32% fish meal were determined. Feeding experiments were conducted in two separate trials I (January to May 2013) and II (October 13 to January 14) as the experimental set-up and high number of treatments did not allow a simultaneous performance of both trials in one experiment.

2.1. Trial I: Experimental setup

Juvenile turbot, approx. 15 g in weight, were obtained from Maximus A/S (Bedsted Thy, Denmark). Fish were examined for infectious diseases at the beginning and at the end of the experiments to confirm suitability as experimental animals. Prior to the experiment, fish were acclimatized for 10 weeks in a recirculating aquaculture system (RAS) in the Center for Aquaculture Research (ZAF) at the Institute for Marine Resources (IMARE) in Bremerhaven (Germany). During acclimatization turbot were fed in the morning and the afternoon at a rate of 2.0% BW^{-1} day^{-1} of a commercial dry feed with 55% crude protein and 16% crude fat (R Europa 15, 2 mm diameter; Skretting ARC, Stavanger, Norway). The system had a total water volume of 40 m^3 and was equipped with drum filter, protein skimmer, moving bed biofilter and disinfection unit (Ozon generator; Sander Aquatec GmbH, Uetze-Eltze, Germany). The experiments were 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 photoperiod was maintained at a 12 h light: 12 h dark cycle throughout. Water parameters, such as dissolved oxygen (8.8 ± 0.3 mg l^{-1}), temperature (16.7 ± 0.5 °C) and salinity (30.8 ± 3.3 g l^{-1}) were monitored constantly with a SC 1000 Multiparameter Universal Controller (Hach Lange GmbH, Düsseldorf, Germany). Ammonia, nitrite and nitrate were measured daily before feeding (0.05 ± 0.09 mg l^{-1} NH_4–N, 0.33 ± 0.18 mg l^{-1} NO_2–N, 390.9 ± 92.1 mg l^{-1} NO_3–N; photometer DR 2800; Hach Lange GmbH, Düsseldorf, Germany).

A total of 1,440 turbot with an initial mean body weight of 48.8 g (±5.2 g) and initial mean standard length of 13.7 cm (±0.6 cm) were randomly allocated to 36 experimental tanks (0.8 m^2 bottom surface, 500 L water volume; 40 individuals $tank^{-1}$; stocking density 50 fish m^{-2} or 2.4 kg m^{-2}). Six feeding groups were assigned to the tanks allowing six replicates per treatment. Over the entire experimental period of 16 weeks fish were hand-fed with floating pellets to apparent satiation twice a day (10:00 and 14:00). All uneaten feed was netted (mash size = 500 μm) out of the tanks 30 min after start of the feeding, dried at 50 °C for 24 h and weighed. The weights of daily recovered pellets were corrected for soluble losses using a factor which was calculated from the difference between dry weight of pellets before and after

recovering. Therefore, pellets (approximately 5 g) of each diet were soaked in system water for 15 min, dried at 50 °C for 24 h and weighed.

2.2. Trial I: Experimental diets

A control diet (CT) was formulated with 77% FM to contain 61% crude protein and 22 MJ kg^{-1} gross energy. The composition and concentration of nutrients (moisture, crude protein, crude fat, ash, phosphorus and calcium) and gross energy of the six formulated diets are presented in Table 1. The other six experimental diets were formulated with regards to an isonitrogenous and isocaloric content and were supplemented with active ingredients of commercially available feed additives: (1) a yeast (*Saccharomyces cerevisiae*) product consisting of 20% beta-1,3/1,6 glucans and 17% mannan oligosaccharides (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-5 V-monophosphate (CMP), disodium uridine-5 V-mono-phosphate (UMP), adenosine-5 V-monophosphate (AMP), disodium inosine-5 V-monophosphate (IMP), disodium guanidine-5 V-monophosphate (GMP)) and ribosomal RNA (Vannagen®, Chemoforma Ltd., Augst, Switzerland) (NR), (4) an acidifier product of potassium diformate containing 35% free formic acid, 35% formate and 30% potassium (Aquaform®, ADDCON/Nordic AS, Porsgrunn, Norway) (PDF) and (5) a probiotic product of bacteria strains *Bacillus subtilis* and *B. licheniformis* (Probiotic-plus.ru, Russia) (BS). The ingredients were mixed using a spiral mixer (WP Kemper President 75 AF-V, Emil Kemper GmbH, Rietberg, Germany) and extruded to floating pellets of 3 mm in diameter using a twin-screw extruder (Bühler 2-Wellen-Extruder DNDL-44, Bühler AG, Uzwil, Schweiz) at temperature of 95–110 °C. Subsequently, pellets were dried (Bühler OTW-25/50, Schweiz) and coated with oil under constant mixing using a wendel mixer (WV 240a, DIOSNA Dierks & Söhne GmbH, Osnabrück, Germany). All diets were sieved at the end to discard fractions below 3 mm.

Table 1
Ingredients, nutrient composition in g kg^{-1} dry matter (DM) and gross energy in MJ kg^{-1} DM of the experimental diets in trial 1.

	Diets					
	CT	GM	AC	NR	PDF	BS
Ingredients [g kg^{-1}]						
Fish meal[a]	777.0	777.0	777.0	777.0	777.0	777.0
Wheat gluten[b]	30.0	29.5	29.5	28.0	30.0	30.0
Wheat starch[b]	110.0	104.5	105.5	110.0	107.0	109.4
GM	0.0	6.0	0.0	0.0	0.0	0.0
AC	0.0	0.0	5.0	0.0	0.0	0.0
NR	0.0	0.0	0.0	2.0	0.0	0.0
PDF	0.0	0.0	0.0	0.0	3.0	0.0
BS	0.0	0.0	0.0	0.0	0.0	0.6
Fish oil[c]	74.0	74.0	74.0	74.0	74.0	74.0
Vitamin/mineral mixture[d]	7.0	7.0	7.0	7.0	7.0	7.0
Titanium dioxide[e]	2.0	2.0	2.0	2.0	2.0	2.0
Nutrient composition[f] [g kg^{-1}]						
Moisture	34	29	34	34	33	35
Crude protein	614	621	611	617	618	604
Crude fat	149	147	146	148	152	149
Crude ash	136	134	137	134	139	138
Calcium	30	29	29	29	29	31
Phosphorus	21	21	21	21	21	22
Gross energy [MJ kg^{-1}][g]	22	22	22	22	22	22

CT = control, GM = b-glucan/MOS, AC = alginic acid, NR = nucleotides/RNA, PDF = potassium diformate and BS = *Bacillus* spp. Additive concentrations were recommended by manufacturers and literature (Burrells et al., 2001a,b; Lückstädt, 2008; Merrifield et al., 2011).

[a] Köster Marine Proteins GmbH, Hamburg, Germany.
[b] Kröner Stärke, Ibbenbüren, Germany.
[c] Vereinigte Fischmehlwerke Cuxhaven GmbH & Co KG, Cuxhaven, Germany.
[d] Spezialfutter Neuruppin GmbH & Co. KG, Neuruppin, Germany.
[e] Kronos Titan GmbH & Co.OHG, Nordenham, Germany.
[f] 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), ash (VDLUFA Bd. III 8.1); ICP-mass spectrometry: calcium and phosphor (PM DE01_018).
[g] Bomb calorimeter (6100, Parr Instrument GmbH, Frankfurt a. M., Germany).

2.3. Trial II: Experimental setup

The experimental set-up was identical to trial 1 (see Section 2.1). Water parameters, such as dissolved oxygen (9.3 ± 0.5 mg l^{-1}), temperature (17.3 ± 0.5 °C) and salinity (28.6 ± 1.4 g l^{-1}) were monitored constantly. Ammonia, nitrite and nitrate were measured in a three days interval before feeding (0.01 ± 0.02 mg l^{-1} NH_4–N, 0.04 ± 0.03 mg l^{-1} NO_2–N, 80.6 ± 16.7 mg l^{-1} NO_3–N; photometer DR 2800).

900 turbot individuals with an initial mean body weight of 95.8 g (±17.7 g) and initial mean standard length of 18.0 cm (±1.1 cm) were used for this experiment and randomly placed into the experimental tanks (25 individuals $tank^{-1}$; stocking density 31.3 fish m^{-2} or 3.0 kg m^{-2}). Each of the six feeding groups contained six replicates (n = 36 tanks). Feeding was done as described for trail 1 (see 2.1) over a period of 12 weeks.

2.4. Trial II: Experimental diets

A high FM control diet with 58% FM (C-HF) and a low FM control diet with 32% FM (C-LF) were formulated to contain 56% crude protein and 22 MJ kg^{-1} gross energy (Table 2). Protein content in C-LF was partly replaced with soy bean concentrate and wheat gluten with an inclusion of 56% PP. The reduction of FM to 32% was chosen as studies showed that growth performance in turbot was significantly decreased with a FM

Table 2
Ingredients, nutrient composition in g kg^{-1} dry matter (DM) and gross energy in MJ kg^{-1} DM of the experimental diets in trial 2.

	Diets					
	C-HF	C-LF	GM	AC	NR	BS
Ingredients [g kg^{-1}]						
Fish meal[a]	585.0	320.0	320.0	320.0	320.0	320.0
Soy protein concentrates[a]	125.0	250.0	250.0	250.0	250.0	250.0
Corn gluten[b]	30.0	40.0	40.0	40.0	40.0	40.0
Wheat gluten[c]	20.0	147.0	146.7	146.8	146.9	147.0
Wheat starch[c]	184.0	160.0	154.3	155.2	158.1	159.4
GM	0.0	0.0	6.0	0.0	0.0	0.0
AC	0.0	0.0	0.0	5.0	0.0	0.0
NR	0.0	0.0	0.0	0.0	2.0	0.0
BS	0.0	0.0	0.0	0.0	0.0	0.6
Fish oil[d]	45.0	72.0	72.0	72.0	72.0	72.0
Vitamin/mineral mixture[e]	10.0	10.0	10.0	10.0	10.0	10.0
Titanium dioxide[f]	1.0	1.0	1.0	1.0	1.0	1.0
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

C-HF = high fish meal control, C-LF = low fish meal control, GM = b-glucan/MOS, AC = alginic acid, NR = nucleotides/RNA and BS = *Bacillus* spp. Additive concentrations were recommended by manufacturers and literature (Burrells et al., 2001a,b; Merrifield et al., 2011).

[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), 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).

content below 40% and a PP level above 30%, respectively (Bonaldo et al., 2011; Day and Plascencia González, 2000). The other four experimental diets were formulated on the basis of control diet C-LF and were supplemented with commercially available feed additives: (1) GM, (2) AC, (3) NR and (4) BS (for details see 2.2). All diets were formulated with regards to an isonitrogenous and isocaloric content. Composition and concentration of crude nutrients, minerals and gross energy of the six formulated diets are presented in Table 2. The preparation of the diets was identical to trial 1 (see 2.2). Diets were extruded to floating pellets of 5 mm in diameter.

2.5. Sampling, measurements and calculations

All fish were individually weighed and the total length was measured at the beginning and the end of the experiment as well as at 4-week intervals. Prior to weighing fish were starved for 24 h. Deriving from weight and length measurements weight gain, specific growth rate (SGR) and body condition factor (K) were determined for each fish according to the formulae:

(1) Weight gain (g) = final weight − initial weight,
(2) SGR (% body weight day^{-1}) = [ln(final weight) − ln(initial weight)]/feeding days × 100,
(3) K (%) = 100 × final body weight × final body $length^{-3}$.

The actual total feed intake (FI_{total}) was determined by subtracting the dried feed remnants ($F_{uneaten}$) from feed offered ($F_{offered}$) after correcting for soluble losses during feeding:

(4) FI_{total} (g) = $F_{offered}$ − ($F_{uneaten}$ × $factor_{soluble\ loss}$).

Daily feed intake (DFI) and feed conversion ratio (FCR) were calculated according to the formulae:

(5) DFI (% BW day^{-1}) = 100 × FI_{total}/[(initial weight + final weight)/2]/feeding days,
(6) FCR = FI_{total}/weight gain.

For a determination of the hepatosomatic index (HSI) in trial I, livers of 72 individuals (two fish per tank = 12 fish per treatment) were sampled after 16 weeks and, in trial II, livers of 108 individuals (three fish per tank = 18 fish per treatment) were sampled after 12 weeks of feeding. Before sampling, fish were killed with an overdose (500 mg/L^{-1}) of the anesthetic tricaine methane sulfonate (MS 222; Sigma-Aldrich Co. LLC., Munich, Germany). Liver weight and fish weight were recorded and the HSI was calculated for each fish according to the formula:

(7) HSI (%) = (liver weight/final body weight) × 100.

2.6. Whole body composition

At experimental outset 10 fish and at the end of the experiment six fish per dietary treatment (n = 6), respectively, were freeze-dried (Alpha 1–4 LSC, Martin Christ GmbH, Osterode a. H., Germany) and homogenized (grinder GRINDOMIX GM 200, Retsch GmbH, Haan, Germany) for an analysis for their respective proximate composition. The gross energy was determined using a bomb calorimeter (6100, Parr Instrument GmbH, Frankfurt a. M., Germany). Analysis for moisture, crude lipid (CL) and ash were carried out by Intertek Food Services GmbH (Bremen, Germany) following the VDLUFA protocols (Bd. III 3.1, Bd. III 5.1.1 and Bd. III 8.1). Total nitrogen content was determined by the Kjeldahl method (L 06.00-7 (mod.)). CP content of the fish body was calculated by multiplying N by 6.25.

2.7. Statistics

Data are presented as mean ± standard deviation (S.D.) for each treatment. The Sigma plot 11 for Windows (Systat Software Inc., San Jose, CA, USA) software package was used for statistical evaluations. Data of growth parameters, feed utilization, whole body composition and condition parameters (K, HSI) were tested for normality distribution by Shapiro-Wilk test. If normality or homogeneity of variances was confirmed, multiple comparisons were done by one-way analysis of variance (ANOVA) followed by the post hoc Tukey's Honestly Significant Difference (HSD) test or Dunn test. The non-parametric Kruskal–Wallis test was used when the normality assumption was not met. Differences between set of comparisons were considered significant at a probability of error at $p < 0.05$.

3. Results

3.1. Trial I

3.1.1. Mortalities, growth performance and body composition

Mortality was low (0.4 to 2.2%, $p > 0.05$; Table 3) and turbot remained otherwise healthy throughout the experiment. Mean initial weight (by treatment) ranged from 48.6 ± 0.6 g to 49.0 ± 0.3 g. Final fish weight (g) and weight gain (g) ranged from 231.8 ± 50.2 (PDF) to 251.5 ± 52.1 (GM) and 183.2 ± 12.1 (PDF) to 202.9 ± 12.2 (GM), respectively (Table 3). SGR (%) of fish ranged from 1.39 ± 0.04 (PDF) to 1.47 ± 0.04 (GM). No significant differences in growth performance were detected between dietary treatments ($p > 0.05$). Fish DFI (% BW d^{-1}) ranged from 0.86 ± 0.02 (PDF) to 0.90 ± 0.03 (BS) across all treatments (Table 3). FCR of fish ranged from 0.73 ± 0.01 (CT) to 0.76 ± 0.02 (BS). Results of DFI and FCR showed no significant differences among all dietary treatments ($p > 0.05$). Fish K (%) ranged from 2.16 ± 0.10 (AC) to 2.21 ± 0.04 (CT) and fish HSI (%) ranged from 1.07 ± 0.13 (AC) to 1.32 ± 0.14 (GM) across all treatments (Table 3). K and HSI did not significantly differ between dietary treatments ($p > 0.05$).

Crude protein (% dry matter (DM)) of the whole body composition ranged from 69.7 ± 1.1 (BS) to 72.3 ± 2.0 (PDF) (Table 4). Crude lipid (% DM) ranged from 11.2 ± 4.4 (CT) to 13.7 ± 0.8 (BS) (Table 4). Gross energy (MJ kg^{-1} DM) ranged from 20.0 ± 1.4 (CT) to 21.5 ± 0.8 (BS) (Table 4). All results were not significantly different among dietary treatments ($p > 0.05$).

3.2. Trial II

3.2.1. Mortalities, growth performance and body composition

Turbot were healthy throughout the experiment and mortality rates were low (maximum 0.7%, $p > 0.05$; Table 5). Weight (g) at experimental outset ranged between 95.7 ± 0.1 and 95.9 ± 0.8. Highest final weight (299.5 ± 92.0 g), weight gain (203.7 ± 21.8 g) and SGR (1.35 ± 0.09%) were observed in fish fed the high FM-diet (C-HF) (Table 5). Growth performance of fish was significantly different compared to fish fed the low FM-diets ($p < 0.01$). Final weight, weight gain and SGR of fish fed the low FM-diets ranged from 246.3 ± 71.1 (NR) to 257.5 ± 70.4 (AC), 150.3 ± 15.3 (NR) to 161.4 ± 16.7 (AC) and 1.12 ± 0.08 (NR) to 1.17 ± 0.08 (AC), respectively (Table 5). No significant differences were achieved between the low FM treatments ($p > 0.05$). DFIs (% BW d^{-1}) ranged from 0.85 ± 0.08 (AC) to 0.95 ± 0.05 (C-HF) (Table 5). The differences between fish DFIs of all treatments were not significant ($p > 0.05$). FCRs of all dietary treatments ranged from 0.83 ± 0.08 (GM) to 0.78 ± 0.01 (C-HF) (Table 5). No significant differences were found among all treatments ($p > 0.05$). Fish K (%) ranged from 1.97 ± 0.08 (BS) to 2.07 ± 0.04 (C-HF) and fish HSI (%) ranged from 1.19 ± 0.14 (C-HF) to 1.40 ± 0.19 (BS) across all treatments (Table 5). K and HSI showed no significant differences between all dietary treatments ($p > 0.05$).

Table 3
Growth performance and feed utilization of turbot fed experimental diets for 112 days in trial 1.

	CT	GM	AC	NR	PDF	BS
Initial weight, g	49.0 ± 0.3	48.6 ± 0.6	48.9 ± 0.5	49.0 ± 0.2	48.6 ± 0.6	48.8 ± 0.2
Final weight, g	251.4 ± 53.7	251.5 ± 52.1	240.7 ± 63.9	242.3 ± 53.9	231.8 ± 50.2	249.2 ± 60.3
Weight gain, g	202.4 ± 20.9	202.9 ± 12.2	191.5 ± 29.4	193.4 ± 19.2	183.2 ± 12.1	200.5 ± 27.6
SGR, % day^{-1}	1.46 ± 0.07	1.47 ± 0.04	1.42 ± 0.11	1.42 ± 0.08	1.39 ± 0.04	1.45 ± 0.10
DFI, % day^{-1}	0.88 ± 0.03	0.89 ± 0.01	0.88 ± 0.03	0.88 ± 0.02	0.86 ± 0.02	0.90 ± 0.03
FCR	0.73 ± 0.01	0.74 ± 0.01	0.75 ± 0.03	0.74 ± 0.02	0.74 ± 0.01	0.76 ± 0.02
Mortality, %	0.0	0.0	2.2	0.4	0.9	0.9
K, %	2.21 ± 0.04	2.18 ± 0.02	2.16 ± 0.10	2.18 ± 0.07	2.17 ± 0.07	2.20 ± 0.07
HSI, %	1.25 ± 0.22	1.32 ± 0.14	1.07 ± 0.13	1.15 ± 0.14	1.11 ± 0.24	1.14 ± 0.21

CT = control, GM = b-glucan/MOS, AC = alginic acid, NR = nucleotides/RNA, PDF = potassium diformate and BS = *Bacillus* spp. SGR = Specific growth rate, DFI = daily feed intake, FCR = feed conversion ratio, K = condition factor, HSI = hepatosomatic index. Each value is mean ± S.D. (n = 6). No significant differences were identified ($p > 0.05$).

Crude protein (% DM) of the whole body composition ranged from 67.2 ± 5.1 (NR) to 72.5 ± 0.4 (BS) (Table 6). Crude lipid (% DM) ranged from 12.0 ± 2.6 (C-HF) and 12.0 ± 4.8 (AC) to 15.6 ± 2.3 (NR). Gross energy (MJ kg^{-1} DM) ranged from 20.8 ± 1.1 (C-LF) to 21.8 ± 0.9 (BS) (Table 6). All results were not significantly different among dietary treatments ($p > 0.05$).

4. Discussion

Diet additives, classified as functional feeds, have recently attracted extensive attention and investment within the aquaculture industry. However, the cost of most additives and the challenge of incorporating them into extruded feeds have to be considered by evaluating benefits against investment. This study has for the first time shown that growth and feed utilization is not positively influenced by either of the five types of diet additives in growing turbot from 50 g weight up to 300 g in a recirculating system. Additive inclusion in extruded diets failed to improve performances of turbots fed either FM-based diets or diets containing partial FM replacement by soy and wheat proteins.

The current results stand in contrast to much of the literature. Li et al. (2008) reported improved growth in turbot, which had an initial weight of 151.3 ± 11.3 g, when fed a pelleted diet supplemented with a commercial yeast product (containing 20% b-glucan and 20% MOS, 1.3 g kg^{-1} in diet) for 72 days. However, SGR results (0.75–0.84% day^{-1}) were in general low compared to values (1.39–1.46% day^{-1}) in this study. Yoo et al. (2007) also observed a positive effect on weight gain, SGR and feed efficiency ratio in olive flounder (*Paralichthys olivaceus*) which was fed diets containing yeast b-glucan, derived from *Saccharomyces cerevisiae*, for 7 weeks. Growth performance was highest at 1 and 1.5 g kg^{-1} b-glucan inclusion which is similar to the concentration of 6 g kg^{-1} GM (20% b-glucan/17% MOS) applied in this study in one of the diets. However, fish had an initial weight of 9.2 g and were much smaller compared to turbots in this study. Likewise, improved weight gain, SGR and FCR were reported in mirror carp (*Cyprinus carpio*) fed a diet with levels of 10 and 20 g kg^{-1} yeast b-glucan (Kühlwein et al., 2014). Dietary yeast MOS (2 and 4 g kg^{-1} diet) enhanced growth performance in European sea bass (*Dicentrarchus labrax*) and promoted growth, FCR and survival in rainbow trout (*Oncorhynchus mykiss*) (Staykov et al., 2007; Torrecillas et al., 2012).

Further investigations observed growth enhancing properties using extracts of brown algae or yeast derived nucleotides as diet additives. Dietary inclusion of algae extracts (5 g kg^{-1} diet) and yeast nucleotides (1.5 and 2 g kg^{-1} diet) had a positive effect on performances in beluga (*Huso huso*), malabar grouper (*Epinephelus malabaricus*), Atlantic salmon (*Salmo salar*) and rainbow trout (Ahmadifar et al., 2009; Burrells et al., 2001b; Heidarieh et al., 2011, 2012; Lin et al., 2009; Tahmasebi-Kohyani et al., 2012). Other studies demonstrated that inclusion of potassium diformate (2 and 3 g kg^{-1} diet) and probiotic *Bacillus* strains (*B. subtilis* and/or *B. licheniformis*) stimulated growth in nile tilapia (*Oreochromis niloticus*), rohu (*Labeo rohita*), carp and trout (Abu Elala and Ragaa, 2014; Bagheri et al., 2008; He et al., 2011; Kumar et al., 2006).

In contrast, some investigations could not confirm a positive performance of diet additives in other fish species. Research demonstrated that b-glucan or MOS enriched diets did not improve growth in dentex (*Dentex dentex*), tilapia, Asian catfish (*Clarias batrachus*), channel catfish (*Ictalurus punctatus*), hybrid tilapia (*O. niloticus* ♀ × *O. aureus* ♂) and Atlantic salmon (Efthimiou, 1996; Grisdale-Helland et al., 2008; He et al., 2009; Kumari and Sahoo, 2006; Lara-Flores et al., 2003; Welker et al., 2007; Whittington et al., 2005). Moreover, supplementation with brown algae extracts, yeast nucleotides and potassium diformate did not support growth compared to the unsupplemented diets in red drum (*Sciaenops ocellatus*) and tilapia (Li et al., 2005; Merrifield et al., 2011; Zhou et al., 2009).

Refstie et al. (2010) proved that supplementation with MOS (2 g kg^{-1} diet) in a FM reduced diet, containing soy bean and sunflower meal (SBM + SFM) as substitutes, improved growth performance in salmon. However, the same MOS concentration in a SBM diet and b-glucan (0.5 and 1 g kg^{-1} diet) inclusion in both SBM + SFM and SBM diets fail to increase the potential of both PP-based diets. Similarly, growth performance and feed utilization of turbot and gilthead sea bream (*Sparus aurata*) remained unaffected by nucleotide (0.3 and 1 g kg^{-1} diet) and MOS (2 and 4 g kg^{-1} diet) supplementation in a FM- and SBM-based diet (Dimitroglou et al., 2010; Peng et al., 2013).

In the present study, turbots have fed on high quality diets in trial I and have been reared under optimal conditions during the experiment. Although diet additives did not improve growth performance under favorable rearing conditions, these additives may have beneficial impacts

Table 4
Proximate whole body composition in % dry matter basis and gross energy in MJ kg^{-1} dry matter of initial fish samples and turbot fed the control and experimental diets over 112 days in trial 1.

	Initial fish (n = 10)	CT	GM	AC	NR	PDF	BS
Proximate body composition							
Dry matter	21.2	20.3 ± 1.5	21.1 ± 0.4	23.3 ± 3.3	22.9 ± 1.3	24.2 ± 0.3	24.3 ± 0.3
Crude protein	65.4	72.2 ± 3.9	71.9 ± 3.3	70.5 ± 2.5	71.6 ± 3.0	72.3 ± 2.0	69.7 ± 1.1
Crude lipid	14.5	11.2 ± 4.4	13.4 ± 3.7	13.5 ± 3.8	12.1 ± 0.8	12.2 ± 3.4	13.7 ± 0.8
Crude ash	17.6	17.6 ± 1.0	14.7 ± 0.9	16.7 ± 1.9	15.7 ± 1.0	16.7 ± 2.7	16.2 ± 0.1
Gross energy (MJ kg^{-1})	21.3	20.0 ± 1.4	20.9 ± 1.4	21.3 ± 0.9	20.5 ± 0.2	20.5 ± 1.7	21.5 ± 0.8

CT = control, GM = b-glucan/MOS, AC = alginic acid, NR = nucleotides/RNA, PDF = potassium diformate and BS = *Bacillus* spp. Each value is mean ± S.D. (n = 6). No significant differences were identified ($p > 0.05$).

Table 5
Growth performance and feed utilization of turbot fed experimental diets for 84 days in trial 2.

	C-HF	C-LF	GM	AC	NR	BS
Initial weight, g	95.8 ± 0.1	95.8 ± 0.1	95.9 ± 0.8	95.9 ± 0.2	95.9 ± 0.3	95.7 ± 0.1
Final weight, g	299.5 ± 92.0[a]	254.2 ± 77.9[b]	251.9 ± 69.6[b]	257.5 ± 70.4[b]	246.3 ± 71.1[b]	254.0 ± 72.0[b]
Weight gain, g	203.7 ± 21.8[a]	158.4 ± 32.2[b]	156.1 ± 19.7[b]	161.4 ± 16.7[b]	150.3 ± 15.3[b]	158.6 ± 20.8[b]
SGR, % day^{-1}	1.35 ± 0.09[a]	1.15 ± 0.15[b]	1.15 ± 0.09[b]	1.17 ± 0.08[b]	1.12 ± 0.08[b]	1.16 ± 0.10[b]
DFI, % day^{-1}	0.95 ± 0.05	0.86 ± 0.07	0.88 ± 0.06	0.85 ± 0.08	0.86 ± 0.05	0.89 ± 0.05
FCR	0.78 ± 0.01	0.81 ± 0.04	0.83 ± 0.08	0.79 ± 0.09	0.82 ± 0.03	0.83 ± 0.07
Mortality, %	0.0	0.0	0.7	0.0	0.7	0.0
K, %	2.07 ± 0.04	2.01 ± 0.07	2.00 ± 0.06	1.99 ± 0.10	2.03 ± 0.05	1.97 ± 0.08
HSI, %	1.19 ± 0.14	1.25 ± 0.17	1.36 ± 0.20	1.28 ± 0.26	1.21 ± 0.19	1.40 ± 0.19

C-HF = high fish meal control, C-LF = low fish meal control, GM = b-glucan/MOS, AC = alginic acid, NR = nucleotides/RNA and BS = *Bacillus* spp. SGR = specific growth rate, DFI = daily feed intake, FCR = feed conversion ratio, K = condition factor, HSI = hepatosomatic index. Each value is mean ± S.D. (n = 6). Different superscript letters within a line denote significant differences ($p < 0.05$).

on fish in challenging situations, for instance under conditions of immunodepression related to environmental stress, as demonstrated in previous studies (Burrells et al., 2001b; El-Boshy et al., 2010; Kumar et al., 2006; Santarém et al., 1997; Tahmasebi-Kohyani et al., 2012; Torrecillas et al., 2012; Yeh et al., 2008). Some authors hypothesize that these functional additives are able to enhance mechanism of the immune system that in turn leads to resistance against pathogens and diseases (Dalmo and Bøgwald, 2008; Merrifield et al., 2010; Ringø et al., 2012).

Survival, achieved SGR and FCR of turbots feeding FM-based diets and PP-based diets were higher or similar compared to those observed in other studies (Árnason et al., 2009; Bonaldo et al., 2011; Regost et al., 1999; Schram et al., 2009; Van Ham et al., 2003). Decreased growth may be caused by a deficiency of phosphorous in diets or by poor utilization of plant proteins offered and a limitation of essential amino acids. In general, reduced DFI for diets high in plant proteins indicate that these are less attractive and palatable than diet treatments containing a high level of FM. Further investigations can confirm performance loss and reduced feed intake in turbots with increasing PP content in diets containing overall crude protein levels of 50–54% (Regost et al., 1999), 53–51% (Bonaldo et al., 2011), 50% (Day and Plascencia González, 2000), 57–62% (Fournier et al., 2004) and 59% (Nagel et al., 2012).

Researchers suggest that palatability (Arndt et al., 1999; Freitas et al., 2011; Kissil et al., 2000), lack of nutrients (Gatlin et al., 2007), unfavorable amino acid profiles (Li et al., 2008) and antinutritional factors (ANFs) (Francis et al., 2001) of plant meals or concentrates are responsible for reduced feed intake and poor feed conversion in fish species. This may explain performance loss of turbots fed PP-based diets in this study. Previous studies already revealed negative effects of diets containing soy bean meal (SBM) or soy protein concentrate (SPC) on feed consumption and growth performance in turbot (Bonaldo et al., 2011; Day and Plascencia González, 2000) and other fish species (Davis et al., 2005; Kasper et al., 2007; Kaushik et al., 1995; Kissil et al., 2000). However, during the process of SPC production most antinutritional factors should be destroyed, only phytate may be concentrated with the protein fraction (Gatlin et al., 2007). High phytate concentrations reduce the availability of phosphorus as it is bound in or by phytic acid. It seems possible that a limitation of phosphorus and/or essential amino acids (EAAs, e.g. lysine) is responsible for reduced growth rates in turbots (Kaushik, 1998; Peres and Oliva-Teles, 2008; Riche and Brown, 1996).

Whole body composition of turbots remained unaffected by additive inclusions in both, high and low FM diets as in earlier studies comparing FM- or PP-based diets supplemented with some additives (Dimitroglou et al., 2010; Heidarieh et al., 2012; Kühlwein et al., 2014; Merrifield et al., 2011; Ng et al., 2009). The observed growth decline of fish fed high levels of soy bean and wheat proteins cannot be explained by reduced development of fillet muscles caused by lower protein retention or reduced fat storage. In contrast, other authors observed an influence of additives on crude lipid (Baruah et al., 2007; Li et al., 2005) or crude protein and lipid content (Abdel-Tawwab et al., 2008; Bagheri et al., 2008; Bairagi et al., 2004; Lara-Flores et al., 2003) in whole body composition. Additive and PP inclusion also did not influence HSI values and, consequently, did not seem to promote an increased or decreased fat retention in liver. Liver index of Senegalese sole (*Solea senegalensis*) was higher in fish fed a PP-based diet compared to FM-based diets, although HSI decreased with reduced fat content in FM diets (Valente et al., 2011). However, no evidence of increased fat storage in liver was found in other fish species feeding high levels of PP (Chatzifotis et al., 2008; Dimitroglou et al., 2010; Hansen et al., 2013; Lekva et al., 2010).

At present, there is no single definite mode of action and explanation of why or how dietary supplementation with the examined additives causes improved growth in aquatic animals and it is not clear why they affect growth in some species and not in others. Besides, additives that have been successful at improving growth performance in one study proved to be ineffective for the same species in another investigation. The potential effect may depend on the dosage of additives applied and the method of administration, short- or long-term and oral, immersion or injection (Anderson and Siwicki, 1994; Dalmo and Bøgwald, 2008; Jeney and Anderson, 1993; Nikl et al., 1993; Peddie et al., 2002; Selvaraj et al., 2005). In addition, culture conditions, for instance physical and chemical water parameters, stocking density and feeding rate, as well as size and age class have an influence on performances in turbot and have to be considered when comparing results (Blanquet and Oliva-Teles, 2010; Foss et al., 2009; Imsland et al., 2001; Irwin et al.,

Table 6
Proximate whole body composition in % dry matter basis and gross energy in MJ kg^{-1} dry matter of initial fish samples and turbot fed the control and experimental diets over 84 days in trial 2.

	Initial fish (n = 10)	C-HF	C-LF	GM	AC	NR	BS
Proximate body composition							
Dry matter	20.4	25.1 ± 6.3	20.4 ± 1.7	21.5 ± 0.4	20.7 ± 1.8	22.0 ± 1.0	19.0 ± 5.2
Crude protein	72.8	68.3 ± 2.3	69.7 ± 2.7	71.6 ± 1.8	72.0 ± 3.3	67.2 ± 5.1	72.5 ± 0.4
Crude lipid	4.4	12.0 ± 2.6	12.4 ± 2.3	13.1 ± 3.1	12.0 ± 4.8	15.6 ± 2.3	13.8 ± 1.6
Crude ash	23.4	18.1 ± 2.4	17.7 ± 2.4	15.4 ± 1.0	16.5 ± 1.2	16.6 ± 3.2	15.0 ± 1.2
Gross energy (MJ kg^{-1})	17.5	20.9 ± 0.6	20.8 ± 1.1	21.7 ± 0.6	21.6 ± 1.2	21.5 ± 1.0	21.8 ± 0.9

C-HF = high fish meal control, C-LF = low fish meal control, GM = b-glucan/MOS, AC = alginic acid, NR = nucleotides/RNA and BS = *Bacillus* spp. Each value is mean ± S.D. (n = 6). No significant differences were identified ($p > 0.05$).

1999; van Bussel et al., 2012; Van Ham et al., 2003). Under optimal holding conditions, diet additives probably do not have beneficial impacts in turbot during the grow-out phase. However, additives may be valuable growth and immunity promoters, especially in earlier life stages of fish, to improve survival during critical life phases and to overcome environmental stressors in fish farms. In particular, there is still a dearth of information about the effects of diet additives on performances in fish of size classes above 200 g and in fish that have been offered additives over a long-term period more than 4 months.

In conclusion, diet additives (yeast BG/MOS, yeast nucleotides/RNA, alginic acid, potassium diformate and strains of *Bacillus* spp.) failed to improve growth performance and feed utilization in turbots, weighing between 50 and 250 g, fed FM-based diets (78% FM) in RAS holding. Likewise, yeast BG/MOS, yeast nucleotides/RNA, alginic acid and strains of *Bacillus* spp. failed to improve growth and feed conversion in turbots (100–250 g) offering PP-based diets which contain 25% SPC and 15% wheat gluten. Reduced growth performance due to 45% FM protein substitution can partially be explained by a deficiency of calcium and phosphorus and some EAAs in PP-based diets resulting in decreased protein turnover. Results indicate that these additives are not economically viable for inclusion in commercial turbot on-growing diets when culturing turbots under optimal conditions. Further research is required to determine the size and level of optimal conditions where additives become superfluous.

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