Effects of marine protein-, marine oil- and marine-free diets on the growth performance and innate immune responses of Atlantic salmon (*Salmo salar*, L.) post-smolts.

C. Metochis[1], V.O. Crampton[2], K. Ruohonen[2], A. El-Mowafi[2], J.G. Bell[1], A. Adams[1], K.D. Thompson[3]

[1] *Institute of Aquaculture, University of Stirling, Stirling FK9 4LA, Scotland, UK*

[2] *EWOS Innovation, N-4335, Dirdal, Norway*

[3]*Moredun Research Institute, Pentlands Science Park, Bush Loan, Penicuik, UK*

Corresponding author: Christoforos P. Metochis, Institute of Aquaculture, University of Stirling, Stirling FK9 4LA, UK. Tel: (+44) 1786 466597; Email: c.p.metochis@stir.ac.uk , xristoforosmetoxis@gmail.com

Abbreviations

ABP, animal by-products; ANF, anti-nutrient factors; BAPNA, N-a-benzoyl-DL-arginine-p-nitroanilide; CO, canola oil; DMSO, dimethyl sulfoxide; EDTA, ethylene diamino tetra-acetic acid; FCR, feed conversion ratio; MB, marine based; MF, marine-free; FM, fishmeal; FO, fish oil; G-CFB, gelatine-complement fixation buffer; HKM, head kidney macrophages; HSWB, high salt wash buffer; LC-PUFA, long chain polyunsaturated fatty acids; LRT, likelihood ratio test; LSWB, low salt wash buffer; MB, marine based; NQC, Norwegian quality cut; PBS, phosphate buffered saline; RO, rapeseed oil; SBM, soybean meal; SPB, sodium phosphate buffer; SPC, soy protein concentrate; VP, vegetable protein; VO, vegetable oil.

Keywords: Atlantic salmon, post-smolts, innate immunity, growth, fish-free diets

Abstract

Atlantic salmon post-smolts of an average of 940g were fed six diets including two marine based commercial diets one with partial inclusion of vegetable proteins (VPs) and oils (VOs) (2011/12 EU standards) (MB) and a second with partial inclusion of VPs, land animal-by product (ABP) proteins and VOs (non-EU standards) (MBABP), a fully vegetable protein (VP) diet; a fully algal and VOs (VO) diet; a fishery-free vegetable-based (VP/VO) diet; and a fishery-free diet with a mix of VPs and ABP proteins and a mix of algal and vegetable oils (MFABP). Growth was assessed at Days 104 and 175, whereas fillet proximate composition, haematology and innate immune responses were assessed upon termination. Overall, MB salmon was the best performing group for the full period in terms of feed intake and overall weight gain. MB and VP salmon exhibited the highest FCRs compared to the other groups, while VP salmon exhibited the highest condition factor (*K*) and VO salmon the lowest *K* compared to the other groups. Fillet proximate composition did not present differences among the 6 groups. MB salmon demonstrated the highest plasma lysozyme activity compared to the other groups while MFABP, VP and VP/VO salmon demonstrated higher plasma anti-protease activity in contrast to MB salmon. The dietary groups did not present differences in plasma protein, total IgM or natural haemolytic activity while unaltered head kidney macrophage respiratory burst activity was also observed. Overall, diets free from marine proteins or oils and/or both were satisfactorily utilised by salmon without compromising their immune capacity, although longer adaptation periods are required.

1. Introduction

The stagnating supplies of marine proteins and oils from wild fisheries, have led the aquafeed sector to seek for alternative protein and lipid sources. Numerous alternatives to fishmeals (FMs) and fish oils (FOs) are available from other sources, mainly grains, oilseeds, material recovered from the processing of terrestrial livestock and unicellular organisms (yeasts,

moulds, bacteria and microalgae). The use of plant derived feedstuffs as sustainable alternatives to marine meals and oils in Atlantic salmon feeds however, has been the main focus of salmon nutrition, as these ingredients have high global availability at competitive prices in comparison to FM and FO, and premium nutritional properties for most farmed salmonids. On the contrary, the use of terrestrial animal by-products (ABPs), although of great potential has not yet been explored to a similar extend in salmon diets, despite the fact that they have been used in many salmon producing countries including Australia, Canada and Chile. The main reason for that were the legal restrictions established by the European Union (EU), regarding the use of the greater majority of animal derived products, aiming the eradication of transmissible spongiform encephalopathies (TSE) (EU 2001, 2003). However, the rules regarding the use of non-ruminant ABPs such as poultry, feather and porcine blood meals in commercial aquafeeds have been revoked for the past few years (van Dyck 2012).

Vegetable proteins in aquafeeds should be low in oligo- and poly-saccharides, largely free from antinutritional factors (ANFs), have a high protein content, favourable amino acid profile, high nutrient digestibility and be acceptable by the fish (Naylor et al. 2009). Plant protein concentrates, wheat and corn gluten meals possess most of these characteristics (Naylor et al. 2009). On the contrary, ABPs are free from ANFs and therefore their application in salmon feeds could be more desirable compared to their plant protein counterparts. As yet, several studies on various salmonids have assessed the potential of some of these ABPs with promising results (Higgs et al. 1979; Steffens 1994; Twibell et al. 2012; Hatlen et al. 2014). Moreover, it has been proven that close to 100% dietary FM replacement with premium plant and animal-proteins is possible for Atlantic salmon, with no negative effects on growth and feed intake when the dietary amino acid profile is well balanced (Espe et al. 2006; Torstensen et al. 2008). While studies have investigated the effects of moderate FM substitution with plant feedstuffs on Atlantic salmon immune responses (Krogdahl et al. 2000; Bransden et al. 2001)

73 the current knowledge about the effects of complete FM replacement on these is unexplored.
74 Studies on partial FM replacement however, demonstrated that moderate levels of dietary SPC
75 in salmon diets promoted gut immune responses such as lysozyme and total IgM levels and
76 resistance to *Aeromonas salmonicida* (Krogdahl et al. 2000). Moreover, Bransden et al. (2001)
77 showed that partial FM substitution with dehulled lupin meal (DLM) or a blend of DLM with
78 hydrolysed poultry feather meal (HPFM) in Atlantic salmon diets does not compromise salmon
79 growth, immune responses or resistance to *Vibrio anguillarum*.

80 Similarly to FM, several studies have revealed that FO can be completely substituted
81 by selected single or mixed VOs in Atlantic salmon diets (Bell et al. 2001, 2002; Torstensen et
82 al. 2005, 2008). In the present trial, diets with complete replacement of FO were supplemented
83 with algal oil as a source of n-3 PUFAs while the n-6/n-3 C_{20} PUFA ratio was kept relatively
84 constant (~1) among FO and FO-free diets. Carter et al. (2003) reported no changes on the
85 growth performance of Atlantic salmon fed on diets containing a mixture of canola oil (CO)
86 and FO or CO and algal (traustochytrids) oil. While, studies on partial or complete replacement
87 of either FM or FO in salmon diets have been widely undertaken, the impact of a combined
88 complete replacement of both, on salmon growth performance and immune responses has not
89 yet been investigated.

90 Currently FM inclusion in commercial salmon diets range from 15% to 55% while FO
91 levels range from 3% to 40%. These variations largely depend on the country feeds are
92 manufactured, and partially reflect differences in the employed production systems, local
93 regulations or legal restrictions as well as differences among the farmed salmon breeds
94 (DeSilva et al. 2012). The present study focused on the innate immune responses and
95 performance of Atlantic salmon post-smolts, fed on two commercial FM- and FO-based feeds,
96 combined with either blends vegetal proteins and oils solely, or a mix of vegetal and ABP
97 proteins with VOs, and fish fed on fully VP or VO and algal oil diets or two feeds with complete

FM and FO substitution with vegetal products (VPs, VOs and algal oils) or a combination of the above with ABPs, was compared. For the present trial, the FM used was tested against many other FM sources and was found to be consistently the highest performing source, while protein and lipid sources utilised in the present trial were selected based on their premium quality.

2. Materials and Methods

2.1. Diets and growth trial

Six different feeds with different levels of FM and FO substitution (Table 1) were tested for their effects on growth performance and health status of Atlantic salmon post-smolts. Two commercial FM and FO based EWOS diets, the first with partial inclusion of vegetable protein concentrates and oils according to the EU standards of 2011-12, namely European marine based (MB) diet, and a second with partial addition of a mixture of VPCs, land ABP proteins and VOs according to non-EU standards, denoted as marine based with inclusion animal by-products diet (MBABP), were used as control treatments. In the VP diet the FM was completely substituted by VPCs, while complete replacement of FO by VOs and algal lipids (the latter used as a source of long chain n-3 PUFAs) was applied in VO diet. Higher levels of FO were included in the VP-based diets in order to compensate for the residual amounts of lipid found in FM. Complete substitution of marine proteins and lipids was tested using two different diets, a fully vegetarian one with complete replacement of FM and FO with VPCs and a combination of VOs and algal oils denoted as VP/VO diet and a second one combining also the inclusion of land animal by-product proteins (poultry and porcine blood meal) namely MFABP diet. The raw materials used to replace FM, as well as the FM itself, were previously included in a routine program to measure protein digestibility and were selected for use in the study due to their high scores on this quality aspect (Crampton, personal communication). Diets with partial or total substitution of FM with alternative protein sources were supplemented with synthetic amino

123 acids and premixes with a starch binder in order to balance nutrients. All diets were formulated
124 to meet the nutrient requirements of salmon according to NRC (1993). Diets were pelleted
125 through extrusion (EWOS Innovation, Dirdal, Norway).

126 Growth trials were conducted at EWOS Innovation facilities at Dirdal, Norway.
127 Unvaccinated Atlantic salmon post-smolts (S0 smolts) of 550g mean body weight, from a
128 commercial SalmoBreed AS (Bergen, Norway) strain selected for improved growth
129 performance were allocated in 24 seawater supplied tanks, until a total of 55 fish were in each
130 tank. Fish were acclimatised to the experimental tanks for 84 days prior to the start of the
131 experiment due to technical issues (delays in the delivery of some of the raw materials used in
132 the experimental feed formulations and thus the manufacturing of the trial feeds), during which
133 time they were fed a commercial diet (EWOS OPAL 500) and later allocated their experimental
134 diet. Daily feeding throughout this period was based on appetite control. Uneaten pellets during
135 this time were counted every day. The system consisted of cylindrical fibreglass tanks with a
136 water volume of 3.0 m^3. Each tank was supplied by running seawater pumped from the nearby
137 fjord at 50m depth (salinity range of 29 ± 1.3 g × l^{-1} and temperature ranging from 6.3 ºC in
138 March (lowest temperature recorded) to 9.1 ºC in August (25^{th}) (highest temperature recorded)
139 (Average water temperature 7.7 ± 1.4 ºC) at a flow rate of 0.8 l × kg × $biomass^{-1}$× min^{-1}.

140 A continuous lighting regime was used during the acclimation and feeding trial period.
141 For the feeding trial, quadruplicate tanks of fish were provided one of the 6 experimental
142 treatments. The fish were weighed prior to the acclimation period (16^{th} of December 2010), at
143 the start of the trial (day 0) (10^{th} of March 2011), at days 104-105 (22^{nd} and 23^{rd} of June 2011)
144 and at the end of the study (days 175-176) (2^{nd} and 3^{rd} of September 2011). The average weight
145 of salmon at the start of the feeding trial was approximately 940g. During the feeding trial
146 period, fish were given pellets of 5mm. Two different dietary batches were used for the full
147 duration of the study. The first batch was given to the fish during the first period of the study

148 from the 10th of March until the 21st of June whereas the second lot was used for the second
149 part of the trial from the 24th of June until the 31st of August. Fish were fed four times daily
150 (feeding times: 01:00, 07:00, 19:00, and 22:00) using an automatic feeding system (Exact;
151 Storvik Aqua, SV, Sundalsøra, Norway). The daily amount of feed was equally distributed
152 within these four feed intervals. Feeding period for each feed interval was about 30-60 min
153 depending on the total feed amount per day. Feed doses were delivered every 60 sec to the
154 tanks for each feeding period whilst the total amount of feed delivered at each feeding time of
155 the feeding period was 3-7 g. The specific feeding rate was the same for acclimation and trial
156 period. The level of daily feed ratio was about 0.5-1.5 of total biomass. To ensure that all fish
157 (or at least as many fish as possible) received adequate feed each day, the daily feed ration was
158 adjusted accordingly so that the amount of uneaten feed laid between 15-30% of the total feed
159 amount offered. An average of 25% "overfeeding" was obtained for the full trial period.
160 Uneaten feed was collected using waste feed collectors. Therefore, estimates of the actual feed
161 consumption and thereby FCRs were possible. The growth trial was conducted for a total of
162 196 days, at which time all fish were removed from the tanks and weighed twice (days 125-
163 126 and days 194-197) after being anaesthetised (MS222, 2g × l^{-1}). On days 125-126 twenty
164 fish from each tank were euthanised with an overdose of MS222 (7 g × l^{-1}) to keep biomass
165 densities below 90 kg × m^{-3} in the tanks resulting in a final number of 35 fish × $tank^{-1}$ (starting
166 mean biomass density of 52 kg × m^{-3} (55 post-smolts); intermediate mean biomass density of
167 67 kg × m^{-3} after the removal of 20 salmon (35 salmon); final mean biomass density of 87 kg
168 × m^{-3} (35 salmon)).

169 ***2.2. Sample collection***

170 For immunological analyses, blood was withdrawn from the caudal vein of 6 fish from each
171 tank, on days 194 and 195 from start of the experiment, using 1 ml syringes rinsed with heparin
172 (10 IU × ml^{-1}); Sigma-Aldrich, Dorset, UK) while pools of Norwegian quality cut (NQC) fillet

samples from 4 fish per tank were obtained for proximate composition analyses. A heparinised capillary tube per sampled fish was filled with blood from the syringe for haematocrit observations. Haematocrit determination was performed for 6 fish per tank. Haematocrit values were measured after centrifugation at 6000 revolution per minute (rpm) for 25 min. Blood from three individuals was used to determine total leucocyte and differential leucocyte numbers. A 10^{-3} dilution of blood in L-15 was used to determine total leucocyte counts. The cells were counted in four squares of a haemocytometer per sample and expressed as:

$$Number\ of cells \times ml^{-1} = N \times DilutionFactor \times 10^4$$

where N is the average number of counted blood cells.

For the determination of differential leucocyte counts 3 blood smears were obtained (for the determination of the percentages of the different leucocyte types in each blood sample initially and their transformation into numbers of cells × ml^{-1} of blood according to total leucocyte numbers). The cells on the blood smears were left to air dry and were stained with Rapid Romanowsky stain (Raymond A lamb, Eastbourne, UK) in the Institute of Aquaculture, University of Stirling. The slides were later examined at × 400 magnification for the determination of differential leucocyte proportions. Two blood pools from three individuals per tank (6 individuals in total) were obtained in 1.5 ml eppendorf tubes (400 µl of blood × sampled $fish^{-1}$ and thus 1200 µl of blood × $tube^{-1}$). The blood was left to clot overnight at 4°C and the next day the pooled plasma was aliquoted into 7 eppendorf tubes (about 40-50 µl) and stored at -80°C until use for the assessment of salmon immune responses. Head kidney samples (approximately 5 mm) from three individuals (derived from the same tank), were aseptically removed according to Secombes (1990), pooled in plastic bjoux vials containing 5ml ice-cold L-15 medium containing 40 µl heparin (10 IU × ml^{-1}) and used for respiratory burst assays. Two pools per tank were used to determine the level of superoxide anion (O^{-2}) produced by head kidney macrophages.

198 ***2.3. Calculations***

199 Feed intake is the calculated amount of food ingested by fish per treatment expressed in g

200 Feed Conversion Ratio (*FCR*):

201 $$FCR = \frac{Feed\ Intake\ (FI)\ (g)}{Wet\ weight\ gain\ (g)}$$

202 Thermal Growth Rate:

203 $$TGC = \left(\frac{\sqrt[3]{W1} - \sqrt[3]{W0}}{(t \times T)}\right) \times 100$$

204 Weight gain (*WG*):

205 $$WG\ (\frac{g}{day}) = \frac{Wet\ weight\ gain(g)}{Number\ of\ days}$$

206 Condition Factor (*K*):

207 $$K = \frac{Fish\ weight\ (g)}{Fish\ length\ (cm)^3}$$

208

209 In the above formulae W is the weight of the sampled fish in grams; W0 and W1 are the initial

210 and the final fish mean weights in grams.

211 ***2.4. Isolation of head kidney macrophages (HKM) and estimation of HKM respiratory burst***

212 ***activity***

213 For the isolation of head kidney macrophages, the head kidney was teased through a 100µm

214 nylon mesh (BD Falcon; BD Biosciences, Franklin Lakes, NJ, USA) into 2.5 ml Leibovitz

215 medium (L-15; Sigma-Aldrich) containing 40 µl of heparin (10 IU × ml^{-1}). The mesh was

216 rinsed with 2.5 ml of the medium and placed on ice. The O^{-2} production by head kidney

217 macrophages was measured by the conversion of NBT (Sigma-Aldrich) to formazan, following

218 the method of Secombes (1990) with some modifications described by Korkea-aho et al.

219 (2011).

220 ***2.5. Determination of plasma protein concentration and lysozyme activity***

221 Plasma protein content was determined using the Pierce BCA (bicinchoninic acid) Protein
222 Assay kit (Thermo Scientific, IL, USA) based on the conversion of Cu^{2+} to Cu^{1+} under alkaline
223 conditions (Biuret reaction) using BSA as standard. Plasma lysozyme activity was based on
224 the lysis of lysozyme sensitive *Micrococcus lysodeikticus* as described by Korkea-aho et al.
225 (2011).

226 ***2.6. Measurement of plasma natural haemolytic activity***

227 The assay used was based on a method described by (Langston et al., 2001) with modifications.
228 Briefly sheep red blood cells (SRBC) (Oxoid, UK) were used as target cells at a final
229 concentration of 2.5×10^8 cells $\times$ ml^{-1} of blood. Plasma was diluted in double serial dilutions
230 in 0.1 % gelatine-complement fixation buffer (0.1% G-CFB) **(**1 complement fixation tablet
231 (Oxoid, UK) and 0.1g of gelatin (Sigma-Aldrich) in 100 ml of warm distilled water**)** and 25 µl
232 added to each well of a non-absorbent U-well micro-plate (Sterilin) in duplicate. Ten µl 0.5 %
233 SRBC suspension was added to each plasma dilution. Controls on each plate comprised 0.1 %
234 anhydrous Na_2CO_3 (v/v) (100 % lysis) replacing plasma. G-CFB replacing plasma (0 % lysis)
235 and plasma blanks (duplicate wells of plasma dilutions with CFT-G buffer replacing SRBC
236 suspension). The micro-titre plates were incubated at 22°C for 90 min with constant shaking
237 and the reaction terminated by the addition of 140 µl G-CFB with 20mM EDTA, followed by
238 centrifugation to spin down the remaining SRBC. After centrifugation 100 µl of the supernatant
239 from each well was transferred to a new flat-bottomed 96-well non-absorbent micro-titre plate
240 (Sterilin). The absorbance of the wells was read at 450 nm using a micro-plate reader (Synergy
241 HT; BioTek Instruments, Winooski, VT, USA) and the percentage lysis of SRBCs calculated.
242 The absorbance values of samples were corrected by subtracting the absorbance of the sample
243 blank control (0 % haemolysis). A graph of log x (x = concentration of plasma) (ordinate axis)
244 *vs* log y/ (1-y) (y = % SRBC haemolysis) (abscissa axis) was drawn and after estimating the

volume of plasma giving 50 % haemolysis (H_{50}), and the $H_{50} \times ml^{-1}$ of plasma calculated by dividing the dilution factor of plasma with the estimated plasma volume causing lysis to the 50% of the RBCs in the wells expressed in ml.

2.7. Total plasma Immunoglobulin M (IgM)

The level of plasma IgM in experimental salmon was determined using indirect enzyme linked immunosorbent assay (ELISA) (Magnadottir and Gudmundsdottir, 1992), with modifications. Briefly, two replicate rows of a 96-well an Immulon™ 4HBX plate (Thermo Scientific, Maine, USA) were coated with 100 µl × $well^{-1}$ serial dilution of purified IgM (Aquatic Diagnostics, Stirling, Scotland) in 0.05M sodium carbonate/bicarbonate buffer, pH 9.6 (starting from 0.32 mg × ml^{-1} – 0.00016 mg × ml^{-1}) to form a standard curve of IgM concentration *vs.* absorbance at 450 nm. To the remainder of the wells 100 µl of a fold 1/500 and 1/1000 dilution of plasma from experimental fish was added, diluted in 0.05M sodium carbonate/bicarbonate buffer, pH 9.6, using two replicate wells for each dilution. The plates were then incubated overnight at 4°C and washed 5 times with low salt wash buffer **(**LSWB; 0.02 M Trizma base, 0.38 M NaCl, 0.05% (v/v) Tween 20, pH 7.2**)**. The wells were blocked with 250 µl of 3 % w/v dried skimmed milk (Marvel, Dublin, Ireland) in water and the plates were incubated for 120 min at 21°C. The casein solution was removed before adding 100 µl of mouse anti-trout/salmon IgM (F11-monoclonal anti trout/salmon IgM - Aquatic Diagnostics, Stirling, Scotland) solution (1:66) in 1 % BSA in LSWB for 1 h at 21°C. Plates were then washed with 5 washes of high salt wash buffer **(**HSWB; 0.02 M Trizma base, 0.5 M NaCl, 0.01 % (v/v) Tween 20, pH 7.4**)** and incubated for 5 min on last wash before adding 100 µl × $well^{-1}$ goat anti-mouse immunoglobulin-G labelled with horseradish peroxidase (HRP) (Sigma/Aldrich) diluted 1: 4000 in conjugate buffer) incubating for 60 min at 21°C. Plates were washed with 5 washes of HSWB, incubating for 5 min on last wash and the reaction was developed by adding 100 µl × $well^{-1}$ of substrate/chromogen **(**i.e. 15 ml substrate buffer containing 5 µl hydrogen peroxide

and 150 µl trimethyl-benzidine **(TMB)** di-hydrochloride**)** and incubating for 10 min at 22°C. The reaction was stopped with 50 µl × well^{-1} of 2M H_2SO_4 and plate read at 450 nm after 5 seconds in a micro-plate reader (Biotek Synergy HT).

2.8. Plasma anti-protease activity

The method used was designed to detect anti-protease activity in trout plasma, and was based on the method described by (Ellis, 1990), modified for use in microtitre plates. A hundred micrograms per millilitre of trypsin solution was prepared by adding 1 ml of 25 mg × ml^{-1} of trypsin stock solution (Invitrogen, UK) in 249 ml 0.1 M Tris.HCl (pH 8.2). Plasma samples were diluted two-fold in the Tris.HCl buffer in round-bottomed 96 well plates (Sterilin), giving final plasma volumes of 2.5, 1.25, 0.625 and 0.313 µl. In a flat-bottomed 96 well plate, 5 µl of diluted samples were added to 15 µl trypsin and incubated for 5 min; duplicates were used where enough plasma was available. Finally, 200 µl of chromogen solution in distilled water (0.1% Nα-Benzoyl- L -arginine 4-nitroanilide hydrochloride or simply BAPNA (Sigma-Aldrich)) was added to each well. Wells containing only BAPNA solution and Tris.HCl buffer without the addition of plasma samples served as a zero reference. The plates were then incubated for 30 min at 22°C before centrifuging them for 6 min at 750 × g. One hundred microliters from each well was transferred to wells of a flat bottom 96-well plate and the absorbance measured with a micro-plate reader (Biotek Synergy HT) set on a 5 min kinetic run, reading every 1 min at 410 nm. Tryptic activity was a measure of the difference in values at 5 min from the ones at time 0 divided by 5 (units expressed as change of 0.001 units of absorbance at 410 nm × min^{-1}). The 75 % inhibition value was calculated from the blank samples, which represent the 100 % inhibition of tryptic activity and reference samples which represent the 0 % inhibition of trypsin. The volume of plasma required to achieve 75 % inhibition of trypsin activity was calculated from a graph of % trypsin inhibition against the volume of plasma used. The units of trypsin inhibited at a percentage of 75 % per ml of plasma

were obtained by multiplying the estimated value of tryptic activity by 1000; as a unit of trypsin activity was the amount of trypsin causing decrease in absorbance of 0.001 and dividing this number by the volume of plasma required to inhibit the activity of trypsin at a percentage equal to 75 %. The quotient was then multiplied by 1000 to transform µl to ml; so 75 % trypsin inhibition was expressed in units $TI_{75} \times min^{-1} \times ml^{-1}$.

2.9. Dietary and NQC fillet composition analysis

The assessment of dietary lipid and FA composition was conducted by The Nutrition Analytical Services (NAS) of the Institute of Aquaculture, University of Stirling and were run in duplicates. Dietary crude fat was determined using two different methodologies. Firstly dietary lipid content was determined following acid hydrolysis using a Soxtec System 1047 hydrolysing unit (Tecator Application note 92/87) followed by exhaustive Soxhlet extraction using petroleum ether (40–60°C boiling point) on a Soxtec System HT6 (Tecator application note 67/83) as described by Bell et al. (2001). In addition, dietary lipid fraction was determined according to the Folch method (Folch et al. 1957) with non-lipid impurities removed by washing with 0.88% (w/v) KCl. The lipid weight was determined gravimetrically after evaporation of solvent under nitrogen and desiccation under vacuum for at least 16 h. Dietary Fatty acid methyl esters (FAME) were prepared from total lipid by acid catalyzed transesterification as described by Christie (2003) and FAMEs extracted and purified as described by Tocher & Harvie (1988). FAMEs were separated and quantified by Gas Liquid Chromatography (GLC) (Carlo Erba Vega 8160, Milan, Italy) using a 30 m × 0.32 mm capillary column (CP Wax 52CB, Chrompak, London, UK). Hydrogen was used as carrier gas and temperature programming was from 50 to 150°C at 40°C × min^{-1} and then to 230°C at 2.0°C × min^{-1}. Individual methyl esters were identified by comparison with known standards and by reference to published data (Ackman 1980). Peak data was processed using Chromcard for Windows (version 1.19) computer package (Thermoquest Italia S.P.A., Milan, Italy). Dry

320 weight and ash contents of the diets were determined after oven-drying the samples to constant
321 weight and by ashing dried samples in an oven at 550°C (AOAC 1990). Dietary nitrogen was
322 determined by Eurofins Scientific (Norway) after total combustion using a Nitrogen-Analyser
323 (Perkin Elmer, 2410 Ser. II, Norwalk, CT, USA), crude protein content calculated assuming
324 that proteins contain 16% N. Amino acid composition of the feed raw materials was analysed
325 by near infrared reflectance (Fontaine et al. 2001) and was also performed by Eurofins
326 Scientific (Norway). Amino acid composition of compound feed was analysed according to
327 (Llames & Fontaine 1994) while dietary elemental composition was determined via Inductively
328 Coupled Plasma - Mass Spectroscopy (ICP-MS) on dietary ash (Shearer 1994).

329 Pools of homogenised NQC fillet samples (1 pool of 4 individuals per tank) for
330 chemical analysis were frozen and then thawed before blending (whole). Dry matter,
331 moisture, ash and crude protein levels were determined according to standard methods (AOAC
332 1990) by oven drying to constant weight. Crude protein from dry NQC samples was estimated
333 via application of the method described by Kjeldahl, using a Tecator Kjeltec System. Lastly,
334 crude fat from dried NQC carcass was determined using petroleum ether (40–60°C boiling
335 point) on a Soxtec System HT6 (Tecator application note 67/83) (Christie 2003).

336 ***2.10. Statistics***

337 The statistical analysis was carried out with the help of the R language (R Core Development
338 Team, 2014) and its lme4 package (Bates et al., 2014). Similar statistical analyses are presented
339 by Espe et al. (2012) and Hartviksen et al. (2014). To investigate the effect of the diets on the
340 haematological and immunological responses, the data were fitted in two different models
341 without (only the tank effect was added) and with the feed variable (tank effect nested within
342 the dietary effect), which were then nested and compared with a likelihood ratio test (LRT).

343 Feed intake (FI) and feed conversion ratio (FCR) over the full trial period and during
344 first and second study period were modelled as ordinary linear models since there was no

345 multilevel structure (only one observation per tank). Gutted weights are available from a
346 subsample of fish at the end of the trial. Daily WG for the same period was modelled with the
347 help of splines to allow the identification of non-linear responses of the diets in time. Since
348 there were three weight points available, the degree of freedom for the spline was constrained
349 to 2. Two models were then fitted without and with the dietary effect, and compared with LRT.
350 The modelling of the condition factor was conducted by fitting a length-weight
351 relationship and adding the treatment as a covariate to the model. Since whole fish (ungutted)
352 weights were available at the end of the trial, these were used as a predictor, in order for the
353 model to adjust for an average-sized sampled fish and for a direct comparison to be possible.
354 Lastly, two nested models were fitted, without and with the dietary effect, and compared with
355 a LRT as above.
356 Composition percentages were modelled with an ordinary linear model (only one
357 observation per tank) using an arcsin transformation to the responses which were expressed as
358 a percentage of wet weight and adding the mean weight of the sample as a covariate. Sample
359 mean weights were mean-centred before the analysis so that the results are easy to interpret as
360 for the average-sized sampled fish.
361 Models demonstrating possibilities (*P* values) of 0.1 were selected for the description
362 of data. For the modelled immune responses affected by the dietary treatments, the results are
363 summarised as graphs with the mean response and 95% confidence interval. Confidence
364 intervals were solved by a posterior simulation from the statistical model with 1500 random
365 draws (Gelman and Hill, 2007). Differences among dietary treatments were revealed when the
366 95% confidence intervals for a certain response of a dietary group did not overlap with the
367 mean values of the same response from another group.

368 **3. Results and Discussion**

369 ***3.1. Diets***

370 Total replacement of FM or FO and/or both marine ingredients in the experimental feeds was
371 done in order to meet or exceed salmon known nutrient requirements (NRC 1993). In the
372 present study the selection of the protein and oil sources was based on previous studies
373 performed by EWOS, reporting high protein and energy digestibility (Crampton personal
374 communication) as well as adequate growth (Crampton et al. 2010; Hartviksen et al. 2014;
375 Hatlen et al. 2014). Nonetheless, the dietary amino acid profiles of the six experimental diets
376 differed as a consequence of FM substitution with alternative protein sources (Table 2)
377 reflecting the amino acid composition of the different ingredients used. Lower levels for most
378 indispensable amino acids (IAA) were observed in the treatments that were free from marine
379 proteins (VP, VP/VO and MFABP diet) in comparison to the marine protein based diets, with
380 the exception of leucine and phenylalanine which were found at higher levels in FM-free feeds.
381 The changes among IAA were less pronounced to those reported by Torstensen et al. (2008)
382 regarding the differences between diets with partial substitution of FM and FO with plant
383 derived ingredients compared to a fully marine based control feed. Mambrini and Kaushik
384 (1994) and Green et al. (2002) reported that IAA: DAA ratio could affect several performance
385 parameters in fish. Herein, dietary IAA: DAA ratios were kept constant among treatments. The
386 dietary FA concentrations of the experimental diets are presented in Table 3. Lower amounts
387 of saturated and monounsaturated FAs and higher levels of total n-6 and n-3 polyunsaturated
388 FAs were observed for the VO-based diets. For the last group of FAs both marine based diets
389 presented higher 20:5n-3 (eicosapentaenoic acid-EPA) levels while 22:6n-3 (docosahexaenoic
390 acid-DHA) was higher for the VO-based feeds due to the inclusion of algal oils, characterised
391 by increased levels in the aforementioned FAs which is in accordance to previous algal oil
392 feeding studies performed by Carter et al. (2003) and Miller et al. (2007). Furthermore, n-3 to
393 n-6 PUFAs ratio demonstrated a gradual decrease in the diets in the following order:
394 MB=MBABP>VP>VO>VP/VO=MBABP.

3.2. Growth and chemical composition of NQC fillet samples

The results of this study demonstrated negligible mortality (<1%) in all dietary groups during the full experimental period, which is a primary indication that all of the diets fulfilled the nutrient requirements of salmon (Table 4).

However, salmon performance in terms of expected WG and FI for the first and full period of the study were largely influenced by the initial size differences of the fish assigned to the different diets. Reassignment of salmon populations in the tanks prior to the initiation of the feeding trial, could have given even more sound and clear results regarding the overall performance of salmon. However, comparable to the present study, were also the differences in salmon starting weights before the commencement of a similar commercial feeding study conducted by EWOS and reported by Crampton et al. (2011), utilising a commercial EWOS marine based and a low FM diet in Atlantic salmon post-smolts reared in sea cages. All the above, highlight the difficulties when conducting large scale scientific studies involving salmon of large size like the present one. However, this should not detract from the significance of this investigation as important conclusions could still be drawn from it.

Salmon fed the control MB diet presented higher FI than the rest of the groups (Fig. 1B) (since the expected mean FI values of the MB group did not overlap with the 95% C.I. of the other dietary groups). Furthermore, VP-fed salmon exhibited higher expected FI than the MBABP, MFABP and VP/VO groups, while the latter group, also demonstrated lower FI than VO-fed salmon. Studies have reported that total or partial replacement of FM in salmonid diets can negatively affect the FI in fish (Gomes et al. 1995; De Francesco et al. 2004; Kaushik et al. 2004; Espe et al. 2006) and this could be due to the lack in certain FM components acting as feeding stimulators (Kousoulaki et al. 2012). Moreover, self-selecting feeding trials have shown that rainbow trout prefer diets containing FO over those with VO, suggesting that some fish do actively select feeds based on the oil origin (Geurden et al. 2005, 2007). Herein, the VP

420 diet was supplemented with higher levels of FO in order to compensate the lack of residual
421 fish-derived lipid found in the FM fraction of the marine based diets resulting in a FA profile
422 which was more closely related to that of the marine based diets. Liland et al. (2012), proposed
423 that dietary FA composition might be a regulating component of Atlantic salmon appetite. This
424 could explain the numerically lower FI in VO-fed fish in comparison to the VP group and the
425 absence of differences between MBABP salmon in contrast to the former group, despite the
426 size difference at the start of the study. Hence the suggestion made by Liland et al. (2012)
427 seems to be valid for Atlantic salmon post-smolts. In contrast to the present findings, Carter et
428 al. (2003) and Miller et al. (2007) reported unaffected FI in juvenile Atlantic salmon fed diets
429 containing just algal oils, a combination of algal and VO compared to salmon fed fully or
430 partially FO based diets. Unaffected growth was also reported for the aforementioned dietary
431 groups compared to the FO-fed fish, which is in agreement with our results.

432 For the same period the MB control group exhibited higher expected weight gain (WG)
433 than the majority of the dietary groups except for the VO-fed group (Fig. 1A). The majority of
434 the other groups exhibited no differences in WG. The only exception was the VP/VO salmon
435 which exhibited lower WG in contrast to the VO-fed fish. The initial size discrepancies
436 promoting contrasting FIs and thus further size differences among the latter groups of salmon
437 seem to be the main reason for the last observation. Higher FCR values were obtained for the
438 MB and VP salmon, during the first period of the feeding trial (Fig. 1C), revealing the lower
439 efficiency of salmon in the utilisation of dietary nutrients from the two aforementioned feeds,
440 compared to the other dietary treatments.

441 Both expected FIs and WGs demonstrated no differences among the six dietary groups
442 during the second period. Nevertheless, a significant increment in FI was observed for the
443 MBABP, MFABP and VP/VO groups (the expected mean FI values for these groups during
444 the second period did not overlap with the 95% C.I. of the FI values obtained during the first

445 period), while a rather substantial but not significant increase was also witnessed for the VO
446 group for this period compared to the initial phase. Furthermore, a significant reduction in
447 expected WG was observed for the MB group at the second period in comparison to the initial
448 one while no significant differences were witnessed for all other groups. Increased feed
449 consumption and thus growth (defined as "compensatory growth") after periods of restricted
450 FI have been demonstrated in Atlantic salmon (Johansen et al. 2001; Torstensen et al. 2008).
451 The above findings demonstrate that Atlantic salmon requires long adaptation periods before
452 accepting any diet with high levels of alternative protein and lipid sources as previously
453 reported by Torstensen et al. (2008).

454 Overall, MB salmon had a higher overall FI by the end of the trial compared to most of
455 the other dietary groups, except VP salmon (Fig. 1B). In addition, higher overall FI was
456 observed for VP-fed salmon in comparison to the MBABP salmon and the marine-free
457 (MFABP and VP/VO) groups of salmon. The above observations highlight the importance of
458 the oil fraction on the acceptability of aquafeeds by salmonids (Geurden et al. 2005; 2007;
459 Liland et al. 2012), as lower FI was obtained for the VO group regardless of the greater initial
460 size of these fish compared to VP salmon. Furthermore, higher WG was observed for the MB
461 salmon compared to the MBABP, MFABP and VP/VO fed salmon (Fig. 1A). FCR values for
462 the full duration of the trial were found to be higher for the MB and VP salmon in contrast to
463 all other dietary groups while no differences were observed between the former groups (Fig.
464 1C). Therefore, among the two marine based groups, MB salmon exhibited lower feed
465 efficiency while MBABP salmon with intermediate growth performance values demonstrated
466 better efficiency in the utilisation of dietary nutrients. Excluding VP salmon, the low FCR
467 values demonstrated for the majority of the experimental groups during the full study period,
468 indicate that judicious selection of alternatives to FM and FO and careful formulation of salmon
469 feeds in order to satisfy their nutrient requirements could promote adequate growth even when

both marine-derived proteins and lipids are fully excluded. Espe et al. (2006) reported equal FCR values in Atlantic salmon fed a FM-based compared to FM-free diets, which in the case of the present study was true only for the MBABP compared to the VP and the two marine-free dietary groups. However, in the latter study marine based by-products were included in the experimental diets in order to improve their acceptance by the fish. Contrary to our findings, were also the higher FCR values reported in Atlantic salmon post-smolts fed on diets where marine and plant derived ingredients from commercial salmon diets were partially substituted by terrestrial ABPs (Hatlen et al. 2013, 2014). Similar to our results, unaffected FCRs were also reported in Atlantic salmon fed low marine ingredient diets compared to a fully marine dietary group (Torstensen et al. 2008). Moreover, most FO replacement studies for Atlantic salmon diets demonstrated unaffected FCRs for VO- in comparison to FO-fed salmon which are partially in agreement with the present findings (Bell et al. 2002; Torstensen et al. 2005; Karalazos et al. 2007).

Condition factor (*K)* values at the end of the trial ranged between 1.25 and 1.75 for the majority of the fish, describing salmon with fairly good to excellent quality (Barnham and Baxter 1998). Expected *K* values were found to be higher for the VP group, while salmon fed the VO diet exhibited lower *K* values compared to the rest of the groups. Moreover MBABP, salmon presented higher *K* values than the MB group and salmon maintained on the marine free diets (MFABP and VP/VO). Furthermore, higher *K* was obtained for VP/VO salmon in comparison to the MB salmon, while no difference was noticed between the latter group and MFABP salmon (Fig. 1D). Since *K* factor describes the relationship between the full (ungutted whole fish) weight and salmon length, the high values obtained for VP-fed salmon is an indication of thicker bodies whereas the low *K* values observed for the VO-fed fish point at much leaner fish compared to the other groups. In general, Atlantic salmon post-smolts of 2.5 kg (mean final salmon weight for all groups) are characterised by increased muscle growth,

hepatic and visceral fat deposition (Shearer 1994). Therefore, the increased *K* values exhibited for the salmon maintained on the VP diet could actually be an indirect indication of higher hepatic and visceral fat accumulation, as no differences in fillet fat levels were observed among the dietary groups. Despite the fact that the opposite trend was illustrated for the VO-fed group, the assumption of lower adiposity in these fish requires a more thorough investigation. Studies by Ruyter et al. (2006) and Jordal et al. (2007) demonstrated that high dietary VO or fully VO-based diets could induce visceral and/or hepatic adiposity, while Torstensen et al. (2011) reported increased visceral adiposity in salmon fed diets with high levels of VPs and VOs respectively. Since fat levels in the liver, intestine and pancreas were not estimated in the current study no further comments could be made on this matter. In contrast to our findings, Espe et al. (2006) demonstrated unaltered *K* values in Atlantic salmon fed VP-based diets. Furthermore, Torstensen et al. (2008) reported lower *K* values in Atlantic salmon maintained on diets with high levels of VPs and moderate or high supplementation with VOs compared to fish fed a marine-based diet or a diet containing a moderate inclusion of VPs and high inclusion of VOs.

Proximate analysis of the NQC samples revealed no differences in moisture, crude protein, lipid and ash levels among the six dietary groups, suggesting similar levels of nutrient accumulation in the salmon fillets. Similarly, previous studies have reported unaffected fillet composition in salmonids fed fully VO-based feeds (Karalazos et al., 2007; Turchini and Francis, 2009). Contrary to our findings, a body of literature has demonstrated reductions in the lipid content and subsequent increases in the protein levels, in the fillets of VO-fed salmonids (Bell et al., 2002, 2001; Jokumsen and Alsted, 1990). The latter findings combined with the presence of increased hepatic fat levels, have triggered the hypothesis that VOs could induce adiposity (Bell et al., 2002). Recently, Torstensen et al. (2011) demonstrated that diets with high levels of both VPs and VOs could promote visceral adiposity and metabolic

imbalance which could affect salmon health. Based on the present data, the only indication for increased visceral adiposity as was previously mentioned were the high *K* values demonstrated for the VP group while no similar assumptions could be made for the other groups.

3.3. Haematology and innate immune responses

The haematological and immunological responses of salmon at the end of the trial are summarized in Table 5. No significant differences were revealed for the majority of the estimated haematological parameters. However, lower expected haematocrits were obtained for the MB group compared to the MBABP, MFABP and VP/VO groups, while the latter two groups of salmon presented higher values in comparison to the VO-fed group (Fig. 2A). Therefore, it is apparent that the elimination of the FM fraction from the diets resulted in increased haematocrit, while the elimination of both fractions promoted even higher haematocrit which could imply improved health status for the aforementioned groups. Most of the existent reports of FM and FO substitution with alternative feed ingredients are contradictory to the present findings. Twibell et al. (2012) reported lower haematocrit levels in coho salmon and rainbow trout (*Oncorhynchus mykiss*) fed on VP and ABP in combination with VO diets compared to salmon fed MB diets. Furthermore, complete replacement of dietary FM with ABPs did not significantly affect haematocrit levels in previous feeding trials with coho salmon (Higgs et al. 1979) or rainbow trout (Steffens 1994) compared with fish fed a FM control diet. On the other hand Hemre et al. (1995; 2005) reported decreased haematocrit levels in Atlantic salmon fed on diets with increased substitution of FM with soybean products or increased dietary inclusion of crude fibre which is the case at high levels of FM replacement with most plant derived feed proteins. In addition, Thompson et al. (1996) reported unaffected haematocrit in Atlantic salmon fed on diets with complete substitution of FO with sunflower oil (SO). Nonetheless, haematocrit values for all dietary salmon groups were found to be within normal ranges varying from 43-60%, indicating healthy fish (Hardie et al. 1990; Waagbø et al.

1994; Thompson et al. 1996) without compromised blood oxygen carrying capacity, since there was lack of anaemia which could be related to iron or other mineral deficiencies.

No differences in total and differential leucocyte numbers were detected among the six dietary groups of Atlantic salmon post-smolts, indicating the modulation of similar levels and patterns in leucocyte production in the 6 groups of fish. Thompson et al. (1996) reported that Atlantic salmon parr fed on diets with complete replacement of FO with sunflower oil (SO) did not exhibit differences in total and differential circulating leucocyte levels. On the contrary Rumsey et al. (1994), showed that rainbow trout fed on soy proteins presented increased numbers of circulating leucocytes.

No differences regarding credible plasma haemolytic activity, plasma protein and total IgM and expected respiratory burst activity in stimulated and non-stimulated head kidney macrophages, were observed among the six dietary salmon groups. Contrary to this, reduced levels of total plasma IgM were reported by Jalili et al. (2013) in rainbow trout fed on diets with total substitution of FM with VPs. Furthermore, Jalili et al. (2013) and Sitjà-Bobadilla et al. (2005) reported decreased alternative complement activity in rainbow trout and gilthead sea bream fed diets with 100% substitution of FM with VPs. In agreement with the present findings, no differences in HKM respiratory burst activity were observed in feeding trials where Atlantic salmon and rainbow trout were fed on FM-based diets supplemented only with soybean oil or linseed oil (Kiron et al. 2004; Seierstad et al. 2009). Furthermore, Carter et al. (2003) demonstrated no changes in total immunoglobulin and protein levels, anti-protease activity and circulating leucocytes respiratory burst activity for Atlantic salmon fed diets with complete replacement of FO with canola oil (CO) or 2 blends of CO and FO or CO and algal oil which are in line with the present findings. Similar results were also obtained by (Thompson et al. 1996) who demonstrated no differences in plasma complement, anti-protease and HKM

569 respiratory burst activities of Atlantic salmon fed full soybean oil diets compared to FO fed
570 salmon.

571 Higher expected lysozyme activity was demonstrated for MB salmon compared to all
572 other dietary groups (Fig. 2B). No differences regarding lysozyme activity were witnessed
573 amongst the MBABP group compared to the rest of the experimental groups. Reductions in
574 lysozyme activity could render fish susceptible to diseases (Saurabh & Sahoo 2008). Several
575 disease resistance selection studies, however, have observed a negative correlation between
576 survival rate and lysozyme activity in Atlantic salmon challenged against several bacterial
577 diseases (Røed et al. 1993; Fevolden et al. 1994; Lund et al. 1995), demonstrating that the
578 resistance of salmon against diseases might be more dependent on other immune responses or
579 their efficiency in detoxifying from the by-products of immune activation. Moreover, Fevolden
580 et al. (1994) suggested that lysozyme activity following a disease challenge in salmonids, is
581 not a reflection of a superior immune mobilisation, but an indication of stress induction which
582 could increase the susceptibility of challenged salmonids. Therefore, increased stress as a result
583 of the overall higher stocking density (promoted by their increased growth) in the tanks hosting
584 MB salmon, at the first period of the study, could have promoted stress and higher lysozyme
585 activity in these fish. This could also be supported by their inhibited growth performance
586 compared to the other groups during the second study period (Pickering 1993; Plisetskaya &
587 Duan 1994).

588 Furthermore, higher expected anti-protease activity was exhibited for MFABP salmon
589 compared to MB, MBABP and VO salmon while higher anti-protease activity was observed
590 for the VP and VP/VO dietary groups in contrast to the MBABP group (Fig. 2C). Increased
591 plasma anti-protease activity in salmon fed diets containing only plant proteins (VP and
592 VP/VO) or high levels of plant proteins (MFABP) could be a favourable feature against several
593 bacterial infections (Ellis 1990). Several plant extracts used as feed additives in previous

studies, demonstrated an increase in plasma anti-protease activity (Rao & Chakrabarti 2004; Kaleeswaran et al. 2011). It is possible that high levels of plant derived ingredients and more specifically plant protein concentrates even after processing manipulations targeting the improvement of their nutritional quality might contain certain levels of bioactive compounds exerting an immunostimulatory activity to the fish.

The findings of the current study suggest that marine protein-, marine oil- and marine-free diets can be utilised satisfactorily by Atlantic salmon post-smolts, compared to commercial feed formulations, stimulating both adequate growth and innate immune responses. However, longer adaptation periods might be required for salmon to fully accept these diets. Moreover, dietary FO substitution seems to be easier than FM replacement. The future application of such feeds will depend on the availability and prices of these prime protein and lipid alternatives, which currently do not consist a cost-efficient solution for the production of aqua-feeds compared to the feedstuffs currently used in commercial feed formulations. Future studies on similar levels of FM and FO replacement in salmon feeds should focus on the testing of such treatments under the stressful cage-culture conditions and the assessment of salmon resistance and performance against industrially important diseases.

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830

Figure Captions

Figure 1. Expected performance indices at different study points. (A) Feed Intake (FI); (B) Daily weight gain (WG); (C) Feed Conversion Ratio (FCR) of the Atlantic salmon groups over the periods 1 and 2 and the full duration of the trial and (D) condition factor (*K)* of the salmon groups for the full period of the trial. These parameters were affected by the diets and the results are summarised as graphs with the expected mean response and 95% confidence intervals. Confidence intervals were solved by a posterior simulation from the statistical model with 1500 random draws. Significant differences among dietary groups are revealed when the confidence intervals bars for a certain response of a dietary group do not overlap with the mean values of the same response from another group. The effect of feeds on the above growth performance indices was confirmed by fitting a model without the dietary effect (only tank effect) and with it (tank effect nested within it) and comparing the models with a likelihood ratio tests (LRT). FI and FCR are modelled as ordinary linear models since there is no multilevel structure (only one observation per tank) whereas WG modelled with the help of splines to allow non-linear response in time. FI and WG are expressed as g × $fish^{-1}$× day^{-1} to adjust for the different duration of the periods. Diet abbreviations: MB, European commercial marine based diet (2011-12); VP, vegetable protein diet; VO, vegetable oil diet; VP/VO, vegetable protein/vegetable oil diet; MBABP, Non-EU commercial marine based diet with inclusion of animal-by-product (2011-12); MFABP, fish free animal-by-product diet. Significant differences (*P* values < 0.05) between dietary groups are denoted by different letters.

Figure 2. Expected levels of the affected haematological and immunological responses.(A) Haematocrit (%); (B) Lysozyme (Units × min^{-1} × ml^{-1}); Plasma anti-protease activity (Units TI_{75} × ml^{-1}); (%); of the dietary groups of Atlantic salmon post-smolts for the full period of the trial. These parameters were affected by the diets and the results are summarised as graphs with the expected mean response and 95% confidence intervals. Confidence intervals were solved by a posterior simulation from the statistical model with 1500 random draws. Significant differences among dietary groups are revealed when the confidence intervals bars for a certain response of a dietary group do not overlap with the mean values of the same response from another group. The dietary effect on the health responses was confirmed by fitting a model without the dietary effect (only tank effect) and with it (tank effect nested within it) and comparing the models with a likelihood ratio tests (LRT).The modelling for all above parameters Diet abbreviations: MB, European commercial marine based diet (2011-12); VP, vegetable protein diet; VO, vegetable oil diet; VP/VO, vegetable protein/vegetable oil diet; MBABP, Non-EU commercial marine based diet with inclusion of animal-by-product (2011-12); MFABP, fish free animal-by-product diet. Significant differences (*P* values < 0.05) between dietary groups are denoted by different letters.

Figure 1.

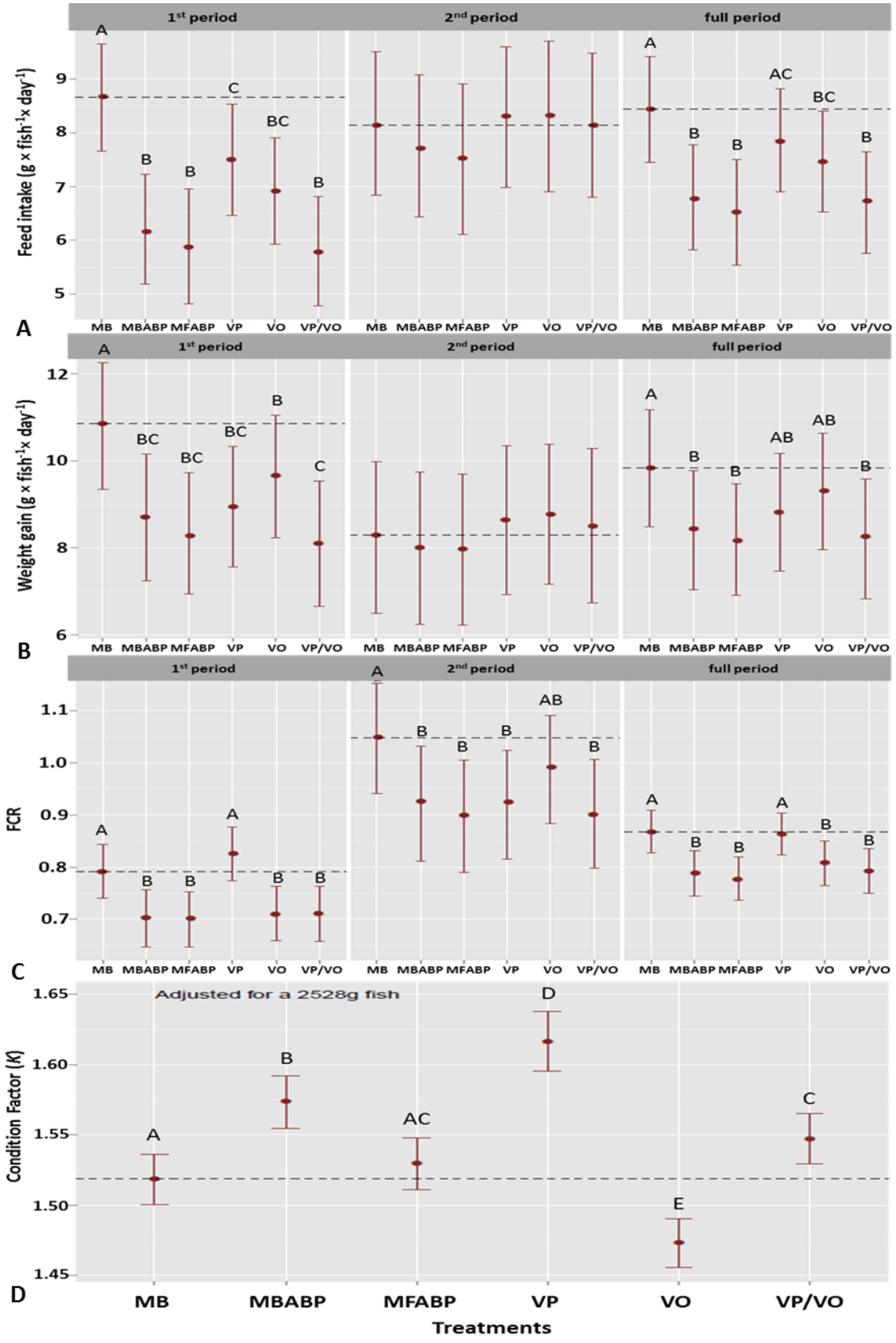
1st period
2nd period
full period
Feed intake (g x fish-1x day-1)
A
Weight gain (g x fish-1x day-1)
B
FCR
C
Adjusted for a 2528g fish
Condition Factor (K)
D
MB
MBABP
MFABP
VP
VO
VP/VO
Treatments

Figure 2.

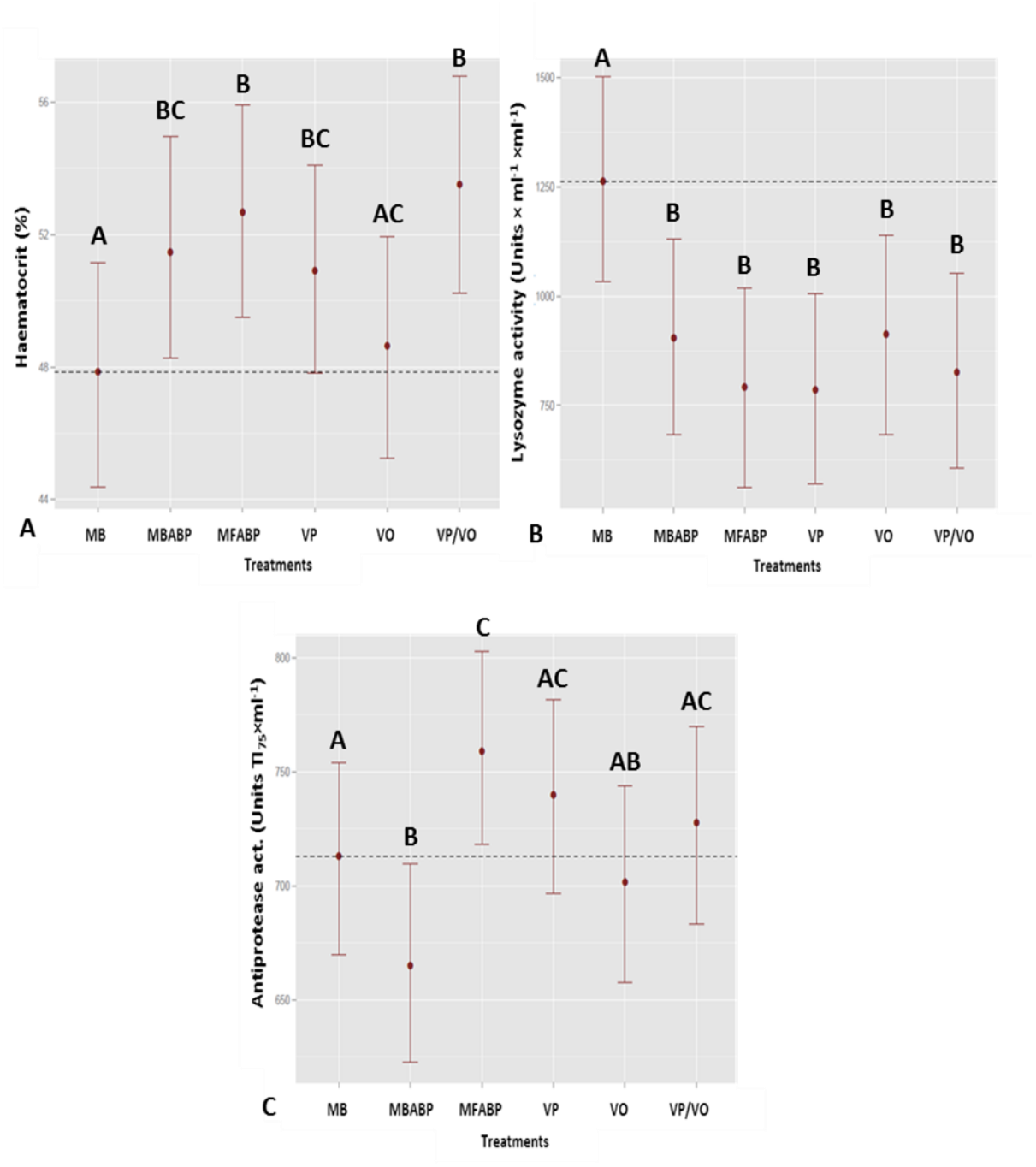
Haematocrit (%)
56
52
48
44
A
BC
B
BC
AC
B
MB
MBABP
MFABP
VP
VO
VP/VO
Treatments
A
Lysozyme activity (Units × ml-1 ×ml-1)
1500
1250
1000
750
A
B
B
B
B
B
MB
MBABP
MFABP
VP
VO
VP/VO
Treatments
B
Antiprotease act. (Units T75×ml-1)
800
750
700
650
A
B
C
AC
AB
AC
MB
MBABP
MFABP
VP
VO
VP/VO
Treatments
C

Table 1 Feed formulations of the six diets.

FEED FORMULATION	DIETS					
Ingredient composition (g × kg^{-1})	**MB**	**MBABP**	**MFABP**	**VP**	**VO**	**VP/VO**
LT Fishmeal[a]	300.0	200.0	-	-	300.0	-
Plant Protein Concentrates[b]	255.3	207.6	386.8	528.1	255.3	528.1
Starch Binder	87.9	85.4	88.6	92.8	87.9	92.8
Animal By-Products[c]	-	155.0	160.0	-	-	-
Amino Acids[d]	9.1	7.4	15.2	1.7	9.1	1.7
Vitamin/Mineral & Pigment Mixes[e]	35.9	35.9	41.9	41.9	35.9	41.9
Fish Oil[f]	148.0	157.6	-	177.0	-	-
Plant Lipids[g]	163.8	150.9	220.3	138.4	239.0	228.2
Algal Lipids[h]	-	-	87.2	-	72.8	87.2

Diet abbreviations: MB, European commercial marine based diet (2011-12); MBABP, Non-EU commercial marine based diet with inclusion of animal-by-products (2011-12); MFABP, fish free animal-by-product diet; VP, vegetable protein diet; VO, vegetable oil diet; VP/VO, vegetable protein/vegetable oil diet. [a] LT Fishmeal (low temperature fishmeal from Egersund Sildeoljefabrikk AS, Egersund, Norway): superior quality FM due to the fact that is treated at lower drying temperatures allowing FM to keep optimal essential amino acid profile, low biogenic amines with an apparent protein digestibility coefficient of 90.2%; [b] Vegetable proteins: includes protein concentrates from soy (Imcopa, - Importação, Exportação e Indústria de Óleos Ltda., Araucária - Paraná, Brazil) and pea (AgriMarin, Stavanger, Norway) and wheat gluten (Henan Tianguan, Nanyang City, China); [c] Animal by-products: includes Poultry by-product meal (Poultry by-product meal, GePro Geflügel-Protein Vertriebs- GmbH & Co, Diepholz, Germany) and porcine blood meal (Daka Proteins, Løsning, Denmark); [d] Amino acids from Evonik Degussa International AG, Hanau, Germany; [e] proprietary of EWOS Innovation; Plant lipids: includes mainly rapeseed oil (Cargill PLC, Lincoln, UK); [f] Fish Oil: Capelin oil (Egersund Sildeoljefabrikk AS, Egersund), Norway; Plant Lipids: includes rapeseed oil; [h] Algal lipids: includes oil from heterotrophically grown algal species (Origin Unknown).

Table 3 Chemical composition of the experimental diets (g × kg^{-1} of dietary wet weight)

Chemical composition (w.w.)	DIETS					
	MB	**MBABP**	**MFABP**	**VP**	**VO**	**VP/VO**
Moisture (g × kg^{-1})	72.65	60.85	81.15	69.20	76.75	85.95
Protein N*6.25 (g × kg^{-1})	390.00	373.00	385.00	373.00	385.50	380.00
Lipid (g × kg^{-1})	374.15	355.60	358.45	352.15	373.45	342.35
Crude fibre (g × kg^{-1})	9.05	9.85	10.85	10.80	8.05	10.80
Ash (g × kg^{-1})	41.80	39.35	36.10	20.45	42.60	16.25
P (g × kg^{-1})	11.10	11.90	11.50	8.20	11.85	9.30
Ca (g × kg^{-1})	13.55	14.25	12.50	8.45	15.10	9.90
Mg (g × kg^{-1})	1.50	1.15	1.15	0.85	1.30	0.80
Zn (mg × kg^{-1})	272.50	281.00	281.00	254.50	258.50	275.50
AMINO ACID COMPOSITION						
Alanine (g × kg^{-1})	20.00	22.50	22.45	19.45	19.95	19.55
Arginine (g × kg^{-1})*	23.95	22.85	23.00	22.10	24.05	22.35
Cysteine (g × kg^{-1})	4.55	4.55	4.40	4.95	4.50	5.00
Glutamate (g × kg^{-1})	68.00	59.90	66.30	78.70	68.40	79.20
Glycine (g × kg^{-1})	19.45	21.65	21.20	13.50	24.25	13.40
Histidine (g × kg^{-1})*	8.85	9.25	7.75	8.55	8.90	8.35
Hydroxyproline(g × kg^{-1})	1.35	2.70	4.50	0.10	1.05	0.10
Isoleucine (g × kg^{-1})*	18.05	16.70	15.65	17.60	17.75	17.20
Leucine (g × kg^{-1})*	29.50	31.65	41.60	43.00	29.30	43.95
Lysine (g × kg^{-1})*	30.55	28.95	27.50	30.00	30.65	29.50
Methionine (g × kg^{-1})*	9.20	8.30	8.00	8.40	9.35	8.55
Ornithine (g × kg^{-1})	0.15	0.15	0.20	0.10	0.15	0.10
Phenylalanine (g × kg^{-1})*	16.85	16.90	18.40	20.10	16.90	20.30
Proline (g × kg^{-1})	20.60	20.55	23.90	23.70	20.35	25.60
Serine (g × kg^{-1})	17.70	17.50	17.90	17.75	17.75	18.95
Threonine (g × kg^{-1})*	15.95	15.45	14.00	14.50	15.80	14.80
Tryptophane (g × kg^{-1})*	4.40	4.20	3.70	3.90	4.05	3.45
Tyrosine (g × kg^{-1})	13.10	13.15	13.65	15.50	13.35	15.35
Valine (g × kg^{-1})*	19.75	19.00	17.00	18.60	19.55	17.95
Sum IAA	177.00	178.66	176.95	185.88	176.15	196.15
Sum DAA	164.90	162.35	170.40	174.35	177.35	183.10
IAA/DAA	1.07	1.07	1.01	1.07	1.04	1.05

Diet abbreviations: MB, European commercial marine based diet (2011-12); MBABP, Non-EU commercial marine based diet with inclusion of animal-by-products (2011-12); MFABP, fish free animal-by-product diet; VP, vegetable protein diet; VO, vegetable oil diet; VP/VO, vegetable protein/vegetable oil diet.

* Amino acids followed by an asterisk are Indispensable (Essential) Amino Acids (IAA) for salmon and the ones without are dispensable (non-essential) amino acids (DAA).

The data presented are consolidated averages of the two dietary batches used for the study.

Table 3 Fatty acid composition of the experimental diets (g × kg^{-1} of dietary wet weight)

Fatty acid composition (g × kg^{-1})(w.w.)	DIETS					
	MB	**MBABP**	**MFABP**	**VP**	**VO**	**VP/VO**
14:0	31.50	31.50	17.00	32.00	18.00	15.50
15:0	2.50	2.50	1.00	3.00	1.50	1.00
16:0	110.50	117.00	100.00	118.00	89.50	88.50
18:0	25.50	28.50	26.50	30.00	24.00	24.00
20:0	3.50	4.00	4.50	4.00	4.50	4.50
22:0	1.50	2.00	3.00	2.00	3.00	3.00
Sum saturated	143.25	154.00	135.00	157.00	122.50	121.00
16:1n-7	37.00	37.00	5.50	36.00	8.00	2.50
16:1n-9	2.50	2.00	1.50	2.50	1.50	1.00
18:1n-7	29.00	28.50	22.50	28.00	24.00	21.50
18:1n-9	342.00	340.00	434.50	329.00	438.50	436.50
20:1n-7	2.00	2.00	<1.00	2.50	1.00	<1.00
20:1n-9	72.50	68.50	11.00	70.00	20.00	10.00
20:1n-11	3.00	2.50	<1.00	2.50	<1.00	<1.00
22:1n-9	8.50	8.00	3.00	8.50	4.00	3.00
22:1n-11	68.00	64.00	3.00	62.50	14.00	1.00
24:1n-9	6.00	6.50	2.00	6.00	3.50	2.00
Sum MUFAs	570.25	558.75	484.00	547.25	515.25	478.50
18:2n-6	108.50	110.00	165.00	126.00	146.00	172.50
18:3n-6	<1.00	1.00	<1.00	<1.00	<1.00	<1.00
20:2n-6	2.00	2.00	1.00	2.00	1.00	1.00
20:3n-6	<1.00	<1.00	1.00	<1.00	1.00	1.00
20:4n-6	2.50	2.50	2.50	2.50	2.00	2.00
Sum n-6 PUFAs	115.00	116.50	170.50	132.50	151.00	177.50
18:3n-3	45.00	42.50	57.50	41.50	59.50	61.50
18:4n-3	12.00	11.00	1.00	11.00	2.50	1.00
20:3n-3	<1.00	<1.00	<1.00	<1.00	<1.00	2.00
20:4n-3	2.50	2.50	2.00	2.50	2.00	2.00
20:5n-3	33.00	32.50	3.00	32.00	9.00	2.50
22:5n-3	3.00	3.00	1.00	3.50	1.50	1.00
22:6n-3	41.50	42.50	93.00	37.00	89.00	99.00
Sum n-3 PUFAs	138.00	135.00	158.50	128.50	164.50	169.00
n-3/n-6	1.20	1.16	0.93	0.97	1.09	0.95

Diet abbreviations: MB, European commercial marine based diet (2011-12); MBABP, Non-EU commercial marine based diet with inclusion of animal-by-products (2011-12); MFABP, fish free animal-by-product diet; VP, vegetable protein diet; VO, vegetable oil diet; VP/VO, vegetable protein/vegetable oil diet.

The data presented are consolidated averages of the two dietary batches used for the study.

Table 4 Performance factors and NQC proximate composition of Atlantic salmon parr fed the experimental diets.

Performance parameters	DIETS					
	MB	**MBABP**	**MFABP**	**VP**	**VO**	**VP/VO**
Initial weight (g)	992.3±119.8	901.8±58.1	924.8±32.3	940.7±127.2	983.9±66.8	892.9±29.9
Intermediate weight (g)	2041.3±224.6	1858.4±111.9	1856.7±113.1	1927.1±301.2	2014.7±142.4	1795.7±39.3
Final weight (g)	2608.7±268.6	2417.5±171.1	2415.6±197.1	2528.9±417.0	2626.9±286.8	2381.7±11.2
***Feed Intake 1st Period (g×fish^{-1}×day^{-1})*	*8.65±0.75^{A}*	*6.16±0.62^{B}*	*5.87±0.98^{B}*	*7.50±1.79^{C}*	*6.89±0.72^{BC}*	*5.78±0.46^{B}*
Feed Intake 2nd Period (g×fish^{-1}×day^{-1})	*8.11±1.33*	*7.69±1.21*	*7.53±1.23*	*8.29±1.56*	*8.30±1.58*	*8.12±0.70*
Feed Intake full Period (g×fish^{-1}×day^{-1})	*8.38±1.04^{A}*	*6.93±0.91^{B}*	*6.70±1.11^{B}*	*7.90±1.67^{AC}*	*7.60±1.15^{BC}*	*6.95±0.58^{B}*
**Wt gain (g× fish $^{-1}$×day^{-1}) 1st Period*	*10.84±0.52^{A}*	*8.63±0.52^{BC}*	*8.22±0.77^{BC}*	*8,98±1.475^{BC}*	*9.59±0.76^{B}*	*8.10±0.32^{C}*
Wt gain (g×fish $^{-1}$×day^{-1}) 2nd Period	*8.11±2.01*	*7.87±1.77*	*7.87±1.80*	*8.48±1.68*	*8.58±2.09*	*8.25±0.55*
Wt gain (g×fish $^{-1}$×day^{-1}) full Period	*9.74±0.71^{A}*	*8.32±0.78^{B}*	*8.08±0.87^{B}*	*8.78±1.48^{AB}*	*9.18±1.25^{AB}*	*8.16±0.06^{B}*
†FCR 1st Period	*0.80±0.05^{A}*	*0.71±0.03^{B}*	*0.71±0.05^{B}*	*0.83±0.07^{A}*	*0.72±0.03^{B}*	*0.71±0.06^{B}*
FCR 2nd Period	*1.02±0.11^{A}*	*0.99±0.09^{B}*	*0.97±0.08^{B}*	*0.98±0.03^{B}*	*0.98±0.08^{AB}*	*0.98±0.02^{B}*
FCR full Period	*0.91±0.05^{A}*	*0.85±0.05^{B}*	*0.84±0.05^{B}*	*0.90±0.04^{A}*	*0.85±0.03^{B}*	*0.85±0.04^{B}*
‡Condition Factor (K) end of trial	*1.52±0.11^{A}*	*1.54±0.12^{B}*	*1.51±0.11^{AC}*	*1.61±0.12^{D}*	*1.46±0.10^{E}*	*1.52±0.10^{C}*
Mortalities (%) 1st period	*0.3±0.5*	*0*	*0*	*0*	*0.3±0.5*	*0*
Mortalities (%) 2nd period	*0*	*0*	*0*	*0*	*0*	*0*
NQC composition						
Moisture (%)	*65.07±0.23*	*65.19±1.17*	*65.67±1.10*	*64.27±0.57*	*65.75±1.08*	*65.65±0.29*
Protein (%)	*18.46±0.23*	*18.87±0.27*	*18.69±0.51*	*18.82±0.81*	*18.89±0.70*	*18.99±0.28*
Crude Lipid (%)	*13.56±0.21*	*12.57±0.58*	*12.64±0.82*	*13.47±1.03*	*12.07±0.32*	*12.92±0.48*
Ash (%)	*1.37±0.04*	*1.34±0.05*	*1.27±0.06*	*1.38±0.08*	*1.33±0.09*	*1.38±0.11*

The present data are the real mean values for each parameter with their standard deviation (SD). Statistical analysis using linear mixed effect models estimates the expected mean values the above parameters with their 95% confidence intervals (by using 1500 random draws). These are shown in Figure 1. Parameters in *italics* were the ones analysed statistically. Data for the proximate composition of Norwegian quality cut (NQC) fillet samples are referred as means ± SD of 4 pooled samples per diet (1 pool per tank). Data for the performance factors are referred as means ± SD of 4 replicate tanks. Significant differences between the groups due to the use of different diets (*P* values < 0.1) are denoted by different letters (Modelled based statistical analysis).

**Wt gain (Daily Weight gain) (g/fish/day) =Total wt of fish within treatment (g) × (Number of fish within treatment)$^{-1}$× (Number of trial days)$^{-1}$*; ***Feed intake = Amount of food ingested by fish per treatment (g); †Feed Conversion Ratio (FCR) = Feed intake (g) × Overall Weight gain (g)*; *‡Condition Factor (K) = Fish Weight (g) × Fish Length (cm)^{3}*).

Diet abbreviations: MB, European commercial marine based diet (2011-12); VP, vegetable protein diet; VO, vegetable oil diet; VP/VO, vegetable protein/vegetable oil diet; MBABP, Non-EU commercial marine based diet with inclusion of animal-by-products (2011-12); MFABP, fish free animal-by-product diet.

Table 5 Effect of experimental diets on immunological and haematological responses of Atlantic salmon post-smolts.

Haematological & Immune parameters	MB	MBABP	MFABP	VP	VO	VP/VO
Haematocrit (%)	*47.9±5.3^{A}*	*51.4±5.7^{BC}*	*52.7±6.5^{B}*	*50.9±3.7^{ABC}*	*48.6±3.7^{AC}*	*53.5±6.3^{B}*
Leucocytes (× 10^7×ml^{-1})	*10.3±4.0*	*10.2±2.9*	*10.2±2.7*	*11.7±3.7*	*10.4±3.9*	*8.3±1.9*
Lymphocytes (× 10^7×ml^{-1})	*5.2±2.1*	*5.6±1.4*	*4.8±1.4*	*5.8±1.6*	*4.9±1.4*	*4.3±1.3*
Thrombocytes (× 10^7×ml^{-1})	*4.4±1.7*	*4.1±1.7*	*4.8±1.6*	*5.3±1.9*	*4.9±2.8*	*3.6±1.4*
Granulocytes (× 10^7×ml^{-1})	*0.6±0.4*	*0.4±0.2*	*0.5±0.3*	*0.6±0.3*	*0.5±0.5*	*0.5±0.2*
Monocytes (× 10^7×ml^{-1})	*0.05±0.08*	*0.03±0.04*	*0.04±0.05*	*0.05±0.06*	*0.06±0.06*	*0.03±0.03*
Lysozyme activity (units × min^{-1}× ml^{-1})	*1259.8±252.1^{A}*	*913.7±319.5^{B}*	*794.7±372.2^{B}*	*791.9±289.6^{B}*	*912.4±185.1^{B}*	*830.1±317.0^{B}*
Plasma haemolytic activity (units SH_{50}×ml^{-1})	*928±298.2*	*954.6±139.1*	*1043.1±378.3*	*928.5±260.1*	*986.8±444.2*	*906.8±271.4*
HKMs respiratory burst (NBT) ($O.D._{610}$ for 10^5 nuclei)	*0.40±0.15*	*0.53±0.26*	*0.37±0.18*	*0.36±0.22*	*0.40±0.21*	*0.36±0.22*
Stimulated HKMs respiratory burst ($O.D._{610}$ for 10^5 nuclei)	*0.55±0.22*	*0.66±0.29*	*0.51±0.19*	*0.50±0.28*	*0.52±0.23*	*0.49±0.29*
Total plasma protein (mg×ml^{-1})	*66.4±6.3*	*66.1±8.3*	*65.3±6.7*	*66.5±6.3*	*67.9±4.3*	*67.9±5.9*
Plasma antiprotease act. (Units TI_{75} × min^{-1}× ml^{-1})	*713.6±15.9^{A}*	*665.4±66.0^{B}*	*757.9±32.1^{C}*	*740.2±12.0^{AC}*	*701±57.3^{AB}*	*727.5±28.2^{AC}*
Total plasma IgM (mg×ml^{-1})	*6.4±2.8*	*5.8±2.6*	*3.7±2.8*	*5.1±3.1*	*7.9±2.6*	*3.9±2.8*

The present data are the real mean values for each parameter with their standard deviation (SD). Statistical analysis using linear mixed effect models estimates the expected mean values the above parameters with their 95% confidence intervals (by using 1500 random draws). Selected models are shown in Figure 2. Values for immune responses are means ± SD from 8 pools of 3 fish per diet; for haematocrit values are means ± SD from 24 individual fish per diet; and for leucocyte and differential leucocyte counts values are means ± SD from 12 individual fish per diet. Significant differences between the groups due to the use of different diets (*P* values < 0.1) are denoted by different letters (Modelled based statistical analysis).

Diet abbreviations: MB, European commercial marine based diet (2011-12); VP, vegetable protein diet; VO, vegetable oil diet; VP/VO, vegetable protein/vegetable oil diet; MBABP, Non-EU commercial marine based diet with inclusion of animal-by-products (2011-12); MFABP, fish free animal-by-product diet.