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

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4 C. Metochis¹, V.O. Crampton², K. Ruohonen², A. El-Mowafi², J.G. Bell¹, A. Adams¹, K.D.
5 Thompson³

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

7 ² *EWOS Innovation, N-4335, Dirdal, Norway*

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

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

13 *Abbreviations*

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

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

23 **Abstract**

24 Atlantic salmon post-smolts of an average of 940g were fed six diets including two marine
25 based commercial diets one with partial inclusion of vegetable proteins (VPs) and oils (VOs)
26 (2011/12 EU standards) (MB) and a second with partial inclusion of VPs, land animal-by
27 product (ABP) proteins and VO (non-EU standards) (MBABP), a fully vegetable protein (VP)
28 diet; a fully algal and VO (VO) diet; a fishery-free vegetable-based (VP/VO) diet; and a
29 fishery-free diet with a mix of VPs and ABP proteins and a mix of algal and vegetable oils
30 (MFABP). Growth was assessed at Days 104 and 175, whereas fillet proximate composition,
31 haematology and innate immune responses were assessed upon termination. Overall, MB
32 salmon was the best performing group for the full period in terms of feed intake and overall
33 weight gain. MB and VP salmon exhibited the highest FCRs compared to the other groups,
34 while VP salmon exhibited the highest condition factor (*K*) and VO salmon the lowest *K*
35 compared to the other groups. Fillet proximate composition did not present differences among
36 the 6 groups. MB salmon demonstrated the highest plasma lysozyme activity compared to the
37 other groups while MFABP, VP and VP/VO salmon demonstrated higher plasma anti-protease
38 activity in contrast to MB salmon. The dietary groups did not present differences in plasma
39 protein, total IgM or natural haemolytic activity while unaltered head kidney macrophage
40 respiratory burst activity was also observed. Overall, diets free from marine proteins or oils
41 and/or both were satisfactorily utilised by salmon without compromising their immune
42 capacity, although longer adaptation periods are required.

43 **1. Introduction**

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

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

60 Vegetable proteins in aquafeeds should be low in oligo- and poly-saccharides, largely
61 free from antinutritional factors (ANFs), have a high protein content, favourable amino acid
62 profile, high nutrient digestibility and be acceptable by the fish (Naylor et al. 2009). Plant
63 protein concentrates, wheat and corn gluten meals possess most of these characteristics (Naylor
64 et al. 2009). On the contrary, ABPs are free from ANFs and therefore their application in
65 salmon feeds could be more desirable compared to their plant protein counterparts. As yet,
66 several studies on various salmonids have assessed the potential of some of these ABPs with
67 promising results (Higgs et al. 1979; Steffens 1994; Twibell et al. 2012; Hatlen et al. 2014).
68 Moreover, it has been proven that close to 100% dietary FM replacement with premium plant
69 and animal-proteins is possible for Atlantic salmon, with no negative effects on growth and
70 feed intake when the dietary amino acid profile is well balanced (Espe et al. 2006; Torstensen
71 et al. 2008). While studies have investigated the effects of moderate FM substitution with plant
72 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 C₂₀ 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

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

103 **2. Materials and Methods**

104 ***2.1. Diets and growth trial***

105 Six different feeds with different levels of FM and FO substitution ([Table 1](#)) were tested for
106 their effects on growth performance and health status of Atlantic salmon post-smolts. Two
107 commercial FM and FO based EWOS diets, the first with partial inclusion of vegetable protein
108 concentrates and oils according to the EU standards of 2011-12, namely European marine
109 based (MB) diet, and a second with partial addition of a mixture of VPCs, land ABP proteins
110 and VOs according to non-EU standards, denoted as marine based with inclusion animal by-
111 products diet (MBABP), were used as control treatments. In the VP diet the FM was completely
112 substituted by VPCs, while complete replacement of FO by VOs and algal lipids (the latter
113 used as a source of long chain n-3 PUFAs) was applied in VO diet. Higher levels of FO were
114 included in the VP-based diets in order to compensate for the residual amounts of lipid found
115 in FM. Complete substitution of marine proteins and lipids was tested using two different diets,
116 a fully vegetarian one with complete replacement of FM and FO with VPCs and a combination
117 of VOs and algal oils denoted as VP/VO diet and a second one combining also the inclusion of
118 land animal by-product proteins (poultry and porcine blood meal) namely MFABP diet. The
119 raw materials used to replace FM, as well as the FM itself, were previously included in a routine
120 program to measure protein digestibility and were selected for use in the study due to their high
121 scores on this quality aspect ([Crampton, personal communication](#)). Diets with partial or total
122 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 m³. Each tank was supplied by running seawater pumped from the nearby
137 fjord at 50m depth (salinity range of $29 \pm 1.3 \text{ g} \times \text{l}^{-1}$ and temperature ranging from 6.3 °C in
138 March (lowest temperature recorded) to 9.1 °C in August (25th) (highest temperature recorded)
139 (Average water temperature $7.7 \pm 1.4 \text{ °C}$) at a flow rate of $0.8 \text{ l} \times \text{kg} \times \text{biomass}^{-1} \times \text{min}^{-1}$.

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 (16th of December 2010), at
143 the start of the trial (day 0) (10th of March 2011), at days 104-105 (22nd and 23rd of June 2011)
144 and at the end of the study (days 175-176) (2nd and 3rd 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, $2\text{g} \times \text{l}^{-1}$). On days 125-126 twenty
164 fish from each tank were euthanised with an overdose of MS222 ($7\text{g} \times \text{l}^{-1}$) to keep biomass
165 densities below $90\text{kg} \times \text{m}^{-3}$ in the tanks resulting in a final number of $35\text{fish} \times \text{tank}^{-1}$ (starting
166 mean biomass density of $52\text{kg} \times \text{m}^{-3}$ (55 post-smolts); intermediate mean biomass density of
167 $67\text{kg} \times \text{m}^{-3}$ after the removal of 20 salmon (35 salmon); final mean biomass density of 87kg
168 $\times \text{m}^{-3}$ (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 ($10\text{IU} \times \text{ml}^{-1}$); Sigma-Aldrich, Dorset, UK) while pools of Norwegian quality cut (NQC) fillet

173 samples from 4 fish per tank were obtained for proximate composition analyses. A heparinised
174 capillary tube per sampled fish was filled with blood from the syringe for haematocrit
175 observations. Haematocrit determination was performed for 6 fish per tank. Haematocrit values
176 were measured after centrifugation at 6000 revolution per minute (rpm) for 25 min. Blood from
177 three individuals was used to determine total leucocyte and differential leucocyte numbers. A
178 10^{-3} dilution of blood in L-15 was used to determine total leucocyte counts. The cells were
179 counted in four squares of a haemocytometer per sample and expressed as:

$$180 \quad \text{Number of cells} \times \text{ml}^{-1} = N \times \text{DilutionFactor} \times 10^4$$

181 where N is the average number of counted blood cells.

182 For the determination of differential leucocyte counts 3 blood smears were obtained
183 (for the determination of the percentages of the different leucocyte types in each blood sample
184 initially and their transformation into numbers of cells $\times \text{ml}^{-1}$ of blood according to total
185 leucocyte numbers). The cells on the blood smears were left to air dry and were stained with
186 Rapid Romanowsky stain (Raymond A Lamb, Eastbourne, UK) in the Institute of Aquaculture,
187 University of Stirling. The slides were later examined at $\times 400$ magnification for the
188 determination of differential leucocyte proportions. Two blood pools from three individuals
189 per tank (6 individuals in total) were obtained in 1.5 ml eppendorf tubes (400 μl of blood \times
190 sampled fish $^{-1}$ and thus 1200 μl of blood \times tube $^{-1}$). The blood was left to clot overnight at 4°C
191 and the next day the pooled plasma was aliquoted into 7 eppendorf tubes (about 40-50 μl) and
192 stored at -80°C until use for the assessment of salmon immune responses. Head kidney samples
193 (approximately 5 mm) from three individuals (derived from the same tank), were aseptically
194 removed according to [Secombes \(1990\)](#), pooled in plastic bijoux vials containing 5ml ice-cold
195 L-15 medium containing 40 μl heparin (10 IU $\times \text{ml}^{-1}$) and used for respiratory burst assays.
196 Two pools per tank were used to determine the level of superoxide anion (O^{-2}) produced by
197 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
$$FCR = \frac{\text{Feed Intake (FI) (g)}}{\text{Wet weight gain (g)}}$$

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

206 Condition Factor (*K*):

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

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 O² 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 μ l
232 added to each well of a non-absorbent U-well micro-plate (Sterilin) in duplicate. Ten μ l 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 μ l G-CFB with 20mM EDTA, followed by
238 centrifugation to spin down the remaining SRBC. After centrifugation 100 μ l 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

245 volume of plasma giving 50 % haemolysis (H_{50}), and the $H_{50} \times \text{ml}^{-1}$ of plasma calculated by
246 dividing the dilution factor of plasma with the estimated plasma volume causing lysis to the
247 50% of the RBCs in the wells expressed in ml.

248 **2.7. Total plasma Immunoglobulin M (IgM)**

249 The level of plasma IgM in experimental salmon was determined using indirect enzyme linked
250 immunosorbent assay (ELISA) ([Magnadottir and Gudmundsdottir, 1992](#)), with modifications.
251 Briefly, two replicate rows of a 96-well an Immulon™ 4HBX plate (Thermo Scientific, Maine,
252 USA) were coated with $100 \mu\text{l} \times \text{well}^{-1}$ serial dilution of purified IgM (Aquatic Diagnostics,
253 Stirling, Scotland) in 0.05M sodium carbonate/bicarbonate buffer, pH 9.6 (starting from 0.32
254 $\text{mg} \times \text{ml}^{-1} - 0.00016 \text{ mg} \times \text{ml}^{-1}$) to form a standard curve of IgM concentration vs. absorbance
255 at 450 nm. To the remainder of the wells $100 \mu\text{l}$ of a fold 1/500 and 1/1000 dilution of plasma
256 from experimental fish was added, diluted in 0.05M sodium carbonate/bicarbonate buffer, pH
257 9.6, using two replicate wells for each dilution. The plates were then incubated overnight at
258 4°C and washed 5 times with low salt wash buffer (LSWB; 0.02 M Trizma base, 0.38 M NaCl,
259 0.05% (v/v) Tween 20, pH 7.2). The wells were blocked with $250 \mu\text{l}$ of 3 % w/v dried skimmed
260 milk (Marvel, Dublin, Ireland) in water and the plates were incubated for 120 min at 21°C . The
261 casein solution was removed before adding $100 \mu\text{l}$ of mouse anti-trout/salmon IgM (F11-
262 monoclonal anti trout/salmon IgM - Aquatic Diagnostics, Stirling, Scotland) solution (1:66) in
263 1 % BSA in LSBW for 1 h at 21°C . Plates were then washed with 5 washes of high salt wash
264 buffer (HSWB; 0.02 M Trizma base, 0.5 M NaCl, 0.01 % (v/v) Tween 20, pH 7.4) and
265 incubated for 5 min on last wash before adding $100 \mu\text{l} \times \text{well}^{-1}$ goat anti-mouse
266 immunoglobulin-G labelled with horseradish peroxidase (HRP) (Sigma/Aldrich) diluted 1:
267 4000 in conjugate buffer) incubating for 60 min at 21°C . Plates were washed with 5 washes of
268 HSWB, incubating for 5 min on last wash and the reaction was developed by adding $100 \mu\text{l} \times$
269 well^{-1} of substrate/chromogen (i.e. 15 ml substrate buffer containing $5 \mu\text{l}$ hydrogen peroxide

270 and 150 μ l trimethyl-benzidine (**TMB**) di-hydrochloride) and incubating for 10 min at 22°C.
271 The reaction was stopped with 50 μ l \times well⁻¹ of 2M H₂SO₄ and plate read at 450 nm after 5
272 seconds in a micro-plate reader (Biotek Synergy HT).

273 **2.8. Plasma anti-protease activity**

274 The method used was designed to detect anti-protease activity in trout plasma, and was based
275 on the method described by (Ellis, 1990), modified for use in microtitre plates. A hundred
276 micrograms per millilitre of trypsin solution was prepared by adding 1 ml of 25 mg \times ml⁻¹ of
277 trypsin stock solution (Invitrogen, UK) in 249 ml 0.1 M Tris.HCl (pH 8.2). Plasma samples
278 were diluted two-fold in the Tris.HCl buffer in round-bottomed 96 well plates (Sterilin), giving
279 final plasma volumes of 2.5, 1.25, 0.625 and 0.313 μ l. In a flat-bottomed 96 well plate, 5 μ l of
280 diluted samples were added to 15 μ l trypsin and incubated for 5 min; duplicates were used
281 where enough plasma was available. Finally, 200 μ l of chromogen solution in distilled water
282 (0.1% N α -Benzoyl- L -arginine 4-nitroanilide hydrochloride or simply BAPNA (Sigma-
283 Aldrich)) was added to each well. Wells containing only BAPNA solution and Tris.HCl buffer
284 without the addition of plasma samples served as a zero reference. The plates were then
285 incubated for 30 min at 22°C before centrifuging them for 6 min at 750 \times g. One hundred
286 microliters from each well was transferred to wells of a flat bottom 96-well plate and the
287 absorbance measured with a micro-plate reader (Biotek Synergy HT) set on a 5 min kinetic
288 run, reading every 1 min at 410 nm. Tryptic activity was a measure of the difference in values
289 at 5 min from the ones at time 0 divided by 5 (units expressed as change of 0.001 units of
290 absorbance at 410 nm \times min⁻¹). The 75 % inhibition value was calculated from the blank
291 samples, which represent the 100 % inhibition of tryptic activity and reference samples which
292 represent the 0 % inhibition of trypsin. The volume of plasma required to achieve 75 %
293 inhibition of trypsin activity was calculated from a graph of % trypsin inhibition against the
294 volume of plasma used. The units of trypsin inhibited at a percentage of 75 % per ml of plasma

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

300 ***2.9. Dietary and NQC fillet composition analysis***

301 The assessment of dietary lipid and FA composition was conducted by The Nutrition Analytical
302 Services (NAS) of the Institute of Aquaculture, University of Stirling and were run in
303 duplicates. Dietary crude fat was determined using two different methodologies. Firstly dietary
304 lipid content was determined following acid hydrolysis using a Soxtec System 1047
305 hydrolysing unit (Tecator Application note 92/87) followed by exhaustive Soxhlet extraction
306 using petroleum ether (40–60°C boiling point) on a Soxtec System HT6 (Tecator application
307 note 67/83) as described by [Bell et al. \(2001\)](#). In addition, dietary lipid fraction was determined
308 according to the Folch method ([Folch et al. 1957](#)) with non-lipid impurities removed by
309 washing with 0.88% (w/v) KCl. The lipid weight was determined gravimetrically after
310 evaporation of solvent under nitrogen and desiccation under vacuum for at least 16 h. Dietary
311 Fatty acid methyl esters (FAME) were prepared from total lipid by acid catalyzed
312 transesterification as described by [Christie \(2003\)](#) and FAMEs extracted and purified as
313 described by [Tocher & Harvie \(1988\)](#). FAMEs were separated and quantified by Gas Liquid
314 Chromatography (GLC) (Carlo Erba Vega 8160, Milan, Italy) using a 30 m \times 0.32 mm
315 capillary column (CP Wax 52CB, Chrompak, London, UK). Hydrogen was used as carrier gas
316 and temperature programming was from 50 to 150°C at 40°C \times min⁻¹ and then to 230°C at
317 2.0°C \times min⁻¹. Individual methyl esters were identified by comparison with known standards
318 and by reference to published data ([Ackman 1980](#)). Peak data was processed using Chromcard
319 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 Kjeltac 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.

395 **3.2. Growth and chemical composition of NQC fillet samples**

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

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

410 Salmon fed the control MB diet presented higher FI than the rest of the groups (Fig.
411 1B) (since the expected mean FI values of the MB group did not overlap with the 95% C.I. of
412 the other dietary groups). Furthermore, VP-fed salmon exhibited higher expected FI than the
413 MBABP, MFABP and VP/VO groups, while the latter group, also demonstrated lower FI than
414 VO-fed salmon. Studies have reported that total or partial replacement of FM in salmonid diets
415 can negatively affect the FI in fish (Gomes et al. 1995; De Francesco et al. 2004; Kaushik et
416 al. 2004; Espe et al. 2006) and this could be due to the lack in certain FM components acting
417 as feeding stimulators (Kousoulaki et al. 2012). Moreover, self-selecting feeding trials have
418 shown that rainbow trout prefer diets containing FO over those with VO, suggesting that some
419 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

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

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

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

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

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

523 **3.3. Haematology and innate immune responses**

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

545 [1994; Thompson et al. 1996](#)) without compromised blood oxygen carrying capacity, since there
546 was lack of anaemia which could be related to iron or other mineral deficiencies.

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

554 No differences regarding credible plasma haemolytic activity, plasma protein and total
555 IgM and expected respiratory burst activity in stimulated and non-stimulated head kidney
556 macrophages, were observed among the six dietary salmon groups. Contrary to this, reduced
557 levels of total plasma IgM were reported by [Jalili et al. \(2013\)](#) in rainbow trout fed on diets
558 with total substitution of FM with VPs. Furthermore, [Jalili et al. \(2013\)](#) and [Sitjà-Bobadilla et
559 al. \(2005\)](#) reported decreased alternative complement activity in rainbow trout and gilthead sea
560 bream fed diets with 100% substitution of FM with VPs. In agreement with the present
561 findings, no differences in HKM respiratory burst activity were observed in feeding trials
562 where Atlantic salmon and rainbow trout were fed on FM-based diets supplemented only with
563 soybean oil or linseed oil ([Kiron et al. 2004; Seierstad et al. 2009](#)). Furthermore, [Carter et al.
564 \(2003\)](#) demonstrated no changes in total immunoglobulin and protein levels, anti-protease
565 activity and circulating leucocytes respiratory burst activity for Atlantic salmon fed diets with
566 complete replacement of FO with canola oil (CO) or 2 blends of CO and FO or CO and algal
567 oil which are in line with the present findings. Similar results were also obtained by ([Thompson
568 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

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

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

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616 **REFERENCES**

617 Ackman R.G. (1980) Fish Lipids part 1. In: *Advances in Fish Sciences and Technology* (ed. by J.J.
618 Connell), pp. 86–103. Fishing News Books Farnham, UK.

- 619 AOAC (1990) Official Methods of Analysis of the AOAC, Fifteenth. ed. AOAC International,
620 Washington DC.
- 621 Barnham C. & Baxter A. (1998) Condition factor, K, for salmonid fish, Fisheries Notes. Department of
622 Environment & Primary Industries, Melbourne, State of Victoria.
- 623 Bates D., Maechler M., Bolker B. & Walker S. (2014) lme4: Linear mixed-effects models using Eigen
624 and S4. *R package version 1* (4)
- 625 Bell J.G., Henderson R.J., Tocher D.R., McGhee F., Dick J.R., Porter A., Smullen R.P. & Sargent J.R.
626 (2002) Substituting fish oil with crude palm oil in the diet of Atlantic salmon (*Salmo salar*) affects
627 muscle fatty acid composition and hepatic fatty acid metabolism. *Journal of Nutrition* **132**, 222–
628 230.
- 629 Bell J.G., McEvoy J., Tocher D.R., McGhee F., Campbell P.J. & Sargent J.R. (2001) Replacement of
630 fish oil with rapeseed oil in diets of Atlantic salmon (*Salmo salar*) affects tissue lipid
631 compositions and hepatocyte fatty acid metabolism. *Journal of Nutrition* **131**, 1535–1543.
- 632 Bransden, M.P., Carter, C.G. & Nowak, B.F. (2001) Effects of dietary orotein source on growth,
633 immune function, blood chemistry and disease resistance of Atlantic salmon (*Salmo Salar* L.)
634 parr. *Animal Sciences* **73**, 105 – 113.
- 635 Carter, C.G., Bransden, M.P., Lewis, T.E. & Nichols, P.D. (2003) Effects of dietary protein source on
636 growth, immune function, blood chemistry and disease resistance of Atlantic salmon (*Salmo*
637 *salar* L.) parr. *Marine Biotechnology (NY)* **5**, 480–492.
- 638 Christie, W.W., 2003. Isolation, separation, identification and structural analysis of lipids, in: Christie,
639 W.W., Han, X. (Eds.), *Lipid Analysis*. The Oily Press, High Wycombe, pp. 91–102.
- 640 Crampton V.O., Nanton D.A., Ruohonen, K., Skjervold P.-O. & El-Mowafi A.F.A. (2011)
641 Demonstration of salmon farming as a net producer of fish protein and oil. *Aquaculture Nutrition*
642 **16**, 437–446.
- 643 De Francesco M., Parisi G., Médale F., Lupi P., Kaushik S.J. & Poli B.M. (2004) Effect of long-term
644 feeding with a plant protein mixture based diet on growth and body/fillet quality traits of large
645 rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* **236**, 413–429.
- 646 De Silva S., Turcchini, G. & Francis, D. (2012) Nutrition. In: *Aquaculture: farming aquatic animals*
647 *and plants, 2nd Edition* (ed. by J.S. Lucas & P.C. Southgate), pp. 184. Blackwell Publishing Ltd,
648 Southern Gate, Chichester, Sussex, UK.
- 649 Ellis A.E. (1990) Plasma antiproteases. In: *Techniques in Fish Immunology* (ed. by J.S. Stolen, T.C.
650 Fletcher, D.P. Anderson, B.S. Roberston, W.B. van Muiswinkel), pp. 95–99. SOS Publications,
651 Fair Haven, NJ.
- 652 Espe M., Lemme A., Petri, A. & El-Mowafi A. (2006) Can Atlantic salmon (*Salmo salar*) grow on diets
653 devoid of fish meal? *Aquaculture* **255**, 255–262.
- 654 Espe M., Ruohonen K. & El-Mowafi A. (2012) Effect of taurine supplementation on the metabolism
655 and body lipid-to-protein ratio in juvenile Atlantic salmon (*Salmo salar*). *Aquaculture Research*
656 **43**, 349–360.

- 657 EU (2001) Commission Regulation (EC) No 999/2001 of the European Parliament and of the Council
658 of 22 May 2001. Laying down rules for the prevention, control and eradication of certain
659 transmissible spongiform encephalopathies. Brussels, Belgium.
- 660 EU (2003) Commission Regulation (EC) No 1234/2003 of 10 July 2003. Amending Annexes I, IV and
661 XI of regulation (EC) No 999/2001 of the European Parliament and of the council and regulation
662 (EC) No 1326/2001 as regards transmissible spongiform encephalopathies and animal feeding.
663 Brussels, Belgium.
- 664 Fevolden S.E., Røed K.H. & Gjerde B. (1994) Genetic components of post-stress cortisol and lysozyme
665 activity in Atlantic salmon; correlations to disease resistance. *Fish Shellfish Immunology* **4**, 507–
666 519.
- 667 Folch J., Lees M. & Sloane-Stanley G.H. (1957) A simple method for the isolation and purification of
668 total lipids from animal tissues. *Journal of Biological Chemistry* **226**, 497–509.
- 669 Fontaine J., Hörr J. & Schirmer B. (2001) Near-infrared reflectance spectroscopy enables the fast and
670 accurate prediction of the essential amino acid contents in soy, rapeseed meal, sunflower meal,
671 peas, fishmeal, meat meal products, and poultry meal. *Journal of Agriculture & Food Chemistry*
672 **49**, 57–66.
- 673 Gelman A. & Hill J. (2007) Data analysis using regression and multilevel/hierarchical models.
674 Cambridge University Press, Cambridge.
- 675 Geurden I., Corraze G. & Boujard T. (2007) Self-feeding behaviour of rainbow trout, *Oncorhynchus*
676 *mykiss*, offered diets with distinct feed oils. *Applied Animal Behaviour Science* **108**, 313–326.
- 677 Geurden I., Cuvier A., Gondouin E., Olsen R.E., Ruohonen K., Kaushik S. & Boujard T. (2005)
678 Rainbow trout can discriminate between feeds with different oil sources. *Physiology & Behaviour*
679 **85**, 107–114.
- 680 Gomes E.F., Rema P. & Kaushik S.J. (1995) Replacement of fish meal by plant proteins in the diet of
681 rainbow trout (*Oncorhynchus mykiss*): digestibility and growth performance. *Aquaculture* **130**,
682 177–186.
- 683 Green, J.A. & Hardy, R.W. (2002) The optimum dietary essential amino acid pattern for rainbow trout
684 (*Oncorhynchus mykiss*), to maximize nitrogen retention and minimize nitrogen excretion. *Fish*
685 *Physiology & Biochemistry* **27(1)**, 97-108.
- 686 Hardie L.J., Fletcher T.C. & Secombes C.J., 1990. The effect of vitamin E on the immune response of
687 the Atlantic salmon (*Salmo salar* L.). *Aquaculture* **87**, 1–13.
- 688 Hartviksen M., Vecino J.L.G., Ringø E., Bakke A., -M., Wadsworth S., Krogdahl Å., Ruohonen K. &
689 Kettunen A. (2014) Alternative dietary protein sources for Atlantic salmon (*Salmo salar* L.)
690 effect on intestinal microbiota, intestinal and liver histology and growth. *Aquaculture Nutrition*
691 **20**, 381-398.
- 692 Hatlen B., Jakobsen J.-V., Crampton V., Alm M., Langmyhr E., Espe M., Hevrøy E.M., Torstensen
693 B.E., Liland N. & Waagbø R. (2014) Growth, feed utilization and endocrine responses in Atlantic
694 salmon (*Salmo salar*) fed diets added poultry by-product meal and blood meal in combination
695 with poultry oil. *Aquaculture Nutrition* **21**, 714-725.

- 696 Hatlen B., Oaland Ø., Tvenning L., Breck O., Jakobsen J.V. & Skaret J. (2013) Growth performance,
697 feed utilization and product quality in slaughter size Atlantic salmon (*Salmo salar* L.) fed a diet
698 with porcine blood meal, poultry oil and salmon oil. *Aquaculture Nutrition* **19**, 573–584.
- 699 Hemre G.I., Sanden, M., Bakke-McKellep, A.M., Sagstad, A. & Krogdahl, Å. (2005) Growth, feed
700 utilization and health of Atlantic salmon *Salmo salar* L. fed genetically modified compared to
701 non-modified commercial hybrid soybeans. *Aquaculture Nutrition* **11**, 157–167.
- 702 Hemre G.I., Sandnes K., Lie Ø. & Waagbø R. (1995) Blood chemistry and organ nutrient composition
703 in Atlantic salmon, *Salmo salar* L., fed graded amounts of wheat starch. *Aquaculture Nutrition*
704 **1**, 37–42.
- 705 Higgs D.A., Markert J.R., MacQuarrie D.W., McBride J.R., Dosanjh B.S., Nichols C. & Hoskins G.
706 (1979) Development of practical dry diets for coho salmon, *Oncorhynchus kisutch*, using poultry-
707 by-product meal, feather meal, soybean meal and rapeseed meal as major protein sources. In:
708 *Finfish Nutrition and Fish Feed Technology Vol.2.* (ed. by F.E. Halver, K. Tiews), pp. 191–218.
709 Heenemann Verlagsgesellschaft MbH, Berlin.
- 710 Jalili R., Tukmechi A., Agh N., Noori F. & Ghasemi A. (2013) Replacement of dietary fish meal with
711 plant sources in rainbow trout (*Oncorhynchus mykiss*); effect on growth performance, immune
712 responses, blood indices and disease resistance. *Iranian Journal of Fisheries Science* **12**, 577–
713 591.
- 714 Johansen S.J.S., Ekli M., Stangnes B. & Jobling M. (2001) Weight gain and lipid deposition in Atlantic
715 salmon, *Salmo salar*, during compensatory growth: evidence for lipostatic regulation? *Aquac.*
716 *Res.* **32**, 963–974.
- 717 Jokumsen A. & Alsted N. (1990) Ørredvækst i relation til forholdet mellem protein og fedt i foderet.
718 In: *Meddelesler fra Forsøgdambruget* **80**, 1-15.
- 719 Jordal A.-E.O., Lie Ø. & Torstensen B.E. (2007) Complete replacement of dietary fish oil with a
720 vegetable oil blend affect liver lipid and plasma lipoprotein levels in Atlantic salmon (*Salmo*
721 *salar* L.). *Aquaculture Nutrition* **13**, 114–130.
- 722 Kaleeswaran B., Ilavenil S. & Ravikumar S. (2011) Growth Response, Feed Conversion Ratio and
723 Antiprotease Activity of *Cynodon dactylon* (L.) Mixed Diet in *Catla catla* (Ham.). *Journal of*
724 *Animal & Veterinary Advances* **10**, 511–517.
- 725 Karalazos, V., Bendiksen E.Å., Dick J.R. & Bell J.G. (2007) Effects of dietary protein, and fat level and
726 rapeseed oil on growth and tissue fatty acid composition and metabolism in Atlantic salmon
727 (*Salmo salar* L.) reared at low water temperatures. *Aquaculture Nutrition* **13**, 256–265.
- 728 Kaushik S.J., Covès D., Dutto G. & Blanc D. (2004) Almost total replacement of fish meal by plant
729 protein sources in the diet of a marine teleost, the European seabass, *Dicentrarchus labrax*.
730 *Aquaculture* **230**, 391–404.
- 731 Kiron V., Puangkaew J., Ishizaka K., Satoh S. & Watanabe T. (2004) Antioxidant status and nonspecific
732 immune responses in rainbow trout (*Oncorhynchus mykiss*) fed two levels of vitamin E along
733 with three lipid sources. *Aquaculture* **234**, 361–379.
- 734 Korkea-aho T.L., Heikkinen J., Thompson K.D., von Wright A. & Austin B. (2011) *Pseudomonas* sp.
735 M174 inhibits the fish pathogen *Flavobacterium psychrophilum*. *Journal of Applied*
736 *Microbiology* **111**, 266–77.

- 737 Krogdahl A., Bakke-McKellep A.M., Røed K.H. & Baeverfjord G. (2000) Feeding Atlantic salmon
738 *Salmo salar* L. soybean products: effects on disease resistance (furunculosis), and lysozyme and
739 IgM levels in the intestinal mucosa. *Aquaculture Nutrition* **6**, 77–84.
- 740 Langston, A.L., Johnstone, R. & Ellis A.E. (2001) The kinetics of the hypoferraemic response and
741 changes in levels of alternative complement activity in diploid and triploid Atlantic salmon,
742 following injection of lipopolysaccharide. *Fish & Shellfish Immunology* **11**, 333–45.
- 743 Liland N.S., Rosenlund G., Berntssen M.H.G., Brattelid T., Madsen L. & Torstensen B.E. (2012) Net
744 production of Atlantic salmon (FIFO, Fish in Fish out < 1) with dietary plant proteins and
745 vegetable oils. *Aquaculture Nutrition* **19**, 289–300.
- 746 Llames C.R. & Fontaine J. (1994) Determination of amino acids in feeds: collaborative study. *Journal*
747 *AOAC International* **77**, 1362–1402.
- 748 Lund T., Gjedrem T., Bentsen H.B., Eide D.M., Larsen H.J.S. & Roed K.H. (1995) Genetic variation
749 in immune parameters and associations to survival in Atlantic salmon. *Journal of Fish Biology*
750 **46**, 748–758.
- 751 Magnadottir B. & Gudmundsdottir B.K. (1992) A comparison of total and specific immunoglobulin
752 levels in healthy Atlantic salmon (*Salmo salar* L.) and in salmon naturally infected with
753 *Aeromonas salmonicida* subsp. *achromogenes*. *Veterinary Immunology & Immunopathology* **32**,
754 179–89.
- 755 Mambrini, M. & Kaushik, S.J. (1994) Partial replacement of dietary protein nitrogen with dispensable
756 amino acids in diets of Nile tilapia, *Oreochromis niloticus*. *Comparative Biochemistry Physiology*
757 **109A**, 469–477.
- 758 Miller M.R., Nichols P.D. & Carter C.G. (2007) Replacement of fish oil with thraustochytrid
759 *Schizochytrium* sp. L oil in Atlantic salmon parr (*Salmo salar* L) diets. *Comparative Biochemistry*
760 *& Physiology A: Molecular & Integrative Physiology* **148**, 382–92.
- 761 Naylor R.L., Hardy R.W., Bureau D.P., Chiu A., Elliott M., Farrell A.P., Forster I., Gatlin D.M.,
762 Goldberg R.J., Hua K. & Nichols P.D. (2009) Feeding aquaculture in an era of finite resources.
763 *Proceedings of National Academic Sciences USA* **106**, 15103–15110.
- 764 NRC (1993) Nutrient Requirements of fish - National Research Council. National Academy Press,
765 Washington DC.
- 766 Pickering A.D. (1993) Growth and stress in fish production. *Aquaculture* **111**, 51–63.
- 767 Plisetskaya E.M. & Duan C. (1994) Insulin and insulin-like growth factor I in coho salmon
768 *Oncorhynchus kisutch* injected with streptozotocin. *American Journal of Physiology* **267**,
769 R1408–1412.
- 770 R Core Development Team (2014) R: a language and environment for statistical computing |
771 GBIF.ORG [WWW Document]. URL <http://www.r-project.org>. (accessed 11.17.14)
- 772 Rao Y. V. & Chakrabarti R. (2004) Potentiation of antibody production in Indian major carp *Labeo*
773 *rohita*, rohu, by *Achyranthes aspera* as a herbal feed ingredient. *Indian Journal of Clinical*
774 *Biochemistry* **19**, 132–134.

- 775 Røed K.H., Larsen H.J.S., Linder R.D. & Refstie T. (1993) Genetic variation in lysozyme activity in
776 rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* **109**, 237–244.
- 777 Rumsey G.L., Siwicki A.K., Anderson D.P. & Bowser P.R. (1994) Effect of soybean protein on
778 serological response, non-specific defense mechanisms, growth, and protein utilization in
779 rainbow trout. *Veterinary Immunology & Immunopathology* **41**, 323–339.
- 780 Ruyter B., Moya-Falcón C., Rosenlund G. & Vegusdal A. (2006) Fat content and morphology of liver
781 and intestine of Atlantic salmon (*Salmo salar*): Effects of temperature and dietary soybean oil.
782 *Aquaculture* **252**, 441–452.
- 783 Saurabh S. & Sahoo P.K. (2008) Lysozyme: an important defence molecule of fish innate immune
784 system. *Aquaculture Research* **39**, 223–239.
- 785 Secombes C.J. (1990) Isolation of salmonid macrophages and analysis of their killing activity. In:
786 *Techniques in Fish Immunology*. (ed. by J.S. Stolen, T.C. Fletcher, D.P. Anderson, B.S.
787 Robertson, W.B. van Muiswinkel). pp. 137–154. SOS Publications, Fair Haven, NJ.
- 788 Seierstad S.L., Haugland Ø., Larsen S., Waagbø R. & Evensen Ø. (2009) Pro-inflammatory cytokine
789 expression and respiratory burst activity following replacement of fish oil with rapeseed oil in
790 the feed for Atlantic salmon (*Salmo salar* L.). *Aquaculture* **289**, 212–218.
- 791 Shearer K.D. (1994) Factors affecting the proximate composition of cultured fishes with emphasis on
792 salmonids. *Aquaculture* **119**, 63–88.
- 793 Shearer KD, Åsgard T, Andorsdottir G & Aas GH (1994) Whole body elemental and proximate
794 composition of Atlantic salmon (*Salmo salar*) during the life cycle. *Journal of Fish Biology*
795 **44**, 785–797.
- 796 Sitjà-Bobadilla A., Peña-Llopis S., Gómez-Requeni P., Médale F., Kaushik S. & Pérez-Sánchez J.
797 (2005) Effect of fish meal replacement by plant protein sources on non-specific defence
798 mechanisms and oxidative stress in gilthead sea bream (*Sparus aurata*). *Aquaculture* **249**, 387–
799 400.
- 800 Steffens W. (1994) Replacing fish meal with poultry by-product meal in diets for rainbow trout,
801 *Oncorhynchus mykiss*. *Aquaculture* **124**, 27–34.
- 802 Thompson K.D., Tatner M.F. & Henderson R.J. (1996) Effects of dietary (n-3) and (n-6)
803 polyunsaturated fatty acid ratio on the immune response of Atlantic salmon, *Salmo salar* L.
804 *Aquaculture Nutrition* **2**, 21–31.
- 805 Tocher D.R. & Harvie D.G. (1988) Fatty acid compositions of the major Fatty, phosphoglycerides from
806 fish neural tissues; (n-3) and (n-6) polyunsaturated And, acids in rainbow trout (*Salmo gairdneri*)
807 and cod (*Gadus morhua*) brains retinas. *Fish Physiology & Biochemistry* **5**, 229–239.
- 808 Torstensen B.E., Bell J.G., Rosenlund G., Henderson R.J., Graff I.E., Tocher D.R., Lie Ø. & Sargent
809 J.R. (2005) Tailoring of Atlantic salmon (*Salmo salar* L.) flesh lipid composition and sensory
810 quality by replacing fish oil with a vegetable oil blend. *Journal of Agriculture & Food Chemistry*
811 **53**, 10166–10178.
- 812 Torstensen B.E., Espe M., Sanden M., Stubhaug I., Waagbø R., Hemre G.-I., Fontanillas R.,
813 Nordgarden U., Hevrøy E.M., Olsvik P. & Berntssen M.H.G. (2008) Novel production of

- 814 Atlantic salmon (*Salmo salar*) protein based on combined replacement of fish meal and fish oil
815 with plant meal and vegetable oil blends. *Aquaculture* **285**, 193–200.
- 816 Torstensen B.E., Espe M., Stubhaug I. & Lie Ø. (2011) Dietary plant proteins and vegetable oil blends
817 increase adiposity and plasma lipids in Atlantic salmon (*Salmo salar* L.). *British Journal of*
818 *Nutrition* **106**, 633–47.
- 819 Turchini G.M. & Francis D.S. (2009) Fatty acid metabolism (desaturation, elongation and beta-
820 oxidation) in rainbow trout fed fish oil- or linseed oil-based diets. *British Journal of Nutrition*
821 **102**, 69–81.
- 822 Twibell R.G., Gannam A.L., Hyde N.M., Holmes J.S.A. & Poole J.B. (2012) Effects of fish meal- and
823 fish oil-free diets on growth responses and fatty acid composition of juvenile coho salmon
824 (*Oncorhynchus kisutch*). *Aquaculture* **360-361**, 69–77.
- 825 Van Dyck K. (2012) Summary report of the standing committee on the food chain and animal health
826 held in Brussels on 18 July 2012 (Section Biological Safety of the Food chain). Brussels.
- 827 Waagbø R., Glette J., Sandnes K. & Hemre G.I. (1994) Influence of dietary carbohydrate on blood
828 chemistry, immunity and disease resistance in Atlantic salmon, *Salmo salar* L. *Journal of Fish*
829 *Diseases* **17**, 245–258.
- 830

1 **Figure Captions**

2 **Figure 1. Expected performance indices at different study points.** (A) Feed Intake (FI); (B)
3 Daily weight gain (WG); (C) Feed Conversion Ratio (FCR) of the Atlantic salmon groups over
4 the periods 1 and 2 and the full duration of the trial and (D) condition factor (*K*) of the salmon
5 groups for the full period of the trial. These parameters were affected by the diets and the results
6 are summarised as graphs with the expected mean response and 95% confidence intervals.
7 Confidence intervals were solved by a posterior simulation from the statistical model with 1500
8 random draws. Significant differences among dietary groups are revealed when the confidence
9 intervals bars for a certain response of a dietary group do not overlap with the mean values of
10 the same response from another group. The effect of feeds on the above growth performance
11 indices was confirmed by fitting a model without the dietary effect (only tank effect) and with
12 it (tank effect nested within it) and comparing the models with a likelihood ratio tests (LRT).
13 FI and FCR are modelled as ordinary linear models since there is no multilevel structure (only
14 one observation per tank) whereas WG modelled with the help of splines to allow non-linear
15 response in time. FI and WG are expressed as $\text{g} \times \text{fish}^{-1} \times \text{day}^{-1}$ to adjust for the different
16 duration of the periods. Diet abbreviations: MB, European commercial marine based diet
17 (2011-12); VP, vegetable protein diet; VO, vegetable oil diet; VP/VO, vegetable
18 protein/vegetable oil diet; MBABP, Non-EU commercial marine based diet with inclusion of
19 animal-by-product (2011-12); MFABP, fish free animal-by-product diet. Significant
20 differences (*P* values < 0.05) between dietary groups are denoted by different letters.

21 **Figure 2. Expected levels of the affected haematological and immunological responses.**(A)
22 Haematocrit (%); (B) Lysozyme ($\text{Units} \times \text{min}^{-1} \times \text{ml}^{-1}$); Plasma anti-protease activity (Units
23 $\text{TI}_{75} \times \text{ml}^{-1}$); (%); of the dietary groups of Atlantic salmon post-smolts for the full period of the
24 trial. These parameters were affected by the diets and the results are summarised as graphs with
25 the expected mean response and 95% confidence intervals. Confidence intervals were solved
26 by a posterior simulation from the statistical model with 1500 random draws. Significant
27 differences among dietary groups are revealed when the confidence intervals bars for a certain
28 response of a dietary group do not overlap with the mean values of the same response from
29 another group. The dietary effect on the health responses was confirmed by fitting a model
30 without the dietary effect (only tank effect) and with it (tank effect nested within it) and
31 comparing the models with a likelihood ratio tests (LRT).The modelling for all above
32 parameters Diet abbreviations: MB, European commercial marine based diet (2011-12); VP,
33 vegetable protein diet; VO, vegetable oil diet; VP/VO, vegetable protein/vegetable oil diet;
34 MBABP, Non-EU commercial marine based diet with inclusion of animal-by-product (2011-
35 12); MFABP, fish free animal-by-product diet. Significant differences (*P* values < 0.05)
36 between dietary groups are denoted by different letters.

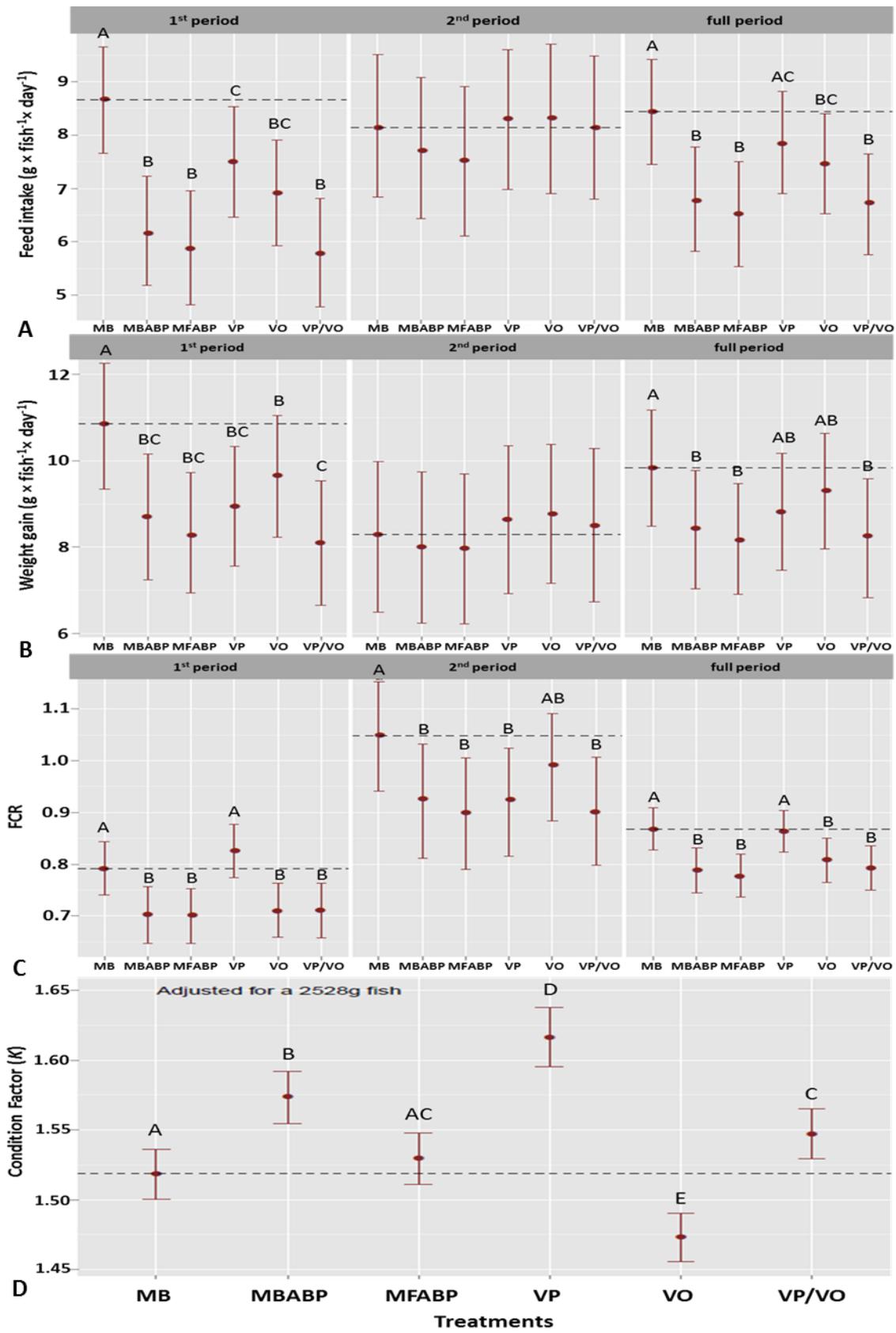
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Figure 1.

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Figure 2.

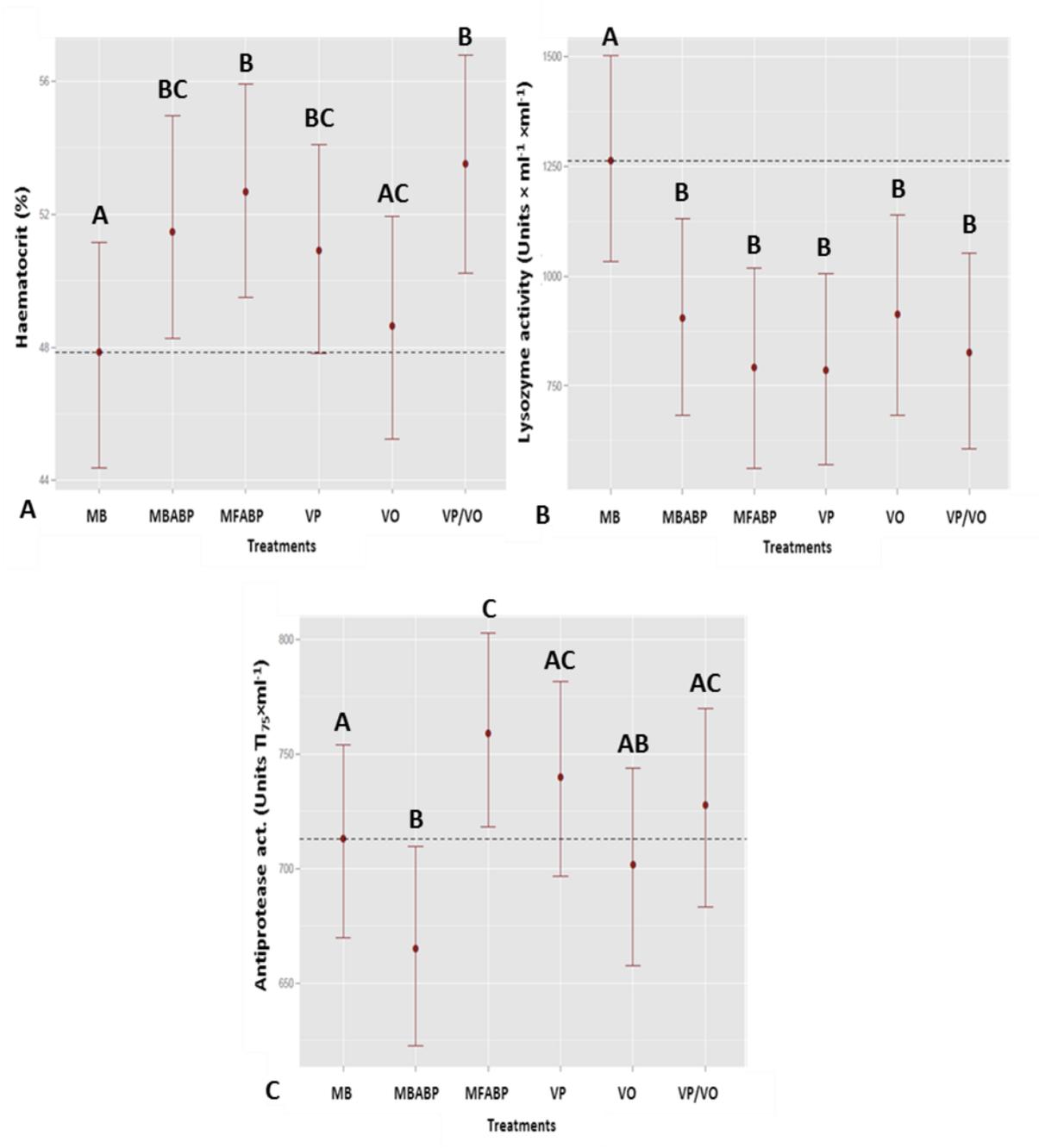


Table 1 Feed formulations of the six diets.

FEED FORMULATION Ingredient composition (g × kg⁻¹)	DIETS					
	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

2 Diet abbreviations: MB, European commercial marine based diet (2011-12); MBABP, Non-EU
3 commercial marine based diet with inclusion of animal-by-products (2011-12); MFABP, fish free
4 animal-by-product diet; VP, vegetable protein diet; VO, vegetable oil diet; VP/VO, vegetable
5 protein/vegetable oil diet. ^aLT Fishmeal (low temperature fishmeal from Egersund Sildeoljefabrikk AS,
6 Egersund, Norway): superior quality FM due to the fact that is treated at lower drying temperatures
7 allowing FM to keep optimal essential amino acid profile, low biogenic amines with an apparent protein
8 digestibility coefficient of 90.2%; ^bVegetable proteins: includes protein concentrates from soy (Imcopa,
9 - Importação, Exportação e Indústria de Óleos Ltda., Araucária - Paraná, Brazil) and pea (AgriMarin,
10 Stavanger, Norway) and wheat gluten (Henan Tianguan, Nanyang City, China); ^cAnimal by-products:
11 includes Poultry by-product meal (Poultry by-product meal, GePro Geflügel-Protein Vertriebs- GmbH
12 & Co, Diepholz, Germany) and porcine blood meal (Daka Proteins, Løsning, Denmark); ^dAmino acids
13 from Evonik Degussa International AG, Hanau, Germany; ^eproprietary of EWOS Innovation; Plant
14 lipids: includes mainly rapeseed oil (Cargill PLC, Lincoln, UK); ^fFish Oil: Capelin oil (Egersund
15 Sildeoljefabrikk AS, Egersund), Norway; Plant Lipids: includes rapeseed oil; ^hAlgal lipids: includes oil
16 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
<i>**Feed Intake 1st Period (g×fish⁻¹×day⁻¹)</i>	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
<i>Feed Intake 2nd Period (g×fish⁻¹×day⁻¹)</i>	8.11±1.33	7.69±1.21	7.53±1.23	8.29±1.56	8.30±1.58	8.12±0.70
<i>Feed Intake full Period (g×fish⁻¹×day⁻¹)</i>	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
<i>*Wt gain (g×fish⁻¹×day⁻¹) 1st Period</i>	10.84±0.52 ^A	8.63±0.52 ^{BC}	8.22±0.77 ^{BC}	8.98±1.47 ^{5BC}	9.59±0.76 ^B	8.10±0.32 ^C
<i>Wt gain (g×fish⁻¹×day⁻¹) 2nd Period</i>	8.11±2.01	7.87±1.77	7.87±1.80	8.48±1.68	8.58±2.09	8.25±0.55
<i>Wt gain (g×fish⁻¹×day⁻¹) full Period</i>	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
<i>†FCR 1st Period</i>	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
<i>FCR 2nd Period</i>	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
<i>FCR full Period</i>	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
<i>‡Condition Factor (K) end of trial</i>	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
<i>Mortalities (%) 1st period</i>	0.3±0.5	0	0	0	0.3±0.5	0
<i>Mortalities (%) 2nd period</i>	0	0	0	0	0	0
NQC composition						
<i>Moisture (%)</i>	65.07±0.23	65.19±1.17	65.67±1.10	64.27±0.57	65.75±1.08	65.65±0.29
<i>Protein (%)</i>	18.46±0.23	18.87±0.27	18.69±0.51	18.82±0.81	18.89±0.70	18.99±0.28
<i>Crude Lipid (%)</i>	13.56±0.21	12.57±0.58	12.64±0.82	13.47±1.03	12.07±0.32	12.92±0.48
<i>Ash (%)</i>	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
<i>Haematocrit (%)</i>	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
<i>Leucocytes ($\times 10^7 \times ml^{-1}$)</i>	10.3±4.0	10.2±2.9	10.2±2.7	11.7±3.7	10.4±3.9	8.3±1.9
<i>Lymphocytes ($\times 10^7 \times ml^{-1}$)</i>	5.2±2.1	5.6±1.4	4.8±1.4	5.8±1.6	4.9±1.4	4.3±1.3
<i>Thrombocytes ($\times 10^7 \times ml^{-1}$)</i>	4.4±1.7	4.1±1.7	4.8±1.6	5.3±1.9	4.9±2.8	3.6±1.4
<i>Granulocytes ($\times 10^7 \times ml^{-1}$)</i>	0.6±0.4	0.4±0.2	0.5±0.3	0.6±0.3	0.5±0.5	0.5±0.2
<i>Monocytes ($\times 10^7 \times ml^{-1}$)</i>	0.05±0.08	0.03±0.04	0.04±0.05	0.05±0.06	0.06±0.06	0.03±0.03
<i>Lysozyme activity (units $\times min^{-1} \times ml^{-1}$)</i>	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
<i>Plasma haemolytic activity (units $SH_{50} \times ml^{-1}$)</i>	928±298.2	954.6±139.1	1043.1±378.3	928.5±260.1	986.8±444.2	906.8±271.4
<i>HKMs respiratory burst (NBT) ($O.D._{610}$ for 10^5 nuclei)</i>	0.40±0.15	0.53±0.26	0.37±0.18	0.36±0.22	0.40±0.21	0.36±0.22
<i>Stimulated HKMs respiratory burst ($O.D._{610}$ for 10^5 nuclei)</i>	0.55±0.22	0.66±0.29	0.51±0.19	0.50±0.28	0.52±0.23	0.49±0.29
<i>Total plasma protein ($mg \times ml^{-1}$)</i>	66.4±6.3	66.1±8.3	65.3±6.7	66.5±6.3	67.9±4.3	67.9±5.9
<i>Plasma antiprotease act. (Units $TI_{75} \times min^{-1} \times ml^{-1}$)</i>	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}
<i>Total plasma IgM ($mg \times ml^{-1}$)</i>	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 \pm SD from 8 pools of 3 fish per diet; for haematocrit values are means \pm SD from 24 individual fish per diet; and for leucocyte and differential leucocyte counts values are means \pm 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.