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The effects of increasing dietary levels of soy protein concentrate (SPC) on the immune responses and disease resistance (furunculosis) of vaccinated and non-vaccinated Atlantic salmon (*Salmo salar* L.) parr

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Abbreviations

AC, alternative complement; ANF, anti-nutrient factors; BSA, bovine serum albumin; CCP, classical complement pathway; dpv, days post vaccination; EAAs, essential amino acids; ELISA, enzyme linked immunosorbent assay; FM, fishmeal; G-CFB, gelatine-complement fixation buffer; HKM, head kidney macrophages; HSWB, high salt wash buffer; i.p., intraperitoneal; LRT, likelihood ratio test; LSWB, low salt wash buffer; PBS, phosphate buffer saline; SRBC, sheep red blood cells; SBM, soybean meal; SPC, soy protein concentrate; TSA, tryptic soy agar; TC, total complement.

Keywords: Atlantic salmon, parr, soy protein concentrate, vaccination, immune function

Abstract

Juvenile salmon, with an initial weight of 9g, were fed three experimental diets, formulated to replace 35 (SPC35), 58 (SPC58) and 80 (SPC80) of high quality fishmeal (FM) with soy protein concentrate (SPC) in quadruplicate tanks. Higher dietary SPC inclusion was combined with increased supplementation of methionine, lysine, L-threonine and phosphorus. The experiment was carried out for 177 days. On day 92 salmon in each tank were bulk weighed. Post weighing eighty salmon from each tank were redistributed in two sets of 12 tanks. Salmon from the first set of tanks were vaccinated, while the second group was injected with phosphate buffer saline (PBS). Salmon were sampled on day 92 (pre-vaccination), day 94 (2 days post vaccination [dpv]/PBS injection [dpPBSinj]) and day 154 (62 dpv/dpPBSinj) of the trial for the assessment of their immune responses, prior to the performance of salmon bulk weights for each tank. On day 154, fish from each tank were again bulk weighed and then seventeen salmon per tank were redistributed in two sets of twelve tanks and intra-peritoneally infected with *Aeromonas salmonicida*. At Day 154, SPC80 demonstrated lower performance (weight gain, specific growth rate and thermal growth coefficient and feed conversion ratio) compared to SPC35 salmon. Reduced classical and total complement activities for salmon fed diets with over 58 % of protein from SPC, were demonstrated prior to vaccination. Reduced alternative complement activity was detected for both SPC58 and SPC80 salmon at 2 dpv and for the SPC80 group at 62 dpv. Total and classical complement activities demonstrated no differences among the dietary groups after vaccination. Numerical increases in classical complement activity were apparent upon increased dietary SPC levels. Increased phagocytic activity (% phagocytosis and phagocytic index) was exhibited for the SPC58 group compared to SPC35 salmon at 62 dpPBSinj. No differences in serum lysozyme activity, total IgM, specific antibodies, protein, glucose and HKM respiratory burst were detected among the dietary groups at any timepoint or state. Mortalities as a result of the experimental infection only occurred in

PBS-injected fish. No differences in mortality levels were demonstrated among the dietary groups. SPC58 diet supported both good growth and health in juvenile Atlantic salmon while SPC80 diet did not compromise salmon' immunity or resistance to intraperitoneally inflicted furunculosis.

1. Introduction

Farmed Atlantic salmon are typically raised in intensive aquaculture production systems and fed nutritionally complete formulated diets. Historically, fish meal (FM) has been the source of protein and essential amino acids for salmon feeds [1]. In 2009, aquaculture' use of global FM production was estimated to be 68%, with salmonid aquafeeds consuming 13.7% [2]. Unless alternative protein sources are used, the reliance of salmon diets on FM may reduce the potential for salmon culture growth, since the worldwide demand for FM is rapidly exceeding supply. Given that plant feedstuffs are readily available, these have received most attention as an alternative to FM [3-6].

Among plant protein ingredients, SPC manufactured through aqueous alcohol extraction of defatted soybeans is a very promising protein source for Atlantic salmon. Alcohol extracted SPC has a protein content, which is very similar to that of FM [7], while its EAA content compares favourably with FM, with the exception of methionine and potentially lysine [8]. Furthermore, lectins, saponins soy antigens and trypsin inhibitors concentrations, which are ANFs, are found at lower the concentrations than those found in conventional SBM [9-11]. Several studies have demonstrated the suitability of SPC as an alternative to FM in Atlantic salmon post smolt diets [12-14]. Moreover, a few studies reported the absence of soybean-induced intestinal inflammation in salmonids receiving diets with even 100% substitution of FM with SPC [15-16]. However, the tolerance of salmon for plant feedstuffs depends on salmon size and stage. Burr et al. [17] demonstrated that early stage Atlantic salmon parr are much more sensitive to dietary vegetal protein inclusion than late stage Atlantic salmon parr.

Previous studies exploring the effects of increasing dietary levels of plant derived ingredients on the immunity of several fish species have reported adverse effects of diets with over 70% of dietary protein from plant derived feedstuffs on immune responses such as the total serum immunoglobulin levels and alternative complement activity in rainbow trout *Oncorhynchus mykiss* [18] and the alternative complement activity of gilthead sea bream *Sparus aurata* [19]. While studies on the use of soybean meals in salmonid diets and their subsequent effects on immune function have been undertaken, only few have investigated the effects of dietary SPC on Atlantic salmon immune responses [1, 16, 20-21]. Briefly, Krogdahl et al. [16] demonstrated an enhancement of lysozyme activity and total IgM levels in the intestinal mucosa of Atlantic salmon smolts, maintained on feeds with 30% of dietary protein from soy products (SBM and SPC), and in turn, enhanced resistance of SPC fed salmon to infection by *A. salmonicida* the causative agent of furunculosis. Moreover, Metochis et al. [21] reported no adverse effects in the immune responses of large size Atlantic salmon parr commercially reared under constant light and intensive feeding with amino acid supplemented diets formulated with up to 80% of protein SPC and constant supplementation of phosphorus compared to a commercial type diet with up to 35% of protein from SPC.

Furunculosis is a highly infectious disease, causing serious fish losses, such as those observed during the epidemic of 1991-1992, which led to the loss of approximately 25% of the total Scottish salmon production [22]. Successful vaccination has enabled the disease to be brought under control and currently the majority of farmed Scottish and Norwegian Atlantic salmon are vaccinated against fununculosis. Thus, vaccination with a commercial *A. salmonicida* vaccine and subsequent infection challenge of the experimental Atlantic salmon parr in this study was used to describe the effects of increasing dietary SPC levels on immune responses upon vaccination and the resistance of Atlantic salmon parr against furunculosis. Since the site(s) of pathogen uptake into fish, is a subject of conjecture and seems likely to

include gills, mouth, anus and/or surface injury [23-26] an i.p. injection of *A. salmonicida* was used for the infection of Atlantic salmon parr in this study.

Generally, soy products and several other vegetable derived products used as FM replacements have been shown to affect a range of immune responses in fishes and these have been interpreted as inflammatory/hypersensitivity or immunostimulatory effects [16, 19-22]. In spite of the fact that commercial application of aquafeeds with higher than 58% of dietary protein from SPC is unlikely, due to the high cost of this feedstuff in comparison to other FM alternatives, the above diets were tested in this framework in order to augment dietary responses, highlighting nutritional deficiencies. Herein, the main objective was to monitor how increased dietary SPC, methionine, lysine and phosphate inclusion (to give similar amino acid/protein ratios and increase the availability of P in diets with higher levels of SPC) affects the immunological responses of naïve and vaccinated (against *A. salmonicida)* Atlantic salmon parr and their protection against furunculosis after i.p. infection with *A. salmonicida*.

2. Materials and Methods

2.1. Diets and fish husbandry

The dietary trial was carried out at the Aquatic Research Facility (ARF), Institute of Aquaculture, University of Stirling and lasted 177 days. The feeding trial started in June, 2013 and ended in December 2013. The fresh water system consisted of twelve 100 l circular tanks supplied with flow-through water at a rate of 1.5 l × min^{-1}. Water temperature was maintained at 12 ± 1ºC (ambient temperature of 12 ± ºC for the first 3 months of the study and application of heating later on Day 115 of the feeding trial to maintain the temperature constant throughout the study), whilst photoperiod was constant to prevent smoltification (12 hours of light: 12 hours of darkness). Dissolved oxygen, ammonia, nitrate, nitrite and pH were monitored and remained within limits recommended for Atlantic salmon. Prior to the trial unvaccinated S1

Atlantic salmon parr (AquaGen QTL eggs - AquaGen Ltd, Kilmacolm, Scotland - selected for improved growth and resistance to IPNV) purchased from Scottish Seafarms Ltd (Dumfries, Scotland, UK) previously maintained on a commercial BioMar (BioMar Fishes Ltd, Grangemouth, Scotland) diet (BioMar Inicio PLUS) were allowed to acclimate for a week within two stock tanks, during which time they were maintained on a commercial EWOS (EWOS Ltd, Westfield, Near Bathgate, Scotland) diet (EWOS micro). The fish were then randomly allocated into the twelve trial tanks, each tank containing 130 individuals in which they were allowed to acclimatize for 7 days. The fish had an average weight of 9.3 g at the start of the trial. The fish were then starved for two days and were subsequently fed a mixture of the commercial feed and the trial diets they were assigned to. The trial diets contained different levels of protein from SPC (35, 58 and 80% of protein from SPC) replacing FM and were manufactured by EWOS Innovation, Dirdal, Norway. Protein/fat ratios were kept constant (~3.0), while methionine, lysine, L-threonine and phosphorus (P) supplementation increased concomitantly with increased dietary SPC inclusion. Each dietary treatment included four replicate tanks. Dietary formulations are presented in Table 1. Parr were acclimatized to the trial feeds for 3 days prior to the start of the trial. The trial lasted for almost 6 months (177 days) during which fish were fed on the diets to satiation twice daily at 09.30 and 16:30 hours. During feeding the outlets of the tanks were blocked. Satiation was judged to have been achieved when almost 30 pellets were not eaten. Uneaten pellets were then collected through siphoning and feed intake was calculated by subtracting the number uneaten from supplied pellets. The average weight of each pellet was calculated by weighing 8 × 500 pellets of each diet. Eight fish per tank were sampled on Day 92 of the feeding trial prior to vaccination with a commercial anti *A. salmonicida* and infectious pancreatic necrosis virus vaccine (Alpha-Ject 2-2, Pharmaq) to monitor their immune status. Briefly, blood from 8 fish per tank was collected from the caudal vein into non-heparinised syringes and then transferred into 1.5ml eppendorf

tubes. The blood was allowed to clot at 4° C and then 250µl of serum from the first four fish were pipetted into an eppendorf tube creating a pool of serum collected from 4 individuals per tank. Two serum pools per tank were obtained, from which multiple aliquots of 50 µl were created for the performance of immunoassays. Moreover, two pools of head kidney samples from 4 fish/tank were obtained for the isolation of head kidney macrophages (HKM) and the determination of HKM respiratory burst and phagocytic activities as described below. After the first sampling the bulk weights of the fish in each tank were measured for the assessment of their growth performance and approximately 80 fish per tank were divided between the original set of tanks and another set of 12 replicate tanks with the ones kept in the original set of tanks being vaccinated with the above vaccine and the salmon parr transferred to the replicate set of tanks being injected with 0.02 M phosphate buffer saline (PBS) (0.15 M NaCl, pH 7.2). Conditions in all tanks were kept constant to the previous period. The remaining salmon parr (~40 fish distributed in a third set of 3 tanks -1 tank per diet-) were used to establish the lethal dose of bacteria giving 70% mortalities of fish intraperitoneally infected with *A. salmonicida* (100µl). Pools of serum samples (2 serum and 2 head kidney pools from 4 fish per tank) were taken at 2 days post vaccination (2 dpv) (serum samples from only vaccinated individuals/head kidney samples from both vaccinated and PBS-injected salmon) (Day 94 of the feeding trial) and at 62 dpv (Day 154 of the feeding trial-sampling of vaccinated and PBS-injected salmon). Measurements of salmon bulk weights in the tanks were recorded on Day 154 for growth evaluation. The fish were weighed to the nearest 0.1g. Prior to any experimental procedure (e.g. weighing, measuring, vaccinating and challenging) all fish were anaesthetized using MS222 (Tricaine Methanosulphonate, Pharmaq Ltd, Fordingbridge, Hampshire, UK) (50 mg × l^{-1}). After the experimental procedure the fish were placed in clean aerated water and allowed to recover (usually within 5 min) before being returned to their tank. Measurements of fish weight and length were made throughout the experiments. Where fish required to be

sacrificed for blood and tissue sampling, they were anaesthetized with MS222 (100 mg × ml^{-1}).

2.2. Disease resistance

On Day 154 of the feeding trial (62 dpv), twenty five salmon from each tank of vaccinated and PBS-injected fish were removed and stocked in another two sets of 12 replicate tanks in the ARF. The tanks used were also circular fiberglass tanks supplied with flow-through fresh water as described above. The fish were housed under a controlled photoperiod (12 h of light: 12 h of darkness) at a controlled temperature of 13-15ºC. Seventy five hours before *A. salmonicida* was administered; a fresh culture of the passaged bacterium was prepared on a blood agar plate. Twenty seven hours before the commencement of the challenge, seven bacterial colonies were cultured in tryptone soy broth (15ºC for 18 h). Subsequently the bacteria were washed twice with sterile PBS with intermediate centrifugation (3500 × g, 10 min). The OD of the bacterial suspension was then adjusted to 1.0 at 610 nm (6×10^8 cfu × ml^{-1}), and serially diluted to 0.25×10^{-7} (corresponding to ~ 2×10^2 cfu × ml^{-1}), which was the dilution found to give approximately 70% mortalities in salmon parr in a pre-challenge trial. Cell densities were confirmed by distributing eight 25 µl drops of each one of the obtained serial bacterial suspensions (1.0×10^{-7}, 0.25×10^{-7}, 0.5×10^{-7}, 1.0×10^{-6}) onto tryptone soy agar plates (TSA) (Sigma-Aldrich) and colonies counted after 48 h. One-hundred microlitres of the 0.25×10^{-7} bacterial suspension (corresponding to and 2×10^1 cfu × $fish^{-1}$) was i.p. injected into each Atlantic salmon after anaesthetizing them (benzocaine, 30 mg × l^{-1}). Specific mortalities were confirmed by culturing kidney swabs onto TSA and checking colonial morphology. The challenge was terminated after 22 days, at which time mortalities had ceased.

2.3. Growth performance assessment

198 Salmon growth performance was assessed, through the application of the following formulae
199 to the data:
200 Feed intake:

201 $$FI\left(\frac{g}{day}\right)per\ fish = \frac{Weight\ of\ feed\ given - Weight\ of\ feed\ collected}{Number\ of\ fish \times Number\ of\ days}$$

202 FCR:

203 $$FCR = \frac{Weight\ of\ feed\ given - Weight\ of\ feed\ collected}{W1 - WO}$$

204 Weight gain:

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

206 Specific Growth Rate:

207 $$SGR = \left(\frac{lnW1 - lnW0}{Number\ of\ days}\right) \times 100$$

208 Thermal Growth Rate:

209 $$TGC = \left(\frac{\sqrt[3]{W1} - \sqrt[3]{W0}}{(t \times T)}\right) \times 100$$

210 In the above formulae W0 and W1 is the initial and the final fish mean weights in grams.

211 ***2.4. Head kidney macrophage isolation, respiratory burst and phagocytic activity by head***
212 ***kidney macrophages***

213 For the isolation of HKMs, the head kidney was teased through a 100 µm nylon mesh
214 (BD Falcon; BD Biosciences, Franklin Lakes, NJ, USA) into 2.5 ml L-15 containing 40 µl of
215 heparin (10 IU × ml^{-1}). The mesh was rinsed with 2.5 ml of the medium and the cell suspension
216 placed on ice. HKM phagocytic activity and levels of O^{-2} production in HKM suspensions by
217 the conversion of nitroblue tetrazolium (NBT; Sigma-Aldrich) to formazan were measured
218 following the method described by Secombes [27], with modifications described by Korkea-
219 Aho et al. [28].

For the measurement of HKM phagocytic activity, duplicate 100-µl cell samples were placed on glass slides and incubated for 1 h at 15°C to allow macrophages to attach. After this time, non-adherent cells were removed by washing the slides three times with L-15 medium. Baker's yeast resuspended in L-15 medium at 5 mg ml^{-1} (100 µl) was added to one of the samples on the microscope slide. An equal volume of L-15 medium was added to the other sample on the same slide as a negative control. Samples were incubated for 1 h at 15°C to allow phagocytosis to proceed. The slides were then washed three times with L-15 medium before 100-µl volumes of 100% methanol were added for 5 min. Slides were washed three times with 70% methanol and stained with rapid Romanowsky stain (Raymond A Lamb, Eastbourne, UK). The slides were viewed at × 1000 magnification, and 100 macrophages were counted per sample. The phagocytic activity was determined as the percentage of macrophages performing phagocytosis (% phagocytosis) and as the number of yeast cells engulfed by each macrophage (phagocytic index).

For the estimation of HKM respiratory burst activity, one hundred µl of macrophage suspension was added to the 96-well plate (Iwaki, Tokyo, Japan), incubating at 15°C for 2 h to allow cell attachment. The supernatant was removed and wells washed three times with L-15. After washing, 100 µl of L-15 containing 1 mg × ml^{-1} NBT was added to three replicate wells, and this together with phorbol myristic acetate (1 µl × ml^{-1} PMA) was added to another three replicate wells, while 100 µl of lysis buffer (citric acid, 0.1 mol× l^{-1}; Tween 20, 1.0 % (v⁄v); crystal violet, 0.05 % (w⁄v); Sigma-Aldrich) was added to two additional replicate wells. The plate was incubated for 60 min at 15°C, the medium removed and cells fixed with 100 % (v⁄v) methanol for 2–3 min before washing three times with 70 % (v/v) methanol. The plates were air-dried before adding 120 µl of 2 M potassium hydroxide (Sigma-Aldrich) and 140 µl of 2 M dimethyl sulfoxide (Sigma-Aldrich) to each well to dissolve the resulting formazan. The absorbance was determined at 610 nm using an automated multi-mode microplate reader

(Synergy HT; BioTek Instruments, Winooski, VT, USA). The number of macrophages attached to the plate was determined by counting the average number of nuclei released by the addition of lysis buffer for two replicate wells. The number of released macrophage nuclei was achieved using a Neubauer chamber, by counting the number of nuclei in the 4 sets of the 16 corner squares from one grid. The total number of nuclei within the 4 sets of squares was then divided by 4 and then multiplied by the dilution factor giving the number of nuclei $\times 10^4 \times ml^{-1}$. The level of respiratory burst was expressed as an absorbance at 610 nm for 10^5 cells $\times$ $sample^{-1}$.

2.5. Determination of serum glucose, protein and lysozyme activity

Serum glucose was determined using a CONTOUR blood glucose monitoring system (Bayer HealthCare LLC) according to manufacturer's instructions. Briefly a CONTOUR strip was inserted accordingly into the Contour blood glucose monitor and then 5µl of serum were pipetted onto the blood receiving end of the CONTOUR strip and held for 5 sec until the test result was displayed on the screen of the monitor. Protein content of serum was determined using a Pierce BCA (bicinchoninic acid) protein determination kit (Thermo Scientific, IL, USA) using bovine serum albumin (BSA) as a standard. Serum lysozyme activity was estimated according to the protocol described by Korkea-Aho et al. [28], based on the lysis of lysozyme sensitive *Micrococcus lysodeikticus*.

2.6. Determination of serum total IgM

The level of IgM in sera of experimental fish was determined using an indirect enzyme linked immunosorbent assay (ELISA) described by Metochis et al. [29].

2.7. Determination of specific antibody against Aeromonas salmonicida

An ELISA was used to measure the specific antibody response of Atlantic salmon to the *A. salmonicida* vaccine using the method outlined by Metochis et al [22]. Briefly, 96-well

Immulon™ 4HBX plates (ThermoScientific, Maine, USA) were coated with 50 µl of 0.05 % w/v poly-L-lysine (Sigma-Aldrich) in 0.05 M sodium carbonate/bicarbonate buffer, pH 9.6 and incubated for 60 min at 21°C. Plates were then washed twice with a LSWB. *A.salmonicida* (Hooke) in 0.1M PBS was added to the wells at 100 µl × well^{-1} and plates incubated overnight at 4°C. Fifty microliters per well of 0.05 % v/v glutaraldehyde in PBS was added to the bacteria and the plate incubated at 21°C for 30 min before washing three times with LSWB. Non-specific binding sites were blocked by incubating plates with 3% w/v skimmed milk powder in water at 21°C for 120 min. After washing the plates three times with LSWB, 100 µl of serially diluted fish serum diluted in 1 % casein (from 1: 50, 1: 200 and 1: 1000) was transferred to the ELISA plate, which was then incubated overnight at 4°C. Both positive (serum pools from challenged salmon survivors which have been vaccinated prior to challenge) and negative controls (serum blanks/ pooled serum from naïve salmon) were also added to each plate. Plates were washed five times with HSWB with a 5 min soak on the last wash. Anti-rainbow trout/Atlantic salmon IgMmonoclonal antibody (F11-monoclonal anti trout/salmon IgM - Aquatic Diagnostics Ltd, Stirling, Scotland) was then added and plates were incubated at 21°C for 60 min. The steps followed until the development of the plates were the same with those described above. The percentage of specific antibody production was estimated by the comparison of positive (pooled serum from vaccinated and challenged salmon, 100 % antibody production) and negative controls (pooled naïve salmon serum, 0% antibody production) and was expressed as percentage of specific antibody production.

2.8. Measurement of alternative, classical and overall complement activity

Salmon antiserum against sheep RBC was produced by immunising fish i.p. with 10^9 sheep red blood cells (SRBC) in PBS (0.15 M phosphate-buffered saline, pH 7.2). Four weeks after priming a booster injection (10^9) was given, and two weeks later, fish were bled. Control fish were injected with PBS. Endogenous complement activity of anti-SRBC salmon serum was

294 inactivated by heating at 50°C for 30 min and the anti-SRBC serum was diluted with 0.1%
295 Gelatin-Complement Fixation Buffer (G-CFB) (1 tablet of Oxoid complement fixation tablets
296 in 100 ml of warm water and 0.1 g of gelatin from Sigma-Aldrich) with 20mM EDTA. Diluted
297 anti-SRBCs were then stored at – 20°C. Sheep blood (Oxoid) was stored at 4°C in Alsever's
298 solution (1: 1) for 1 week before use. The SRBCs were used to determine lysis by the alternative
299 complement pathway (ACP), while SRBCs, sensitized (60 minutes, 37°C) with pooled and
300 diluted (1: 400) salmon anti-SRBC serum, was used for determination of total (TC) and
301 classical complement pathway (CCP) activity. Buffer for the AC was 0.01 M EGTA-Mg-G-
302 CFB and for determination of the total and classical haemolytic activity G-CFB. Tests were
303 done in round-bottomed 96-well microtiter plates (Sterilin).Briefly complement activity
304 determination was based on methods described by Yano et al. [30-31] with modifications.
305 Briefly serum was diluted four times in double serial dilutions accordingly (starting from 1:4
306 for the estimation of AC activity and 1: 16 for the estimation of TC and CC activity) and 25 µl
307 of each dilution was added to each well of a non-absorbent U-well micro-plate (Sterilin) in
308 duplicate. Ten microliters of 0.5 % SRBC suspension was added to each serum dilution.
309 Controls on each plate comprised 0.1 % anhydrous Na_2CO_3 (v/v) (100 % lysis) replacing
310 serum. G-CFB replacing serum (0 % lysis) and serum blanks (duplicate wells of serum
311 dilutions with G-CFB replacing SRBC suspension). The plates destined for the estimation of
312 TC and CCP activity also included, a CC control sensitization of SRBC with non-immune
313 pooled carp serum and a standard complement sample (serum pool) for correction of plate
314 differences were included. Microtitre plates were incubated at 22°C for 90 min with constant
315 shaking and the reaction terminated by the addition of 140 µl G-CFB with 20mM EDTA,
316 followed by centrifugation at 1500 × g to spin down the remaining SRBCs. After centrifugation
317 100 µl of the supernatant from each well was transferred to a new flat-bottomed 96-well non-
318 absorbent micro-titre plate (Sterilin). The absorbance of the wells was read at 450 nm using a

319 micro-plate reader (Synergy HT; BioTek Instruments, Winooski, VT, USA) and the percentage
320 lysis of SRBCs calculated. The absorbance values of samples were corrected by subtracting
321 the absorbance of the sample blank control (0% haemolysis).

322 ***2.9. Diet composition analysis***

323 Dietary crude fat was determined following acid hydrolysis using a Soxtec System 1047
324 hydrolysing unit (Tecator Application note 92/87) followed by exhaustive Soxhlet extraction
325 using petroleum ether (40–60°C boiling point) on a Soxtec System HT6 (Tecator application
326 note 67/83)as described by Bell et al. [32]. Dry weight and ash contents of diets were
327 determined after oven-drying the samples to constant weight (at 100 ºC) and by ashing dried
328 samples in an oven at 550°C [33]. Dietary energy content was determined through bomb
329 calorimetry [33]. For the determination of phytic acid and phytic acid bound P content in the
330 diets a Megazyme Phytate/Total Phosphorus Assay kit (Megazyme, Ireland) was used. Dietary
331 energy content was determined through bomb calorimetry [33].

332 Dietary carbohydrate was determined following a modified Dubois phenol sulphuric
333 method. Dietary fibre was determined after subjecting defatted dietary samples (3 washes with
334 petroleum ether) within pre-weighed organic capsules, to acid (with 1.25% sulphuric acid
335 solution)and alkaline hydrolysis (with 1.25% sodium hydroxide solution) for 35 min each,
336 using a Fibertec system 1020 hot Extractor. Following one last defatting step (3 washes with
337 petroleum ether), the samples were ashed at 600°C in a muffle furnace (Gallenkamp Muffle
338 Furnace) for 4 hours, cooled in a desiccator and reweighed (W2). Extracted fibre was
339 expressed as percentage of the original undefatted sample and calculated according to the
340 formula:

341 $$Fibre(\%) = \frac{(W1 \times 1.0011) - Capsule\ weight - (W2 - 0.0025)}{Sample\ weight\ (g)} \times 100$$

342 Where W1 is the initial weight of the unprocessed dietary sample and W2 is the weight after
343 processing.

344 Dietary minerals and phosphorus were determined using inductively coupled plasma
345 mass spectroscopy (ICP-MS) with collision cell technology (CCT) (Thermo X Series 2).

346 ***2.10. Statistics***

347 Growth and immunological data were examined using a one-way analysis of variance
348 (ANOVA), general linear model, and pairwise comparison (Tukey-test) of means. All
349 statistical tests were performed using Minitab statistical software (Version 17©, University of
350 Stirling, 2016). Differences were considered statistically significant at *p values < 0.05*.
351 Statistical differences were examined between naïve dietary groups prior to vaccination, the
352 vaccinated dietary groups at 2 and 62 dpv and the PBS-injected dietary salmon at 2 and 62
353 dpPBSinj. Serum pools were used as statistical units (two serum and/or HKM pools from 4
354 fish per tank thus two pools per tank resulting in eight pools per treatment). Data are presented
355 as means ± SEM. The analysis of the survival data from the disease challenge trial was
356 performed with Cox regression (Cox proportional hazard modelling) in SPSS statistical
357 software (IBM SPSS Statistics 21, University of Stirling, 2016) using time-to-event (e.g. the
358 time at which a subject in the 30-day challenge period survived – data for each fish were
359 recorded) and status for each fish (dead =1 or alive =0) variables (individual fish per treatment
360 and time-to-event, were used as statistical units – from the former parameters the coefficients
361 *Exp (B)* e.g. hazard ratios, per treatment group were estimated) and the level of protein from
362 SPC in the dietary treatments as the covariate. Differences were considered statistically
363 significant at *p values* < 0.05 while the coefficient *Exp (B)* was also recorded for the assessment
364 of potential positive or negative effects of increased dietary SPC levels in the resistance of
365 salmon to furunculosis (*Exp (B)* or Hazard Ratio is the factor by which the hazard **-the presence**
366 **of mortalities upon infection-** for a dietary group is increased or decreased; *Exp (B)* values =

1 reveal no changes in salmon resistance and/or hazard ratio -*HR*-, values > 1 reveal an increment in *HR* and deterioration of its disease resistance and values < 1 reveal a decrease in *HR* and an improvement of salmon resistance to furunculosis)

3. Results

3.1. Growth performance

Salmon performance data are given in Table 2. Overall, negligible mortalities (< 0.1 %) were observed in the three dietary groups prior (Day 92) and post vaccination/PBS-injection (Day 154) (Table 2). SPC80 salmon exhibited reductions in feed intake and FCR at Day 92. Significant decreases for all growth performance indices (mean weights, WG, SGR, TGC) were observed for both SPC58 and SPC80 salmon in comparison to SPC35 fish prior to vaccination/PBS injection.

At Day 154, reductions in FCR persisted in SPC80 salmon compared to the SPC35 group. In addition decreased mean weights, WG, TGC and SGR were demonstrated for the SPC80 group in comparison to SPC35 salmon at both vaccination states on Day 154, while no differences were detected between SPC58 and SPC35 salmon for any of the performance parameters.

3.2. Immune Responses

The data of the estimated immunological responses are presented in Table 3. Serum proteins were only affected by time or state and not by dietary levels of SPC. A sharp decrease in serum protein levels was exhibited in vaccinated salmon at 2 dpv while at 62 dpv serum protein increased compared to the levels at 2 dpv. Nevertheless, serum protein concentrations were found to be lower than their initial pre-vaccination serum concentrations. On the contrary, serum protein levels in PBS-injected fish doubled at 62 days post PBS injection (62 dpPBSinj) when compared to naïve salmon.

Serum total IgM concentrations did not exhibit any differences among the dietary salmon groups. While serum total IgM in naïve and PBS-injected salmon were found to be similar, in vaccinated salmon total IgM levels demonstrated a decrease at 2 dpv compared to pre-vaccination values, whereas at 62 dpv total IgM increased 4-fold, compared to naïve salmon serum concentrations. Specific IgM was measured at 62 dpv and no differences were detected among the three dietary groups.

Lysozyme activity was another immune response exhibiting no changes among the dietary salmon groups. Both naïve and PBS-injected salmon at Day 154 exhibited similar lysozyme activities. An increment in average lysozyme activity was obtained at 2dpv compared to the pre vaccination levels, followed by a further increase at 62dpv. Serum glucose concentrations did not show any differences among dietary groups. Moreover, similar glucose concentrations were detected in naïve and PBS-injected salmon serum. However at 2 dpv, glucose levels were found to be higher than in pre-vaccinated fish, while at 62 dpv glucose levels in the vaccinated groups were lower compared to naïve salmon.

At Day 63 prior to vaccination/PBS-injection, SRBC haemolysis due to TC and CC activity demonstrated significant reductions in SPC58 compared to SPC35 (both *p* values for CC and TC were equal to 0.02) salmon while no differences were detected between the SPC80 and SPC35 groups. Alternative complement activity on the other hand did not show significant differences among the treatment groups. No differences in the complement activities were observed at 62 days post PBS-injection among the dietary treatments. However, a decreasing trend in AC activity was observed in fish receiving increasing dietary SPC levels (*p* value = 0.06). On the contrary, significantly lower AC activity was observed for SPC80 salmon at both timepoints post vaccination compared to SPC35 (*p values*: 0.02 at 2 dpv and 0.04 at 62 dpv) salmon while SPC58 salmon exhibited lower activity only at 2 dpv (*p value* = 0.02). No

differences in total complement (TC) activity were exhibited among the dietary groups of vaccinated and PBS-injected salmon.

No differences in PMA-stimulated and non-stimulated HKM respiratory burst and phagocytic activity among the dietary groups of salmon prior to vaccination/PBS-injection, were detected. For vaccinated salmon, the estimated values for all the HKM responses among the dietary groups presented no differences, with respiratory burst activity, phagocytic index levels and phagocytosis % increasing sharply at 2 dpv compared to the values recorded prior vaccination and decreasing at 62 dpv below the latter values. For PBS-injected salmon, respiratory burst was similar to the ones obtained for vaccinated salmon at 2 and 62 dpv exhibiting an almost equal increase at 2 dpPBSinj, followed by a subsequent reduction, at levels lower than the ones determined for naïve salmon except for PMA-stimulated HKM, which presented higher activity at 62 days post injection compared to naïve fish. Significantly lower phagocytic activity (phagocytosis % and phagocytic index) were detected between SPC58 and SPC80 salmon compared to the SPC35 group at 62dpPBSinj (*p value* = 0.03).

3.3. Disease resistance trial

No mortalities were observed for any of the dietary groups vaccinated against *A. salmonicida* after challenging them with the bacterium. The raw data of PBS-injected salmon cumulative survival during the experimental infection are presented in Fig. 1. Mortalities were obtained from all dietary groups in PBS-injected and subsequently challenged Atlantic salmon parr. Mortalities from these fish first started to occur at 6 days post challenge, but only for the SPC35 salmon group. On Day 7 post-challenge the first mortalities from SPC58 salmon were observed, while on Day 8, mortalities from the SPC80 group of salmon also started to occur. Mortalities ceased on Day 19 post-challenge. At this time, the mortality rate for the SPC35 group from all tanks corresponded to 44%. A death rate of 34% was observed for the SPC58 while a 29% mortality rate was recorded for SPC80 salmon. Statistical analysis revealed no

significant differences among the dietary salmon groups (*p value* = 0.07) and a slight reduction in the hazard ratio (*Exp (B)* = 0.76 – non-significant improvement of salmon resistance to furunculosis) of salmon fed on the diets the highest SPC levels (SPC80).

4. Discussion

In the present trial, significant reductions for most performance indices (FCR, average weight, weight gain, SGR and TGC) were observed for both vaccinated and PBS-injected SPC80 compared to their SPC35 counterparts while no differences between the latter dietary group and SPC58 salmon at both states were obtained at Day 154 (62 dpv/dpPBSinj). Considering the initial reductions in SPC58 salmon growth in comparison to the SPC35 group (9-30g fish), it is suggested that larger size salmon parr of ~30 g with a more developed digestive system can make a more efficient use of the SPC58 diet compared to smaller size parr of 9g or that salmon receiving this diet requires an adaptation period before accepting and start utilizing efficiently the nutrients in this type of feed [17, 34-36]. On the contrary, the levels of SPC in the SPC80 diet were overwhelming for salmon parr which never manage to recover the initial reductions in growth performance when compared to SPC35 salmon. Growth reductions in fish fed diets with very high levels of SPC are generally attributed to the increased amounts of phytic acid (Table 1), decreasing the digestibility and availability of dietary nutrients [37] and the lower concentrations of several key nutritional components found abundantly in FM and scarcely or absent in plant proteins (including macro and trace minerals, sterols and non-nitrogen compounds), compromising feed acceptability, FCR and growth in fish [13,14, 38-41]. Compromised feed acceptability was apparent during the first period of the study for SPC80 compared to SPC35 salmon, while numerical decreases persisted during the full course of the study. Moreover, decreased FCR was apparent for the SPC80 group at all timepoints.

Herein, the determination of serum protein levels was used to evaluate the general condition of experimental salmon [42], whereas serum glucose was measured for the evaluation

of the general stress status of fish from different dietary groups [43]. Moreover, the assessed immune responses are considered salient components of salmon defence mechanisms against disease. Lysozyme and macrophage phagocytosis followed by oxygen radical production, are important antimicrobial agents [27-28], while complement and total and specific IgM are linked with the neutralisation and opsonisation of various pathogens [44-45]. Thompson et al. [46] suggested that measuring defence mechanisms prior to challenge only represents resting levels. However, measuring immune function after immune stimulation (either through challenge or vaccination) may highlight dietary modifications that are not previously evident. This is further highlighted in mammalian research, where studies have shown that nutrients are preferentially directed towards the immune system, rather than growth, during times of infection [47]. This includes the distribution of amino acids to the liver for synthesis of acute phase proteins [48] and suggests suboptimal nutrient intake prior to infection may result in a diminished immune response. It therefore seems a logical step to evaluate the immune function of the fish after its dietary protein source has been altered. For this reason, salmon parr receiving the experimental diets in the present trial were vaccinated and then challenged with *A. salmonicida*.

While most of these immunological parameters (serum lysozyme, protein, total IgM, head kidney macrophage phagocytic activity and oxygen radical production) have been previously shown to be modulated by dietary change in various teleosts (summarized by Kiron [44]), no differences compared to the commercial type control SPC35 diet occurred prior or post-vaccination in the present study. Similar to the current trial, Bransden et al. [49] reported no differences in lysozyme activity, plasma concentrations of total IgM, total protein or glucose levels of non-immunized salmon parr fed on diets where dehulled lupin meal replaced 40% of FM. Furthermore, in line with the present study, Jalili et al. [18] demonstrated no differences in serum lysozyme levels of non-immunized rainbow trout (*Oncorhynchus mykiss*) fed on diets

490 fully based on mixed plant proteins. Rumsey et al. [20] reported increased lysozyme activity
491 in rainbow trout fed SBM diets. Contrary to this, Fahrangi & Carter [50] revealed decreased
492 serum protein levels in rainbow trout receiving 30, 40 and 50 % of dehulled lupin meal,
493 compared to trout fed 10% dehulled lupin meal, while no changes were detected in serum
494 glucose levels. Increased lysozyme activity at both points after vaccination for all dietary
495 groups of salmon is indicative of both stress induction and immune stimulation and was not
496 apparent in naïve fish. Vaccination is a stressful process, which could explain the sharp increase
497 in serum glucose at 2 dpv [51-52]. Furthermore, the stress-related reduction in fish appetite due
498 to vaccination could explain the concomitant reduction in serum protein and total IgM levels
499 observed at 2 dpv in vaccinated salmon [53]. The observed initial reduction in serum total IgM
500 could also be related to the formation of antibody-antigen complexes, reducing the number of
501 free circulating natural antibodies at 2 dpv [54-55]. The reduction in serum glucose levels at
502 62 dpv, below those of pre-vaccinated or PBS-injected salmon at 62 days post injection
503 demonstrates the reduced responsiveness to acute stressors (e.g. sampling processes such as
504 netting and exposure to anaesthetic) of salmon subjected to higher stress for a prolonged time
505 period (e.g. vaccinated salmon vs PBS-injected salmon due to immune stimulation) [56-57].
506 However, the 4-fold increase in total IgM for all dietary groups at 62 dpv is an indication of
507 the efficacy of vaccination [53, 58], and the absence of polyetiological stress during the study,
508 which could have compromised this response [52]. The increase of total protein is also
509 indicative of salmon appetite recovery at 62 dpv [53]. In a previous study performed by
510 Metochis et al. [22], higher plasma total IgM and lysozyme activity in SPC50 and SPC65
511 compared to SPC35 salmon prior to vaccination (Day 63 of feeding), 7 dpv (Day 70) and 34
512 dpv (Day 97) were demonstrated. Contrarily lower levels to former two groups were
513 demonstrated for SPC80 salmon, similar to those observed in SPC35 fish. While IgM levels
514 prior to and after vaccination followed similar patterns in the two studies, lysozyme activities

515 presented different post vaccination patterns. The observed differences are discussed further
516 below (paragraph 7 of Section 4).

517 No effects of the SPC inclusion levels on specific antibody production were detected,
518 which is in line with the data from the study by Metochis et al. [22], on increasing dietary SPC
519 levels on larger size Atlantic salmon parr. Kiron et al. [59-60] reported no changes in the
520 specific antibody levels of rainbow trout fed on different dietary protein levels, suggesting that
521 specific antibody production does not seem to depend on dietary protein quantity. This could
522 explain why in the present trial, differences in protein intake attributed to the reduced feed
523 intake or the presence of phytic acid which is known to adversely affect protein digestibility,
524 did not affect the production of specific antibodies. Moreover, many studies on the effects of
525 dietary or injectable immuno-stimulants have found no effect on specific antibody production
526 in fish (summarized by Anderson [61] and Gannam & Schrock [62]), which could be an
527 indication that increasing dietary levels of plant proteins with potential immunostimulatory
528 effects, do not promote specific antibody production and that their activity lies on the
529 stimulation of innate immune components.

530 Complement activity appeared to be the most eminently affected immune response by
531 increased dietary SPC inclusions, during the course of the study, in accordance to previous
532 studies [19, 22, 63]. While no changes in AC activity among the dietary groups were observed,
533 lower CC and TC (the sum of alternative, classical complement activities) activities were
534 detected in salmon fed increased dietary SPC levels at Day 92, prior vaccination. In similar
535 studies, increased or unaffected AC activity were reported in gilthead sea bream (*Sparus*
536 *aurata*) and rainbow trout respectively, in naïve fish fed up to 50% of protein from plant
537 ingredients, while decreased activity was observed in fish fed on diets with over 70% of protein
538 from such feedstuffs [18-19]. Alternative complement activity is considered as a major
539 component of salmon' innate immunological defences against diseases, depending on serum

Mg availability for its activation [64-66]. Classical complement activity, on the other hand, is a component of acquired immunity, enhanced upon specific immune stimulation and increased serum antibody presence, having a requirement for both Mg and Ca for its activation [64-66]. Increased dietary Mg levels in higher SPC inclusion diets could have attributed to the absence of differences in AC activity among the dietary groups despite any proposed reductions in mineral uptake due to reduced feed intake and increased dietary phytic acid concentrations [13-14, 37]. On the contrary, lower dietary Ca in high SPC inclusion diets could have influenced reductions in CC activity [64]. Moreover, potential reductions of protein and/or amino acid intake in high SPC inclusion groups (due to higher dietary phytic acid levels) could have influenced metabolic changes, favouring AC protein production over CC proteins, in order not to compromise salmon innate immune response, the first line of defence against diseases [22, 37, 67-69]. Overall, lower complement activities were detected after vaccination compared to pre-vaccination levels while the highest activities were exhibited in PBS-injected salmon at Day 154 allegedly due to their naivety to immune challenges translating to reduced stress, higher feed intake, higher growth and higher circulating protein levels [51, 70]. In general, lower complement activity after vaccination, could be attributed to the formation of complement complexes with the vaccine, as was the case for serum total antibodies, diminishing the concentration of complement proteins in vaccinated salmon sera [53-55]. Moreover, the reduced levels of alternative complement activity for SPC58 and SPC80 compared to SPC35 salmon could highlight suboptimal nutrient uptake prior to vaccination [46]. Suboptimal protein and or amino acid uptake during the primary (pre-injection) period could have also influenced the trends of decreasing AC activity in naïve fish receiving higher dietary SPC levels at Day 154 [46, 67-68]. At 62 dpv average CC complement activity for all groups was recovered at higher levels than at 2dpv revealing stimulation of the specific immunity, in accordance to previous studies [53, 55, 58]. The observed increments in CC

565 activity at 62 dpv were more pronounced for high dietary SPC groups compared to SPC35
566 salmon. In a previous study, Metochis et al. [22] reported significantly higher plasma
567 haemolytic activity in salmon fed high SPC inclusion diets at 97 days post feeding (and 34
568 dpv), while no differences were observed prior to (Day 63) and at 7 dpv. In the same study,
569 salmon haemolytic activity remained fairly constant prior to and at 7 dpv while showing an
570 increase at 34 dpv, similar to the evolution of the TC haemolytic response patterns presented
571 here.

572 While most of the immune related responses measured for HKMs did not show
573 differences among the dietary groups prior to and post vaccination, higher HKM phagocytosis
574 percentages were detected in SPC58 salmon injected with PBS at 62 dpPBSinj compared to
575 their SPC35 counterparts. This shows that inclusion of 58% of amino acid and phosphate
576 supplemented dietary protein from SPC, could have a stimulatory effect on HKM motility
577 which is reduced at higher dietary SPC inclusion levels. Previously, Metochis et al. [22]
578 reported no differences in the respiratory burst activity of Atlantic salmon fed diets with
579 increasing dietary SPC inclusions. However, the HKM respiratory response patterns observed
580 in the former study were different from the ones recorded here and they are discussed below in
581 the next paragraph. Rumsey et al. [20] demonstrated both increased phagocytosis and
582 respiratory burst activity by circulating leucocytes in rainbow trout fed on SBM. However,
583 those findings were attributed to inflammatory and hypersensitivity processes, since SBM
584 ANFs are linked with intestinal inflammation [16]. Previously, Burrells et al. [21] reported that
585 HKM respiratory burst in rainbow trout fed on diets containing 10-50 % of SBM remained
586 unaffected, whereas inclusion levels of up to 80% caused a reduction in HKM responses. Sitja-
587 Bobadilla et al. [19], contrary to the present findings, reported higher respiratory burst activity
588 by HK leucocytes in juvenile sea bream fed on nutritionally balanced diets in which 75% of

589 FM was substituted with a mixture of different plant protein sources and supplemental amino
590 acids.

591 Discrepancies in the way salmon parr immune responses were affected prior to and post
592 vaccination, their magnitude and differences in the responses of salmon fed increasing dietary
593 SPC levels were not in accordance with the previous study by Metochis et al. [22]. Despite, the
594 high relevance in the experimental design between the two studies, differences in the observed
595 immune responses could be attributed to a number of experimental disparities such as: (A) The
596 use of different salmon breeds (AquaGen salmon selected for improved growth and resistance
597 to IPNV vs Salmo Breed salmon selected for higher growth performance previously) [51]; (B)
598 The use of different commercial adjuvanted vaccines (i.e. liquid paraffin vaccine against
599 furunculosis and IPNV vs montanide vaccine against furunculosis previously) [61]; (C) The
600 concentration and source of dietary P (increasing monocalcium phosphate upon increasing
601 dietary SPC vs diets supplemented with constant amounts of dicalcium phosphate previously)
602 [45 and 71]; (D) The timing of sampling (2 and 62 dpv vs 7 and 34 dpv); (E) The photoperiod
603 applied (12 h dark: 12 h light vs constant light previously) [72-73]; (F) The feeding regime
604 applied (non-intensive: fish fed to satiation twice daily vs intensive: fish fed continuously every
605 435 seconds all throughout the day previously) [74-75]; and (G) The developmental stage of
606 salmon parr used (small size vs large size salmon parr previously) (reviewed by [76]).
607 However, despite the observed differences in the patterns and magnitude of the assessed
608 immune responses in the two studies, both of them have proven that a range of innate and
609 specific immune responses in Atlantic salmon parr fed diets with up to 80% of amino acid
610 supplemented protein from SPC were not compromised compared to fish fed a commercial
611 type control feed with 35% of protein from SPC and constant [22] or increasing P
612 supplementation (present study). Another important finding of the present study is that
613 vaccination can highlight differences in immune responses attributed to dietary changes and

614 which might be masked without stimulation of salmon immunity. The latter finding was also
615 apparent from the results of the previous study by Metochis et al. [22].

616 Salmon were challenged against *A. salmonicida*, on Day 154 of the feeding trial and at
617 64 dpv, in order to prove that the observed data on salmon immune responses pointing at a non-
618 compromised health status were actually meaningful. Challenge with *A.salmonicida* resulted
619 in lower mortality levels (44 %), than expected (~70%) according to the pre-challenge.
620 Reduced virulence for this strain of *A.salmonicida* has been previously observed after long-
621 term storage (6-7 months) of these bacteria in beads at -70ºC or in glycerol at -20ºC (Herath,T.,
622 personal communication and Chalmers, L., personal communication). Another factor that
623 could have influenced the lower number of mortalities in the groups during the challenge period
624 compared to the pre-challenge tests was the fact that potential bacterial dose differences
625 (producing 70% mortalities) due to the larger size of the fish at the challenge timepoint, were
626 not taken into account (reviewed by Tatner [76]). The reason was that very low bacterial doses
627 were required to produce 70% mortalities (LD_{70}) in 32g salmon parr increasing the probability
628 of misestimating the LD_{70} for larger size fish. The absence of mortalities in the vaccinated
629 groups were indicative of the promotion of specific immunity by all treatments and the lack of
630 differences in specific immunity among the dietary salmon groups. The data also suggest that
631 increasing dietary SPC levels were not detrimental for the resistance of naïve salmon to
632 furunculosis, confirming the lack of differences in the assessed immune responses. Similarly
633 to the present findings, Bransden et al. [49] reported no differences in the resistance of salmon
634 parr fed diets with 0 or 40% of FM substitution with dehulled lupin meal when experimentally
635 infected with *Vibrio anguillarum*, whereas Jalili et al. [18] found no differences in the mortality
636 of rainbow trout fed diets with 0, 40, 70 and 100% replacement of FM with plant proteins when
637 challenged against *Yersinia ruckerii*. Krogdahl et al. [16] reported increased survival in salmon
638 fed a diet with 30% of dietary protein from SPC compared to fully FM-fed fish challenged

639 through cohabitation with *A.salmonicida*. In the previous study, the proposed reason for the
640 increased survival was the observed increase in IgM levels in the intestinal mucosa of SPC-fed
641 compared to FM-fed salmon. Herein, it was shown diets with even higher dietary SPC levels
642 compared to the ones reported by Krogdahl et al. [16] (control diet in the present study was
643 close to the SPC diet used in the latter study) do not seem to affect the immunity or resistance
644 of salmon intraperitoneally infected with furunculosis.

645 **5. Conclusions**

646 Atlantic salmon parr presented a slow but steady adaptation to the diet with 58% of protein
647 from SPC, presenting similar growth performance to the commercial type control diet at 154
648 days post feeding but that was not the case with the diet with 80% of protein from SPC. On the
649 contrary, replacement of high quality FM protein with high levels of amino acid and phosphate
650 supplemented SPC (80% of dietary protein) did not produce any reductions of the immune
651 responses or the disease resistance of naïve and/or vaccinated salmon against *A. salmonicida,*
652 suggesting no negative effects on immunity. Diets with 58% of dietary protein from SPC
653 produced similar growth and immune responses to the commercial type control feed with 35%
654 of protein from SPC and could be used for the on-growing of salmon parr. However, further
655 challenge experiments against other bacterial, viral and parasitic diseases are required to
656 properly assess the effects of high SPC inclusion diets on the disease resistance of juvenile
657 Atlantic salmon.

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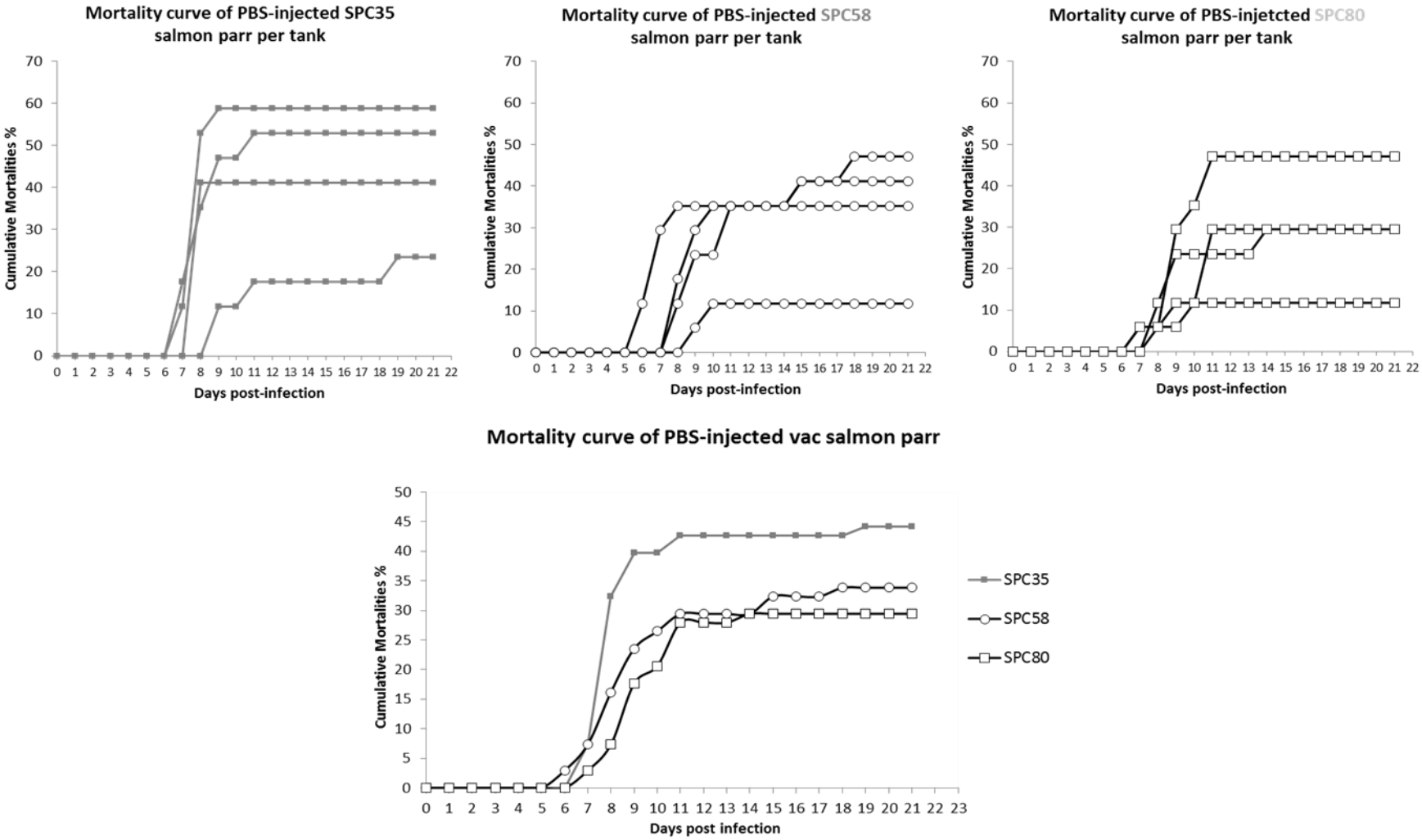
Figure Caption

Figure 1. Observed mortality curves in the quadruplicate tanks of the experimental dietary groups of intra-peritoneally challenged against *A. salmonicida* Atlantic salmon parr (PBS-injected) and cumulative mortality curves for the three dietary groups (PBS-injected).

859

860

Figure 1.



861

862 **Table 1.** Formulation and chemical composition of the experimental diets.

Feed composition (× kg^{-1})	SPC35	SPC58	SPC80	SPC35	SPC58	SPC80
		2mm			3mm	
Fishmeal [a] (g)	449.2	269.8	114.6	449.2	269.8	114.6
SPC [b] (g)	288.3	453.8	598.6	288.3	453.8	598.6
Tapioca [c] (g)	110.0	100.0	90.0	110.0	100.0	90.0
MonoCalcium phosphate [d] (g)	20.0	30.0	40.0	20.0	30.0	40.0
Vitamin and mineral premixes[e] (g)	4.0	4.0	4.0	4.0	4.0	4.0
Vitamin C 35% [e] (g)	1.0	1.0	1.0	1.0	1.0	1.0
Methionine [f] (g)	2.1	4.0	5.5	2.1	4.0	5.5
Lysine 78% [f] (g)	1.8	2.6	3.3	1.8	2.6	3.3
L-Threonine [f] (g)	0.6	0.8	1.0	0.6	0.8	1.0
Nobacithin Powder [g] (g)	10.0	10.0	10.0	10.0	10.0	10.0
Fish Oil [h] (g)	113.0	124.0	132.0	113.0	124.0	132.0
Chemical composition (× kg^{-1})						
Dry matter (g)	959.7	944.1	923.0	923.2	936.1	922.5
In dry matter basis						
Energy (KJ)	222.4	218.9	216.3	223.8	218.8	217.1
Crude protein (g)	531.2	515.2	494.1	532.4	511.8	489.8
Crude fat (g)	182.4	170.6	162.0	177.7	165.4	162.1
Crude prt: Crude fat ratio	29.1	30.2	30.5	29.9	30.9	30.3
Ash (g)	95.5	92.5	89.0	96.1	91.0	87.6
Carbohydrate (g)	289.7	307.9	332.4	287.8	308.0	318.8
Crude fibre (g)	0.1	0.2	0.3	0.1	0.2	0.3
Phytic acid (g)	11.5	14.7	15.4	10.6	13.7	14.2
Phytic acid-bound P (g)	3.0	3.8	3.8	2.7	3.6	3.5
P (g)	16.0	16.1	15.0	15.7	16.1	15.4
Ca (g)	15.1	14.0	11.5	14.8	13.6	11.5
Ca: P ratio	0.9	0.9	0.8	0.9	0.8	0.7
Zn (mg)	295.3	295.3	266.5	280.6	285.9	267.6
Mg (g)	2.3	2.4	2.4	2.1	2.3	2.4
Mn (mg)	84.0	90.0	84.4	82.4	87.8	85.5

863 Abbreviations: SPC 35 - diet with 35% of dietary protein from soy protein concentrate (SPC);
864 SPC 58 - diet with 58% of dietary protein from SPC; SPC 80 - diet with 80% of dietary protein
865 from SPC.
866 * The concentrations of phytic acid and phytic acid-bound P were estimated using a Megazyme
867 Phytate/Total Phosphorus Assay kit (Megazyme, Ireland) following the protocols provided by
868 the company and were then corrected according to the total dietary P values estimated via
869 ICP/MS.
870 [a] Fishmeal (Egersund Sildoljefabrikk, Norway) with an apparent protein digestibility
871 coefficient (ADC protein) of 90.2 %
872 [b] SPC (*62 % crude protein) (Imcopa, Paraná, Brazil) with an apparent protein digestibility
873 coefficient (ADC protein) of 90.8 % (Anti-trypsins <3.0 mg × g^{-1}, Fibre <5.0 mg × g^{-1}, Lectins
874 < 0.1 μg × g^{-1}, Saponins = 0%, Glycinin < 3.0 μg × g^{-1}, β-conglycinin < 1.0 μg × g^{-1})
875 (compositional analyses performed by an authorised external laboratory hired by Imcopa)

[c] Tapioca (Hoff Norske Potetindustrier, Gjøvik, Norway)
[d] Monocalcium Phosphate (Normin AS, Hønefoss, Norway)
[e] Vitamin premix and Mineral premix (EWOS AS, Bergen, Norway)
[f] Amino acids (Evonik Degussa International AG, Hanau, Germany)
[g] Nobacithin: De-oiled lecithin powder (Noba Vital Lipids, Netherlands);
[h] Fish Oil (Egersund Sildoljefabrikk, Norway).

Table 2. Performance data of juvenile Atlantic salmon dietary groups

Growth and survival	SPC35	SPC58	SPC80
Average initial weight (g) (Day 0)	***9.3±0.06***	***9.3±0.16***	***9.3±0.12***
Average intermediate weight (g) (Day 92)	34.2±0.65[a]	32.7±0.56[b]	30.5±0.62[c]
Average Weight (Vac. Fish) (Day 92)	***34.8±4.45***	***32.6±0.94***	***30.2±0.95***
Average final weight (g) (Day 154) Vacc. fish	55.5±6.18[a]	52.6±2.16[ab]	47.4±1.12[b]
Average weight (PBS-inj. fish) (Day 92)	***35.2±1.29***	***33.6±1.02***	***30.6±1.56***
Average final weight (g) (Day 154) PBSinj. fish	59.9±3.10[a]	57.3±1.04[a]	49.5±2.94[b]
Weight gain ($g \times fish^{-1} \times day^{-1}$) (Days 0-92)	0.27±0.01[a]	0.25±0.01[a]	0.23±0.01[b]
Weight gain ($g \times fish^{-1} \times day^{-1}$) (Days 92-154) Vacc. fish	0.33±0.04[a]	0.32±0.02[ab]	0.28±0.01[b]
Weight gain ($g \times fish^{-1} \times day^{-1}$) (Days 92-154) PBSinj. fish	0.40±0.03[a]	0.38±0.02[a]	0.30±0.02[b]
SGR (Days 0-92)	1.50±0.03[a]	1.45±0.03[b]	1.38±0.02[c]
SGR (Days 92-154) Vacc. fish	0.88±0.07	0.90±0.04	0.85±0.02
SGR (Days 92-154) PBSinj. fish	1.00±0.03[a]	1.01±0.06[a]	0.91±0.02[b]
TGC (Days 0-92)	1.03±0.02[a]	0.98±0.02[b]	0.92±0.01[c]
TGC (Days 92-154) Vacc. fish	0.74±0.06	0.74±0.04	0.68±0.01
TGC (Days 92-154) PBSinj. fish	0.86±0.04[a]	0.85±0.05[a]	0.73±0.03[b]
Feed Intake (0-92)	0.22±0.01[a]	0.22±0.01[a]	0.21±0.00[b]
Feed Intake (Days 92-154) Vacc. fish	0.29±0.02	0.28±0.01	0.27±0.02
Feed Intake (Days 92-154) PBSinj. fish	0.38±0.02	0.38±0.01	0.38±0.01
FCR (0-92)	0.82±0.04[a]	0.85±0.03[ab]	0.89±0.01[b]
FCR (Days 92-154) Vacc. fish	0.87±0.06[a]	0.86±0.04[ab]	0.96±0.06[b]
FCR (Days 92-154) PBSinj. fish	0.96±0.03[a]	1.00±0.06[a]	1.24±0.09[b]
Mortalities (%)	0.00±0.00	0.00±0.00	0.01±0.01
Mortalities (%) (Days 92-154) Vacc. fish	0.01±0.01	0.01±0.01	0.01±0.01
Mortalities (%) (Days 92-154) PBSinj. fish	0.00±0.00	0.00±0.00	0.01±0.01

Abbreviations: SPC 35 - diet with 35% of dietary protein from soy protein concentrate (SPC); SPC 58 - diet with 58% of dietary protein from SPC; SPC 80 - diet with 80% of dietary protein from SPC.

Data for growth performance represent means ± SEM for 4 replicate tanks.

Significant differences among dietary groups at each timepoint are given with different superscript letters within each row.

*$Weight\ gain\ (WG)\ (g \times fish^{-1} \times day^{-1})$ = (Final Biomass-Initial Biomass)/ (N × t)

$SGR = [(\ln W1 - \ln W0)/t] \times 100$

$TGC = [(\sqrt[3]{W1} - \sqrt[3]{W0})/(t \times T)] \times 1000$

$Feed\ Intake\ (FI)\ (g \times fish^{-1} \times day^{-1}) = (W_{fg} - W_{fc})/(N \times t)$

$FCR = (W_{fg} - W_{fc})/(W1 - W0)$

where: t = Number of days

T = Average water temperature in ° C

N = Number of fish

$W1$ = Average final weight (g)

$W0$ = Average initial weight (g)

W_{fg} = Weight of feed given (g)

W_{fc} = Cumulative weight of feed collected at the end of each feeding (g)

931 **Table 3.** Immune responses of the dietary groups of salmon parr at different timepoints/states

Pre vaccination	**SPC35**	**SPC58**	**SPC80**
Lysozyme act. (units × min^{-1}× ml^{-1} of serum)	701.9±48.7	656.3±60.0	653.1±48.8
Alternative complement act. (units H_{50} × ml^{-1} of serum)	90.1±14.5	78.9±11.0	99.4±17.9
Classical complement act. (units H_{50} × ml^{-1} of serum)	436.9±46.3[a]	260.9±19.3[b]	314.0±50.0[ab]
Total complement act. (units H_{50} × ml^{-1} of serum)	527.0±49.8[a]	339.7±18.2[b]	413.4±53.5[ab]
HKMs resp. burst (NBT) (OD for 10^5 nuclei)	0.5±0.1	0.7±0.1	0.6±0.1
Stimulated HKMs resp. burst (OD for 10^5 nuclei)	0.6±0.4	0.8±0.1	0.7±±0.2
Phagocytic Index	1.8±0.0	1.9±0.3	1.4±0.2
Phagocytosis (% of HKMs performing phagocytosis)	49.1±4.2	52.9±9.9	46.3±4.6
Total serum protein (mg × ml^{-1} of serum)	33.1±2.9	30.0±3.2	30.9±2.2
Serum glucose (mmol × ml^{-1} of serum)	6.8±0.3	6.5±0.2	6.3±0.3
Total serum IgM (mg × ml^{-1} of serum)	2.8±1.6	1.7±0.5	1.2±0.3
2 days post vaccination			
Lysozyme act. (units × min^{-1}× ml^{-1} of serum)	728.1±30.9	775.6±56.9	735.0±16.7
Alternative complement act. (units H_{50} × ml^{-1} of serum)	95.7±8.1[a]	52.3±15.4[b]	52.0±8.6[b]
Classical complement act. (units H_{50} × ml^{-1} of serum)	240.2±52.7	260.7±59.6	328.1±74.9
Total complement act. (units H_{50} × ml^{-1} of serum)	335.9±52.9	313.0±63.1	380.2±74.1
HKMs resp. burst (NBT) (OD for 10^5 nuclei)	0.7±0.1	0.8±0.1	0.8±0.1
Stimulated HKMs resp. burst (OD for 10^5 nuclei)	1.1±0.1	1.1±0.1	1.3±0.2
Phagocytic Index	5.9±0.3	6.5±0.4	7.0±0.9
Phagocytosis (% of HKMs performing phagocytosis)	76.8±3.5	75.4±2.6	79.4±3.1
Total serum protein (mg × ml^{-1} of serum)	24.4±5.8	26.6±3.5	21.3±2.2
Serum glucose (mmol × ml^{-1} of serum)	9.1±0.8	11.1±1.7	9.7±0.7
Total serum IgM (mg × ml^{-1} of serum)	0.6±0.3	1.2±0.4	0.7±0.3
2 days post PBS-injection			
Lysozyme act. (units × min^{-1}× ml^{-1} of serum)	_	_	_
Alternative complement act. (units H_{50} × ml^{-1} of serum)	_	_	_
Classical complement act. (units H_{50} × ml^{-1} of serum)	_	_	_
Total complement act. (units H_{50} × ml^{-1} of serum)	_	_	_
HKMs resp. burst (NBT) (OD for 10^5 nuclei)	0.6±0.1	0.8±0.1	0.8±0.1
Stimulated HKMs resp. burst (OD for 10^5 nuclei)	1.0±0.2	1.1±0.1	1.0±0.2
Phagocytic Index	4.8±0.4	4.5±0.5	5.3±0.3
Phagocytosis (% of HKMs performing phagocytosis)	68.3±1.6	70.8±1.9	72.3±2.1
Total serum protein (mg × ml^{-1} of serum)	_	_	_
Serum glucose (mmol × ml^{-1} of serum)	_	_	_
Total serum IgM (mg × ml^{-1} of serum)	_	_	_
62 days post vaccination			
Lysozyme act. (units × min^{-1}× ml^{-1} of serum)	867.5±30.2	810.0±113.8	811.9±77.5
Alternative complement act. (units H_{50} × ml^{-1} of serum)	82.3±8.5[a]	69.3±11.5[ab]	47.4±17.5[b]
Classical complement act. (units H_{50} × ml^{-1} of serum)	303.9±35.1	334.2±77.8	513.8±107.8
Total complement act. (units H_{50} × ml^{-1} of serum)	386.2±32.1	403.5±83.3	561.2±107.3
HKMs resp. burst (NBT) (OD for 10^5 nuclei)	0.5±0.1	0.3±0.1	0.3±0.1

Stimulated HKMs resp. burst (OD for 10^5 nuclei)	0.7±0.2	0.4±0.1	0.5±0.1
Phagocytic Index	1.0±0.2	1.1±0.1	0.9±0.1
Phagocytosis (% of HKMs performing phagocytosis)	38.6±5.7	44.9±2.8	36.9±3.6
Total serum protein (mg × ml^{-1} of serum)	31.0±3.0	29.5±2.1	28.0±2.5
Serum glucose (mmol × ml^{-1} of serum)	5.5±0.3	5.4±0.3	5.4±0.2
Total serum IgM (mg × ml^{-1} of serum)	9.0±1.8	8.5±1.9	5.1±0.6
Specific IgM (serum titers)	0.5±0.1	0.3±0.1	0.3±0.1
62 days post PBS-injection			
Lysozyme act. (units × min^{-1}× ml^{-1} of serum)	702.5±49.6	707.5±16.8	711.9±59.6
Alternative complement act. (units H_{50} × ml^{-1} of serum)	209.8±13.6	189.2±34.5	130.6±12.0
Classical complement act. (units H_{50} × ml^{-1} of serum)	383.6±56.7	379.2±44.1	402.0±91.0
Total complement act. (units H_{50} × ml^{-1} of serum)	593.4±59.8	568.3±50.0	532.6±94.2
HKMs resp. burst (NBT) (OD for 10^5 nuclei)	2.5±0.6	2.1±0.3	2.2±0.5
Stimulated HKMs resp. burst (OD for 10^5 nuclei)	4.3±1.4	3.7±0.7	4.5±1.5
Phagocytic Index	$0.3±0.1^{a}$	$0.8±0.1^{b}$	$0.8±0.2^{ab}$
Phagocytosis (% of HKMs performing phagocytosis)	$20.6±5.2^{a}$	$39.6±3.2^{b}$	$37.5±6.1^{ab}$
Total serum protein (mg × ml^{-1} of serum)	61.6±2.3	60.3±6.7	59.3±3.2
Serum glucose (mmol × ml^{-1} of serum)	6.6±0.4	6.1±0.2	6.4±0.3
Total serum IgM (mg × ml^{-1} of serum)	0.8±0.3	0.9±0.2	1.3±0.4

Abbreviations: SPC 35 - diet with 35% of dietary protein from soy protein concentrate (SPC); SPC 58 - diet with 58% of dietary protein from SPC; SPC 80 - diet with 80% of dietary protein from SPC.

Data for growth performance represent means ± SEM for 4 replicate tanks.

Significant differences among dietary groups at each timepoint are given with different superscript letters within each row.