EFFECT OF THE ARACHIDONIC ACID/VITAMIN E INTERACTION ON THE IMMUNE RESPONSE OF JUVENILE ATLANTIC SALMON (*Salmo salar*) CHALLENGED AGAINST *Piscirickettsia salmonis*

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Abstract

Atlantic salmon (*Salmo salar*) were fed 6 experimental diets containing three levels of arachidonic acid (ARA) (0.18, 0.28 and 0.63 % for low, medium and high levels respectively) and two levels of vitamin E (150 and 730 mg kg^{-1} for low and high levels respectively). At the end of the experimental period, fatty acids in the liver and immunity markers (lysozyme activity, respiratory burst and phagocytic activity) were determined and fish subjected to a challenge test against the salmonid ricketsial syndrome (SRS) pathogen. ARA, vitamin E or their interaction did not exert an effect on fish performance, whereas ARA alone clearly increased the deposition of ARA. Dietary vitamin E only enhanced liver vitamin E deposition, while the interaction of ARA and vitamin E influenced lysozyme activity and EPA/ARA ratio pointing out the effect of both nutrients on the fish immune system and metabolism. Only the medium concentration contributed to reducing mortality when the fish were exposed to the SRS pathogen. In conclusion, different levels of supplementation with ARA and vitamin E in the diet had no effect on productivity, but did have effects on immune markers and cumulative mortality when fish were exposed to the SRS pathogen.

Introduction

With the growth of the aquaculture industry, more attention has been paid to fish welfare and health in order to avoid negative impacts on fish performance and consequently economic losses. Use of properly balanced diets seems to be a feasible approach for allowing fish to have improved immunological response and thus greater ability to cope with disease. Although the role of lipids in immune function in fish has been studied, the results are not always conclusive because their effect apparently depends on environmental conditions, the genetic status of the studied organism as well as the interaction among different types of fatty acids and other nutrients (Montero *et al*. 1999; Kiron *et al*. 2004; Bureau *et al*. 2008).

It is known that lipids, in addition to being an excellent source of energy and a vehicle for fat soluble vitamins, are the source of polyunsaturated fatty acids (PUFA) such as di-homo-gamma-linolenic acid (DHGLA; 20:3n-6), arachidonic acid (ARA; 20:4n-6) and eicosapentaenoic acid (EPA; 20:5n-3), which are the precursors of two groups of eicosanoids: those derived from the cyclooxygenase action such as prostaglandins (PG) and thromboxanes (TX) and those derived from the lipoxygenase action such as lipoxins (LX) and leukotrienes (LT) (Bell *et al*. 1995). These compounds are part of the defence systems of the organisms and therefore play a role in certain pathophysiological processes such as resistance to stress stimuli, the immune response and inflammatory processes (Lall 2000). It has been stated that depending on the type of the individual fatty acids, their concentrations and the relationships among them, eicosanoids can have pro- or anti-inflammatory effects. Therefore, diets rich in n-6 fatty acids, primarily

ARA, produce pro-inflammatory effects derived from higher levels of PGE2, LT4 and LX, whereas diets high in n-3 PUFAs, particularly EPA, produce anti-inflammatory effects derived from PGE3 and LT5 (Lall 2000). Although overproduction of PGE2 due to high dietary ARA can also have an immunosuppressant effect (Bell *et al*. 1996), this effect can be mitigated depending on the concentrations of EPA and DHGLA given that both fatty acids have an inhibitory effect on ARA. Because ARA is generally the preferred substrate of lipoxygenases for LT synthesis (Bell *et al*. 1994) and products derived from these enzymes produce stronger immune stimulatory effects than those derived from cyclooxygenases (Hwang 1989), one would expect that incorporating ARA into the diet would contribute to an improved immune response in fish and therefore increased resistance against attack from pathogens. Thus, n-6 fatty acids play an important role in inflammatory processes, stimulating the production of pro-inflammatory cytokines such as the tumour necrosis factor-alpha (TNF-α) and interleukins (IL) 1 and 6 and consequently leading to pro-inflammatory events such as vasodilation and increased vascular permeability (Calder 2002).

Although anadromous teleost fish such as rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) have the ability to elongate and desaturate short-chain fatty acids to long chain PUFA (LC-PUFA) in their fresh water phase and their requirements can be satisfied by fatty acids such as linoleic acid (LA; 18:2n-6) and α-linolenic acid (LNA; 18:3n-3) (Bell & Sargent 2003), it is not clear whether these fatty acids are sufficient for maintaining fish in adequate physiological condition. Thus, supplementation with LC-PUFA, such as ARA, EPA and docosahexaenoic acid (DHA; 22:6n-3) is always appropriate and recommended. Furthermore, Bell *et al*. (2003)

92 suggested the potential impact of ARA in restoring normal immune function in
93 freshwater fish fed low-ARA diets despite of their ability to synthesize LA to ARA.
94 Thus, the use of feeds with an unadjusted n-6/n-3 fatty acids ratio, mainly ARA, can
95 alter the fish immune system and resilience when exposed to pathogens.
96
97 Given that PUFAs are susceptible to oxidation due to their high unsaturation degree,
98 and that oxidation products can be toxic to fish, the high levels of these fatty acids in
99 aquafeeds need to be accompanied by adequate amounts of dietary antioxidants. Among
100 them, vitamin E (α-tocopherol) is a powerful antioxidant that is commonly included in
101 feeds formulation as a form of protection against fatty acid oxidation (Kiron *et al*. 2004;
102 Betancor *et al*. 2011; Hamre 2011). In addition, vitamin E can improve the fish immune
103 response as this nutrient is involved in preventing lipid peroxidation of cell membranes,
104 the regulation of the specific immunity, nonspecific resistance factors and disease
105 resistance capacity (Sahoo & Mukherjee 2002). The relationships among LC-PUFA,
106 vitamin E and the immune response have been studied in rainbow trout (Kiron *et al.*
107 2004; Puangkaew *et al*. 2004, 2005; Trenzado *et al*. 2009), Japanese flounder
108 (*Paralichthys olivaceus*) (Wang *et al*. 2006) and gilthead seabream (*Sparus aurata*)
109 (Montero *et al.* 2004). However most of these studies have not evaluated the effect of
110 the LC-PUFA/vitamin E ratio when fish are challenged against pathogens, and
111 furthermore, no studies have linked an increase of specific dietary fatty acids (such as
112 ARA) with vitamin E requirements. However, there is evidence that vitamin E
113 participates in the conversion of ARA into prostaglandins via cyclooxygenase action in
114 mammals (O'Leary *et al*. 2004) and similar action could also happen in teleost fish.

Furthermore, given that ARA is a minor component of fish cell membranes and that is required in small amounts compared to DHA and EPA, its importance has been underestimated (Bell *et al*. 2003; Xu *et al*. 2010). However, the limited studies on ARA have suggested an important contribution of this fatty acid fish growth, survival and bone formation as well as its influence on modulating the immune response and disease resistance in several teleost fish species (Khozing – Goldberg *et al*. 2006; Van Anholt *et al*. 2004, 2012; Xu *et al*. 2010; Li *et al*. 2012; Boglino *et al*. 2014; Tian *et al*. 2014). Nevertheless, there is not enough evidence linking dietary ARA and vitamin E levels in freshwater fish species. Therefore, it is expected that appropriately balanced incorporation of this fatty acid with vitamin E could improve processes in which ARA is involved such as a favourable immune response.

Salmonid rickettsial syndrome (SRS), caused by *Piscirickettsia salmonis*, is considered to be the most important sanitary problem in the Chilean salmon farming industry, with economic losses up to $100 million per year. Thus, the impact of SRS on salmon farming in Chile could be mitigated by strengthening the immune activity in the fish through an appropriate balance between dietary ARA and vitamin E. In the present study, we aimed to elucidate the interaction between ARA and vitamin E on juvenile Atlantic salmon immune response and their protective effect when fish were challenged with the causative agent of SRS.

Materials and methods

2.1. Experimental fish

One thousand five hundred and thirty juvenile Atlantic salmon were obtained from a local fish farm (Pesquera los Fiordos; commune of Curarrehue, region IX, Chile) and transferred to the Quimey-Co de Sociedad Nalcahue Ltda. fish farm (commune of Pucón, Caburga sector, region IX, Chile). The fish were acclimatised for two weeks and fed a commercial diet (BIOMAR S.A., Chile). Prior to the start of the experimental trial, the fish were distributed among eighteen 100 L tanks at a ratio of 85 fish $tank^{-1}$ with an average weight of 6.5 ± 1.1 g to reach an initial density of 5.5 kg m^{-3}. Tanks had a continuous water supply at a rate of 1.5 L h^{-1} and fish were maintained under a 24 h light photoperiod, being the water the temperature in tanks 9.9 ± 0.2 ºC and dissolved oxygen concentrations above 10 mg L^{-1}.

2.2 Experimental diets and feeding

Six experimental feeds were formulated and manufactured to contain three dietary levels of ARA (1.05, 1.73 and 3.73 % for low, medium and high respectively) and two levels of vitamin E as α-tocopherol acetate (0.12 - 0.19 and 0.72 - 0.76 g kg^{-1}, for low and high respectively). Feeds were isolipidic and isoproteic, and levels of other fatty acids held constant. A set of 5 different oils was employed to obtain the desired levels of ARA altering minimally the levels of other PUFA. In this sense, an ARA-enriched oil was employed in ARA3 diets, whereas a n-6-rich oil (Yardquim oil) was used in ARA2 and ARA3 feeds. All the feeds contained n-3-rich oil (ROPUFA® n-3 INF oil) to help maintain adequate levels on n-3 PUFA as well as olive oil to equalize the fat content of all the feeds. Finally, the diets containing the lowest ARA levels were supplemented with an oil rich in EPA (ROPUFA® n-3 EPA oil) to maintain similar

EPA levels to the other feeds. The diets were labelled as ARA1/E1 (low ARA/low vitamin E), ARA1/E2 (low ARA/high vitamin E), ARA2/E1 (medium ARA/low vitamin E), ARA2/E2 (medium ARA/high vitamin E), ARA3/E1 (high ARA/low Vitamin E) and ARA3/E2 (high ARA/high vitamin E). The diets were formulated and manufactured in the feed pilot plant of the School of Aquaculture of the Catholic University of Temuco (Chile) using a CLEXTRAL experimental extruder, model B21 (Firminy Cedex, France), and oiled by a Dinissen vacuum oiler (Pegasus Menger, 400 l, Sevenum, Holland). Each diet was tested in triplicate for 12 weeks. Fish were fed to apparent visual satiety, ten times per day by hand, and uneaten feed was removed by siphoning, dried and weighed to calculate fish feed intake. VEVODAR® (DSM nutritional products S.A., Puerto Varas, Chile) was used as the source of ARA and Rovimix E-50® for vitamin E (DSM nutritional products S.A., Puerto Varas, Chile). The formulation, proximal composition, fatty acid profile and vitamin E concentration of the diets were analysed in the fish nutrition laboratory of the School of Aquaculture at the Catholic University of Temuco and are shown in Tables 1 and 2.

2.3. Productive indicators

The following productive variables were evaluated:

- Increase in weight (%) = [(final weight – initial weight) / initial weight] × 100.
- Feed conversion factor = increase in weight (g)/total food intake (g).
- Specific growth rate (% day^{-1}) = [(ln final weight - ln initial weight) / days] × 100.
- TGC (thermal growth coefficient) = [(final $weight^{1/3}$ – initial $weight^{1/3}$) × 1000] / ∑ (temperature × days)

- Condition index = (final weight / final length^3) × 100
- Hepatosomatic index = (liver weight (g) / final weight) × 100
- Protein efficiency ratio = gain in body mass (g) / protein intake (g)
- Percent survival (%) = (Nº final specimens/initial number of specimens) × 100

2.4. Analysis and measurements

2.4.1. Collection of blood and tissue samples for analysis.

At the end of the experimental period, fish were starved for 24 hours prior to the final sampling. Six fish per tank (n =18 per treatment) were anesthetized with 20% benzocaine (BZ-20; Veterquimica S.A., Santiago, Chile) and bled by puncturing the caudal vein with 1 mL syringes. Blood samples were immediately placed in Eppendorf tubes containing 5 µL of heparin to prevent blood clotting. The tubes containing blood were centrifuged at 3500 rpm for 10 minutes at a constant temperature of 4 °C to separate the plasma from the cells. Fresh plasma was frozen at -80 °C until lysozyme activity was determined.

Then, fish were sacrificed in 10 L containers with an overdose of benzocaine together with ice to maintain a temperature of 4 ºC in order to obtain fresh samples. Liver samples were collected for determination of fatty acids (6 fish tank^{-1}, n = 18 per treatment), and samples stored at -80 ºC until further analysis. Kidney samples were also obtained for immune parameters analysis and quickly frozen at -80 °C until further analysis.

2.4.2 Total lipids and fatty acid analysis.

Total lipids were extracted using a chloroform:methanol mixture (2:1) according to the method by Folch *et al*. (1957). Fatty acids were methylated according to the method proposed by Morrisony Smith (1964). Fatty acids were separated using a Hewlett Packard 5890 series II Plus (Wilmington NC, USA) gas chromatograph with a capillary column of 30*0.25*0.20 mm (SPTM 2380, SUPELCO, Bellafonte, PA USA). Helium gas was used as a carrier. Fatty acids were identified by comparison to a SUPELCO 37 fatty acid standard (Sigma Aldrich, St. Louis, MO, USA). Fatty acids are expressed as percentage of the total fatty acids identified.

Vitamin E analysis

The free α-tocopherol level of the diets and liver tissues were determined at the Quality Control Laboratory, DSM Nutritional Products S.A. (Puerto Varas, Los Lagos Region, Chile), according to the company analytical methods and procedures for vitamins and carotenoids. Analysis equipment included an HPLC Perkin Elmer series 200 (Shelton, CT, USA) with auto sampler, a UV-visible detector, a Link interface 600 series data system recollection "Totalchrom Workstation" version 6.2.1., and a column Lichrosorb Si 60.5 μm * 25 * 4 mm Alltech brand- Grom (Germany). Briefly, the HPLC conditions used for the vitamin assessment were as follows: reflux extraction with potassium hydroxide solution in methanol and continuation with a hexane:toluene (1:1) solution, room temperature conditions, and a mobile phase with a hexane:dioxane (97:3) solution injected (20 μL) through an automatic nozzle. The results are expressed as mg kg^{-1} of free α-tocopherol.

2.4.3. Immune parameters

- *Extraction of leucocytes to determine phagocytosis and respiratory burst*

Leucocytes from the anterior portion of the kidney were used to determine phagocytic activity and respiratory burst following the method proposed by Sakai *et al*. (1996). Leucocytes were extracted from the same specimens used for blood sampling. Kidneys were aseptically removed and placed in L-15 medium (Sigma, St. Louis, MO, USA) supplemented with 10% foetal bovine serum, 0.2% heparin (Sigma, St. Louis, MO, USA) and 0.1% penicillin/streptomycin (Sigma, St. Louis, MO, USA). Maceration was conducted and the resulting cellular suspension was layered onto a 34/51% Percoll gradient (Pharmacia, Uppsala, Sweden). After centrifugation, the interphase cells were isolated, washed in Hanks medium and number adjusted to 10^7 cells mL^{-1}. Viable phagocytic cells were quantified by trypan blue exclusion (viability > 95%).

- *Leucocytes respiratory burst activity*

To measure intracellular superoxide anion production, the method developed by Yin *et al*. (2006) was used. The leucocyte suspension was distributed into 96-well plates and the reduction of nitroblue tetrazolium (NBT) was evaluated. Untreated leucocytes were used as a negative control and leucocytes treated with 0.5 mg mL^{-1} phorbol myristate acetate (PMA) were used as a positive control. The cell culture was maintained at 18º C for 18 h. The extract, PMA treatments and cell monolayer were washed twice with Hank's saline solution (HBSS, Sigma). Then, 100 µL of NBT solution (1 mg mL^{-1} in RPMI 1640) were added per well and incubated for 60 min at 18º C. After incubation,

the NBT solution was removed and the cells were fixed with methanol for 10 min. The cells were air-dried and the formazan in each well was dissolved in 120 µL of 2 M KOH and 140 µL of dimethyl sulphoxide (Sigma). Optical density was measured with a Multiscan spectrophotometer (Spectra, Count, Pakard USA) at 630 nm.

- *Phagocytic activity*

The phagocytic activity of the leucocytes was determined by the direct count method, which involves co-incubating leucocytes isolated from the anterior kidney with yeast cells stained with Congo red in 96-well plates. The mixtures were incubated at 18 °C for 3 hours and the percentage of leucocytes with phagocytosed cells was counted and considered a direct measure of the level of immune stimulation of the cells. The counts involved a total of 15 optical fields in triplicate for each specimen tested.

- *Lysozyme activity*

The turbidimetric assay for measuring lysozyme (Parry *et al*. 1965) was performed in a 96-well plate. A volume of 200 µL of a suspension of *Micrococcus lisodeikticus* (0.2 mg mL^{-1} in 50nM sodium phosphate buffer) and 5, 10, 20 or 40 µL of blood plasma were mixed in different wells, and then absorbance recorded at 520 nm at 1, 3, 6 and 9 min with a microplate reader (Elx808BioTex USA). One unit of activity was defined as the amount of sample causing a 0.001 min^{-1} decrease in absorbance.

2.4.4. Bacterial challenge test

281 At the end of the experimental period, 18 specimens per treatment (6 fish per tank) were
282 transported from the hatchery of the Catholic University of Temuco to the Aquadvise
283 Experimental Unit of Fundación Chile (Puerto Montt, region X, Chile). Fish were
284 marked with PIT-tags system (Trovan®, Trovan Ltd., United Kingdom) and placed in a
285 720 L tank with a turnover rate of 1.2 L h^{-1}. The oxygen concentration was maintained
286 between 90 and 100% saturation and the water temperature at 14.5 ºC. The temperature
287 was higher because the SRS challenge model requires 14.5 to 15 ºC to develop optimum
288 pathogen replication in the fish. The acclimatisation period lasted 6 days, after which
289 the fish were challenged by intraperitoneal injection using a strain of *Piscirickettsia*
290 *salmonis* (SRS) as a salmonid pathogen at a dose of 0.2 mL SRS per fish at a 10^{-2}
291 dilution considering previous LD50 results. During the SRS challenge, all the fish of the
292 same treatment were kept in the same tank. The inoculum was provided by the
293 "Fundación Ciencia para la Vida" (Science for Life Foundation). To verify the cause of
294 death, head kidney of deceased fish were analysed by the direct immunofluorescence
295 technique SRS FluoroTest Direct (Bios Chile, Santiago, Chile) wherein monoclonal
296 antibodies are conjugated to fluorescein isothiocyanate (FITC). This test is highly
297 sensitive and specific for detecting *Piscirickettsia salmonis* in tissue samples (Marshall
298 et al., 2007).
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300
301 *2.5 Statistical analysis.*
302 The results were statistically analysed using a two-factor ANOVA after prior
303 confirmation of theoretical assumptions of data normality through histograms and
304 homogeneity of variances by Bartlett's test. Once these theoretical assumptions were
305 satisfied, analyses of variance were performed to determine significant differences

among the treatments at the 95% confidence level. If there were differences, Duncan's test was then used to differentiate the means among the three different ARA levels, revealing those treatments that exhibited significant differences (Sokal & Rohlf 1995). For vitamin E levels, a student t-test was employed to denote significant differences between the two vitamin levels. The arcsine transformation was applied to data expressed as percentages to achieve homoscedasticity. The statistical software MS STATISTICA 12 was used in the statistical analyses.

Results

3.1. Growth and dietary utilisation.

Fish growth in terms of final weight did not differ ($P>0.05$) among the different experimental groups or their replicates. All fish groups increased their weight up to three times relative to initial weight, reaching an average value between 21.8 and 23.9g. The specific growth rate (SGR) varied between 1.27 and 1.37% per day. The conversion factor rate (CF) was similar among the experimental diets, ranging from 1.01 to 1.13. Hepatosomatic index (HSI) values were within the normal range (0.95 to 1.11) without showing significant differences ($P>0.05$). None of the evaluated performance parameters showed significant differences ($P>0.05$) when subjected to statistical analysis (Table 3).

3.2 Liver fatty acids and vitamin E contents

Table 4 shows the fatty acid composition and measured vitamin E content in the liver of juvenile Atlantic salmon fed the six dietary treatments. The levels of vitamin E

concentrations in the liver were directly proportional to its dietary incorporation, and higher (P<0.05) in the high vitamin E group. Fish fed diets containing a high concentration of ARA showed significantly higher (P<0.05) accumulation of this fatty acid than fish fed low and medium levels of ARA, together with a reduction in the EPA/ARA ratios (Table 4). Besides, there was no interaction between the two nutrients, ARA concentration did not affect (P>0.05) the vitamin E concentration in liver tissue and vitamin E dietary levels did not affect (P>0.05) the ARA content in the tissue. The only interaction between both nutrients was found in the EPA/ARA ratios (P<0.05; Table 4), whereas ARA levels did affect C14, total monounsaturated, EPA, DHA (P<0.005), as well as ARA (P<0.01) percentages according to the two-way ANOVA performed.

3.3 Fish health: Immune parameters and challenge test.

No significant differences (P>0.05) or interactions between ARA and vitamin E were found for phagocytic activity in fish fed any of the dietary treatments (Table 5). However, a significant effect (P<0.05) of vitamin E was found on lysozyme activity, with the group fed ARA2/E1 displaying significantly (P<0.05) higher lysozyme enzymatic activity (Table 5), although no differences were observed with fish fed ARA3/E2. Besides, ARA levels significantly (P<0.05) affected leucocyte respiratory burst activity with the lowest (P<0.05) activity corresponding to fish fed the lowest ARA level (Table 5). Furthermore, an interaction between ARA and vitamin E levels was observed in lysozyme activity (P<0.01; Table 5), whereas individual ARA did exert an effect on the leucocytes respiratory burst (P<0.01; Table 5).

354

355 Regardless of the level of dietary vitamin E, fish fed diets containing low concentrations
356 of ARA displayed cumulative mortality between 56 and 65% when exposed to the SRS
357 pathogen (Fig. 1). In all cases where the concentration of ARA in the diet was low or
358 high, cumulative mortality was below 50%. However, when the ARA concentration in
359 the diet was medium and the vitamin E concentration was high, higher survival rates
360 were achieved. It was determined that 100% of the recorded mortality was confirmed
361 for the presence of the SRS pathogen.

362

363 **Discussion**

364 Despite of the relevance of vitamin E, studies assessing its relationship with PUFA,
365 particularly ARA, and how this relationship may affect fish growth are scarce. In the
366 present study 6 different experimental feeds containing two levels of vitamin E and
367 three levels of ARA were fed to Atlantic salmon to evaluate the single effect of these
368 nutrients as well as their interaction on fish performance, liver composition and
369 immunological response after challenge with SRS. ARA and vitamin E supplementation
370 did not affect the growth of juvenile Atlantic salmon in terms of length or weight,
371 showing that there was no independent or synergistic effect between the two nutrients
372 on fish performance. This indicates that low dietary concentrations of ARA (circa 1% of
373 the total fatty acids) and vitamin E (circa 150 mg kg^{-1}) were sufficient for supporting
374 adequate fish growth. These results are in line with previous studies where increases in
375 ARA levels over 12 times in terms of total fatty acids together with constant levels of
376 vitamin E did not enhance any of the studied growth performance parameters in Atlantic
377 salmon post-smolts (Glencross *et al*. 2014).

EPA, ARA and DHGLA are fatty acids precursors of eicosanoids *via* the actions of lipoxygenases and cyclooxygenases. It is known that eicosanoids derived from ARA, mainly LTB4 and PGE2, are pro-inflammatory, whereas those derived from EPA and DHGLA have an anti-inflammatory action (Hwang 1989). Thus it is expected that altering the EPA/ARA ratio in cell membranes may alter the production of eicosanoids and modulate the inflammatory response (Bell *et al*. 1995). Some studies have shown that ARA is the preferred precursor for the synthesis of eicosanoids in fish, competing with EPA (Bell *et al*. 1994). In the present study, the changes in liver fatty acid composition largely reflected that of the diets, showing variations in the ARA contents, which were increased with increasing ARA dietary levels. Although in the present study only fatty acids from total lipids were determined, a previous study in Atlantic salmon showed that the ARA content of phospholipids fatty acids of several tissues was correlated to the ARA dietary intake (Betancor *et al*. 2014). Thus, taking into account that the percentage of neutral and polar lipids in Atlantic salmon fed fish oil-profile diet is around 30 and 60% respectively (Torstensen *et al*. 2004), a considerable amount of ARA will be available in the Atlantic salmon membranes for its bioconversion to reactive eicosanoids.

Furthermore, dietary ARA influenced the deposition of other fatty acids such as oleic acid (18:1n-9), EPA and DHA. It must be noted that liver is considered an important site for LC-PUFA synthesis and lipid metabolism in Atlantic salmon (Monroig et al. 2010). Hepatic levels of ARA did not differ between fish fed low and medium ARA levels which could be attributed to enhanced biosynthesis from 18:2n-6. Besides, as the same Δ6 and Δ5 –desaturase and fatty acyl elongase enzymes participate in the synthesis

of both the n-3 and n-6 LC-PUFA (Zheng et al. 2005), the activation of one pathway could also enhance the final and intermediate products of the other, what could account for the increased EPA and DHA contents found in low ARA-fed fish. Enhanced conversion from 18:2n-6 to 20:3n-6 was greater than for 18:4n-3 to 20:4n-3 in Atlantic salmon fed diets devoid of n-3 LC-PUFA, which resulted in significantly higher concentrations of ARA in liver (Miller et al. 2008).

Dietary ARA also elicited an effect in leucocytes respiratory activity, medium and high levels leading to up-regulation of the respiratory burst activity. However ARA did not trigger an effect on any of the other health-related parameters, which could indicate that up-regulation of burst activity alone is not sufficient to enhance bactericidal activity against SRS by Atlantic salmon leucocytes. Similarly, yeast β-glucans, known to have an immune stimulatory effect in fish (Magnadóttir 2006) only elicited macrophages respiratory burst, not eliciting any effect on bactericidal activity (Jørgensen and Robertsen, 1995)

Low levels of ARA combined with high or low levels of vitamin E lead to low concentrations of ARA in the liver, low stimulation of macrophages as well as cumulative mortality between 56 and 65% from the SRS pathogen. Thus an increase in vitamin E did not contribute to stimulate the immune response in the presence of low levels of ARA, nor improved the response against the SRS pathogen as indicated by the two-way ANOVA analysis. Blazer & Wolke (1984) found that T and B lymphocytes function in rainbow trout was suppressed under vitamin E deficiency and that this vitamin requirement may increase during immune stimulation. Furthermore, a combination of medium levels of ARA with high or low levels of vitamin E not only improved macrophage activity but also allowed better resistance to the pathogen SRS,

426 decreasing mortality by 40% and 24%, respectively. Accordingly, a reduction in
427 vitamin E concentration in the diet to near 200 mg kg^{-1} can contribute to maintaining an
428 active immune response provided that ARA is maintained at medium levels. In turn,
429 when the ARA concentration in the diet is high, its concentration in liver tissue
430 increases, the immune response remains high and mortality against the pathogen is
431 maintained only between 45 and 50% regardless of the vitamin E level in the diet.
432 Generally, an increase in ARA in cell membranes enhances the availability of ARA as a
433 substrate for eicosanoid production, resulting in greater availability of PGE2 (Bell &
434 Sargent 2003), which has been shown to have immune stimulatory effects, directly
435 influencing macrophage action (Montero *et al*. 1996). Evidence that ARA incorporation
436 in the diet improves nonspecific immunity has been recently shown in carp
437 (*Ctenopharyngodon idellus*) and in yellow croaker (*Larimichthys crocea*) (Tian *et al*.
438 2014; Xu *et al*. 2014), however none of these studies have monitored indicators of
439 immunity in response to combined ARA and vitamin E or how this is reflected when
440 fish are exposed to a specific pathogen.
441 According to the results of the present study, vitamin E and ARA act synergistically to
442 stimulate lysozyme activity particularly with medium concentrations of ARA and low
443 concentrations of vitamin E. Similarly, high macrophage activity was observed as
444 improved resistance to the pathogen SRS only when ARA was present at a medium
445 concentration in the diet. Thus high supplementation of vitamin E is unnecessary for
446 immune stimulation when dietary ARA level is medium or high. This coincides with the
447 finding of Pungkaew *et al*. (2005), who suggested that high supplementation of vitamin
448 E is not necessary for proper immune stimulation when the level of PUFA is adequate,
449 although these authors did not expose fish to a pathogen which would allow immune

response to be evaluated. Curiously, the EPA/ARA ratio was also affected by the interaction of ARA and vitamin E contents. A relationship between vitamin E and LC-PUFA levels has been described in several fish species (Mourente et al., 2007; Lebold et al., 2011), hypothesizing that tocopherols may influence the biosynthesis of PUFA, especially n-3 PUFA, through alteration of cellular oxidation potential or peroxide tone.

In summary, inclusion of ARA in Atlantic salmon feeds improved some indicators of non-specific immunity such as respiratory burst, being this effect independent of the vitamin E concentration. ARA inclusion also seemed to elicit an effect on LC-PUFA biosynthesis by enhancing EPA and DHA levels in fish fed low ARA feeds. Medium inclusion of ARA combined with a high concentration of vitamin E was found to reduce cumulative mortality by 65%. An interaction between ARA and vitamin E was observed on lysozime activity and EPA/ARA ratio probably indicating and effect of vitamin E on LC-PUFA biosynthesis. To conclude, the interactive effect between vitamin E and ARA was shown to be limited, although each nutrient, particularly ARA, proved to exert a marked effect on Atlantic salmon immune response.

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References

Bell, J.G., Tocher, D.R., MacDonald, F.M. & Sargent, J.R. (1994) Effects of diets rich in linoleic (18:2n-6) and α-linolenic (18:3n-3) on the growth, lipid class and fatty acid composition and eicosanoid production in juvenile turbot (*Scophthalmus maximus*). *Fish Physiol. Biochem.*, **13**, 105-118.

Bell, J.G., Castell, J.D., Tocher, D.R., MacDonald, F.M. & Sargent, J.R., (1995) Effects of diferent dietary arachidonic acid: docosahexaenoic acid ratios on phospholipid fatty acid composition and prostaglandin production in juvenile turbot (*Scophthalmus maximus*). *Fish Physiol. Biochem.*, **14**, 139-151.

Bell, J.G., Ashton, I., Secombes, C.J., Weitzel, B.R., Dick, J.R. & Sargent, J.R. (1996) Dietary lipid effects phospholipid fatty acid composition, eicosanoid production and immune function in Atlantic Salmon (*Salmo salar*). *Prostaglandins Leukotrienes Essent. Fatty Acids*, **54**, 173-182.

Bell, J.G. & Sargent, J.R. (2003) Arachidonic acid in aquaculture feeds: Current status and future opportunities *Aquaculture*, **218,** 401-499.

Bell J.G., McGhee F., Campbell P.J. & Sargent J.R. (2003) Rapeseed oil as an alternative to marine fish oil to marine fish oil in diets of post-smolt Atlantic Salmon (*Salmo salar*). Changes in flesh fatty acid composition and effectiveness of subsequent fish oil "wash out". *Aquaculture*, **218**, 15-28.

498

499 Betancor, M.B., Atalah, E., Caballero, M., Montero, D. & Izquierdo, M.S. (2011) α-
500 tocopherol in weaning diets for European sea bass (*Dicentrarchus labrax*) improves
501 survival and reduces tissue damage caused by excess dietary DHA contents. *Aquacult.*
502 *Nutr.*, **17**, 112-122.

503

504 Betancor, M.B., Howarth, F.J.E., Glencross, B.D. & Tocher, D.R. (2014) Influence of
505 dietary docosahexaenoic acid in combination with other long-chain polyunsaturated
506 fatty acids on expression of biosynthesis genes and phospholipid fatty acid
507 compositions in tissues of post-smolt Atlantic salmon (*Salmo salar*). *Comp. Biochem.*
508 *Physiol.*, **172-173 B**, 74-89.

509

510 Blazer, V.S. & Wolke, R.E. (1984) The effects of α-tocopherol on the immune response
511 and non-specific resistance factors of rainbow trout (*Salmo gairdneri* Richardson).
512 *Aquaculture*, **37**, 1–9.

513 Boglino, A., Darias, M.J., Andree, K.B., Estévez, A. & Gisbert, E. (2014) The effects of
514 dietary arachidonic acid on bone in flatfish larvae: the last but not the least of the
515 essential fatty acids. *J. Appl. Ichthyol.*, **30**, 643-651.

516

517 Bureau, D.P., Hua, K. & Harris, A.M. (2008) The effect of dietary lipid and long-chain
518 n-3 PUFA levels on growth, energy utilization, carcass quality and immune function of
519 rainbow trout (*Oncorhynchus mykiss*). *J. World Aquac. Soc.*, **39**, 1-21.

520

521 Calder, P.C. (2002) Dietary modification of inflammation with lipids. *Proc. Nutr. Soc.*,
522 **61**, 345-358.
523
524 Folch, J., Lees, M. & Stanley, G.H.S. (1957) A simple Method for the isolation and
525 purification of total lipids from animal tissue. *J. Biol. Chem.*, **226**, 497-509.
526
527 Glencross, B.D., Tocher, D.R., Matthew, C. & Bell, J.G. (2014) Interactions between
528 dietary docosahexaenoic acid and other long-chain polyunsaturated fatty acids on
529 performance and fatty acid retention in post-smolt Atlantic salmon (*Salmo salar* L).
530 *Fish Physiol. Biochem.*, **40**, 1213-1227.
531
532 Hamre, K. (2011) Metabolism, interaction, requirements and functions of vitamin E in
533 fish. *Aquacult. Nutr.*, **17**, 98 – 115.
534
535 Hwang, D. (1989) Essential fatty acids and immune responses. *FASEB J.*, **3**, 2052-
536 2061
537
538 Jørgensen, J.B. & Robertsen, B. (1995) Yeast β-glucan stimulates respiratory burst
539 activity of Atlantic salmon (*Salmo salar* L.) macrophages. *Dev. Comp. Immunol.*, **19**,
540 43-57.
541
542 Kiron, V., Puangkaew, J., Ishizaka, K., Satoh, S. & Watanabe, T. (2004) Antioxidant
543 status and Nonspecific immune responses in rainbow trout (*Oncorhynchus mykiss*) fed
544 two levels of vitamin E along with three lipid sources. *Aquaculture*, **234**, 361–379.

Khozin-Goldberg, I., Cohen, Z., Pimenta-Leibowitz,M., Nechev, J. & Zilberg, D. (2006) Feeding with arachidonic acid-rich triacylglycerols from the microalga *Parietochloris incisa* improved recovery of guppies from infection with *Tetrahymena sp*. *Aquaculture*, **255,** 142–150.

Lall, S. (2000) Nutrition and Health of Fish. In: Avances en Nutrición Acuícola. (Cruz-Suarez, I. E., Ricque –Marie, D. Tapia-Salazar, M. Olvera-Novoa M.A. & Civwera-Cerecedo R. Eds.). pp. 13-22. Memorias del V Simposium Internacional de Nutrición Acuícola , Mérida , Yucatan México.

Lebold, K.M., Jump, D.B., Miller, G.W., Wright, C.L., Labut, E.M., Barton, C.L., Tanguay, R.L. & Traber, M.G. (2011) Vitamin E deficiency decreases long-chain PUFA in zebrafish (*Danio rerio*). *J. Nutr.*, **141**, 2113-2118.

Li, Q., Ai, Q., Mai, K., Xu, W. & Zheng, Y. (2012) In vitro effects of arachidonic acid on immune functions of head kidney macrophages isolated from large yellow croaker (*Larmichthys crocea*). *Aquaculture*, **330**, 47-53.

Magnadóttir, B. (2006) Innate immunity of fish (overview). *Fish Shellfish Immunol*., **20**, 137-151.

Marshall, S.H., Conejeros, P., Zahr, M., Olivares, J., Gómez, F., Cataldo, P. &

569 Henríquez V. (2007) Immunological characterization of a bacterial protein isolated
570 from salmonid fish naturally infected with *Piscirickettsia salmonis*. *Vaccine*, **25**, 2095-
571 2102.
572
573 Miller, M.R., Bridle, A.R., Nichols, P.D. & Carter, C.G. (2008) Increased elongase and
574 desaturase gene expression with stearidonic acid enriched diet does not enhance long-
575 chain (n-3) content of seawater Atlantic salmon (*Salmo salar* L.). *J. Nutr.*, **138**, 2179-
576 2185.
577
578 Monroig, Ó., Zheng, X., Morais, S., Leaver, M.J., Taggart, J.B. & Tocher, D.R. (2010)
579 Multiple genes for functional Δ6 fatty acyl desaturases (Fad) in Atlantic salmon (*Salmo*
580 *salar* L.): Gene and cDNA characterization, functional expression, tissue distribution
581 and nutritional regulation. *Biochim. Biophys. Acta*, **1801**, 1072-1081.
582
583 Montero, D., Tort, L., Izquierdo, M.S., Socorro, J., Robaina, L., Vergara, J.M. &
584 Fernández-Palacios, H. (1996) Effect of alpha-tocopherol and n-3 HUFA deficient
585 diets on blood cells, selected immune parameters and body composition of gilthead
586 seabream (*Sparus aurata*). In: Modulators of Immune Responses. The evolutionary
587 Trail. (Stolen J.S., Fletcher T.C., Bayne C.J., Secombes C.J., Zelikoff J.L., Twerdok
588 L., Anderson D.P. Eds.) pp. 251-266. SOS Publications, Fair Haven, New Jersey,
589 USA.
590
591 Montero, D., Izquierdo, M.S., Tort, L., Robaina, L. & Vergara, JM. (1999) High
592 stocking density produces crowding stress altering some physiological and biochemical

parameters in gilthead seabream, *Sparus aurata*, juveniles. *Fish Phisiol. Biochem.*, **20**, 53-60.

Montero, D., Socorro, J., Tort, L., Caballero, M.J., Robaina, L.E., Vergara, J.M. & Izquierdo, M.S. (2004) Glomerulonephritis and immunosuppression associated to dietary essential fatty acids deficiency in gilthead seabream (*Sparus aurata*) juveniles. *J. Fish Dis.*, **27**, 297-306.

Morrison, W. & Smith, L. (1964) Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron-fluoride methanol. *J. Lipids Res.*,**5**, 600-608.

Mourente, G., Bell, J.G. & Tocher, D.R. (2007) Does dietary tocopherol level affect fatty acid metabolism in fish? *Fish Physiol. Biochem*., **33**, 269-280.

O'Leary, K.A., De Pascual-Tereasa, S., Needs, P.W., Bao, Y.P., O'Brien, N.M. & Williamson, G. (2004) Effect of flavonoids and Vitamin E on cyclooxygenase-2 (COX-2) transcription. *Mutat. Res.*, **551**, 245-254.

Parry, R.M, Chandau, R.C. & Shahani, R.M. (1965) A rapid and sensitive assay of muramidase. *Proc. Soc. Exp. Biol*., **119**, 384-386.

Puangkaew, J., Kiron, V., Somamoto, T., Okamoto, N., Satoh, S., Takeuchi, T. & Watanabe, T. (2004) Non-specific immune response of rainbow trout (*Oncorhynchus*

617 *mykiss*) in relation to different status of vitamin E and highly unsaturated fatty acids.
618 *Fish Shellfish Immun.,* **16**, 25-39.
619
620 Puangkaew, J., Kiron, V., Satoh, S. & Watanabe T. (2005) Antioxidant defense
621 ofrainbow trout (*Oncorhynchus mykiss*) in relation to dietary n-3 highly unsaturated
622 fatty acids and vitamin E contents. *Comp. Biochem.Physiol*., **140C**, 187-196.
623
624 Sahoo, P.K. & Mukherjee, S.C. (2002) Influence of high dietary α-tocopherol intakes on
625 specific immune response, nonspecific resistance factors and disease resistance of
626 healthy and aflatoxin B_1-induced immunocompromised Indian major carp, *Labeo rohita*
627 (Hamilton). *Aquacult. Nutr.*, **8**, 159-167.
628
629 Sakai, M., Kobayashi, M. & Kawauchi, H. (1996) In vitro activation of fish phagocytic
630 cells by GH, prolactin and somatolactin. *J. Endocrinol.*, **151**, 113-118.
631
632 Sokal, R.R. & Rohlf F.J. (1995) Biometry. In: R.R. Sokal & F.J. Rohlf (Eds.). The
633 principles ad practice of statistics in biological research. New York, W.H Freeman.
634
635 Tian, J., Hong J., Oku, H. & Zhou, J. (2014) Effects of dietary arachidonic acid (ARA)
636 on lipid metabolism and health status of juvenile grass carp, *Ctenopharyngodon idellus.*
637 *Aquaculture,* **430,** 57-65.
638
639 Torstensen, B.E., Frøyland, L. & Lie, Ø. (2004) Replacing dietary fish oil with
640 increasing levels of rapeseed oil and olive oil – effects on Atlantic salmon (*Salmo salar*

L.) tissue and lipoprotein lipid composition and lipogenic enzyme activities. *Aquacult. Nutr.*, **10**, 175-192.

Trenzado, C.E., Morales, A.E., Palma, J.M. & De la Higuera, M. (2009) Blood antioxidant defenses and hematological adjustments in crowded/uncrowded rainbow trout (*Oncorhynchus mykiss*) fed on diets with different levels of antioxidant vitamins and HUFA. *Comp. Biochem. Physiol.*, **149C** , 440-447.

Van Anholt, R.D., Spanings, F.A.T., Koven, W.M., Nixon, O., & Wendelaar, S.E. (2004) Arachidonic acid reduces the stress response of gilthead seabream *Sparus aurata* L. *J. Exp. Biol.*, **207**, 3419–3430.

Van Anholt, R.D., Spanings, F.A.T., Nixon, O., Wendelaar Bonga S.E. & Koven, W.M. (2012) The effects of arachidonic acid on the endocrine and osmoregulatory response of tilapia (*Oreochromis mossambicus*) acclimated to seawater and subjected to confinement stress. *Fish Physiol. Biochem.*, **38**, 703-713.

Wang, Z., Mai, K., Liufu, Z., Ma, H., Xu, W., Ai, Q., Zhang, W., Tan, B. & Wang, X. (2006) Effect of high dietary intakes of vitamin E and n-3 HUFA on immune responses and resistance to *Edwarsiella tarda* challenge in Japanese flounder (*Paralichthys olivaceus*, Temminck and Schlegel). *Aquacult. Res.*, **37**, 681-692.

663 Yin, G., Jeney, G., Racz, T., Pao, X. & Jeney, Z. (2006) Effect of two Chinese herbs
664 (*Astragalus radix* and *Scutellaria radix*) on non-specific immune response of tilapia,
665 *Oreochromis niloticus*. *Aquaculture*, **253**, 39-47.
666
667 Xu, H., Ai, Q. H., Mai, K., Xu, W., Wang, J., Hongming, M., Zhang, W., Wang, X. &
668 Liufu, Z. (2010) Effects of dietary arachidonic acid on growth performance, survival,
669 immune response and tissue fatty acid composition of juvenile Japanese seabass,
670 *Lateolabrax japonicus*. *Aquaculture,* **307**, 75-82.
671
672 Xu, H., Wang, J., Mai, K., Xu, W., Zhang, W., Zhang, Y. & Ai, Q. (2014) Dietary
673 docosahexaenoic acid to eicosapentaenoic acid (DHA/EPA) ratio influenced growth
674 performance, immune response, stress resistance and tissue fatty acid composition of
675 juvenile Japanese seabass, *Lateolabrax japonicus* (Cuvier). *Aquacult. Res., DOI:*
676 *10.1111/are.1232*
677
678 Zheng, X., Torstensen, B.E., Tocher, D.R., Dick, J.R., Henderson, R.J. & Bell, J.G.
679 (2005) Environmental and dietary influence on highly unsaturated fatty acid
680 biosynthesis and expression of fatty acyl desaturase and elongase genes in liver of
681 Atlantic salmon (*Salmo salar*). *Biochim. Biophys. Acta*, **1734**, 13-24.
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685 **Figure legends**

686 Figure 1. Effect of dietary ARA/Vitamin E content on the cumulative mortality of
687 Atlantic salmon (*Salmo salar*) juveniles after challenge test with *Piscirickettsia*
688 *salmonis* (SRS) at a dose of 0.2 mL SRS $fish^{-1}$ at a 10^{-2} dilution.

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693 Table 1. Formulation of experimental diets

Ingredients (g kg^{-1} diet)	**ARA1/E1**	**ARA1/E2**	**ARA2/E1**	**ARA2/E2**	**ARA3/E1**	**ARA3/E2**
Fish meal[a]	619	618	626	625	621	620
Feather meal[b]	50	50	50	50	50	50
Corn Gluten[c]	40	40	40	40	40	40
Wheat meal[d]	140	140	140	140	140	140
Astaxantin[e]	1	1	1	1	1	1
Vitamin premix[f]	2	2	2	2	2	2
Mineral premix[g]	5	5	5	5	5	5
Olive oil [h]	50	50	50	50	40	40
Vevodar ® ARA oil [i]	-	-	-	-	15	15
Yardquim oil[j]	-	-	21	21	21	21
ROPUFA® *n-3* INF oil[k]	83	83	65	65	65	65
ROPUFA® *n-3* EPA oil[l]	10	10	-	-	-	-
Vitamin E (α-tocopherol)[m]	0.015	0.65	0.015	0.65	0.015	0.65

694 [a]Supplied by Alimentos Marinos (ALIMAR) S.A. Jack mackerel meal, Super Prime
695 (Protein 68%, Lipids 9.9%, Ashes 14.5%).
696 [b]Supplied by Härting S.A., Santiago, Chile.
697 [c]Supplied by BIOMAR Chile S.A., Puerto Montt, Chile.
698 [d]Supplied by Molinos Gorbea S.A., Gorbea, Chile.
699 [e]Supplied by DSM Nutritional Product Chile S.A, Puerto Varas, Chile.
700 [f]Supplied by DSM Nutritional Product Chile S.A. (IU/kg or g/kg of premix): Vitamin A
701 1.0 MIU; Vitamin D3, 0.5 MIU; Vitamin E, 0.04 MIU; Vitamin K 3. 4 g; Vitamin B1,
702 4 g; Vitamin B2, 6 g; Vitamin B5, 10 g; Vitamin B6, 2 g; Vitamin B9, 1.6 g; Vitamin
703 B12, 0.00 4g; Niacin, 40 g; Biotin, 0.1 g; Vitamin C 100 g; Choline, 200 g; Inositol 50
704 g.
705 [g]Supplied by BIOMAR Chile S.A (per g mixture: mg; Cu: 8.3 mg; Mn: 67 mg; Co: 1.7
706 mg; y: 1.7; Zn: 200 mg)
707 [h]Carbonell, Spain (SAFA: 17.00 MUFA: 73.16; PUFA n-3: 0.00; PUFA n-6: 8.65).
708 [i]Supplied by DSM Food Specialities (SAFA: 34.16; MUFA : 11.89; n-3 PUFA: 2.62;
709 n-6; 51.33)
710 [j]Supplied by Yardas División Química LTDA (SAFA: 6.30; MUFA: 25.43; PUFA n-3:
711 55.68; PUFA n-6: 12.59).
712 [k]Supplied by DSM Food Specialties (SAFA: 40.71; MUFA: 32.60; n-3 PUFA : 22.98;
713 n-6 PUFA: 5.60).
714 [l]Supplied by DSM Nutritional Product Chile S.A. (SAFA: 38.80; MUFA: 32.60; n-3
715 PUFA: 22.98; n-6 PUFA :5.60).
716 [m]Supplied by DSM Nutritional Product Chile S.A. Rovimix E-50 (1g is equivalent to
717 500 mg of α-tocopherol acetate)
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Table 2. Chemical (g kg^{-1}) and fatty acids (% of total fatty acid identified) composition of the experimental diets.

	ARA1/E1	**ARA1/E2**	**ARA2/E1**	**ARA2/E2**	**ARA3/E1**	**ARA3/E2**
Proximal composition						
Protein (N × 6.25)	553	553	558	556	556	558
Nitrogen free extract	120	117	118	122	121	114
Ash	102	102	102	102	102	102
Lipid	219	220	215	213	216	220
α-tocopherol acetate	**0.12**	**0.76**	**0.19**	**0.73**	**0.19**	**0.72**
Fatty acid composition						
C14:0	3.91	3.57	3.02	3.10	3.16	3.05
C15:0	0.58	0.43	0.45	0.46	0.46	0.45
C16:0	21.81	21.24	18.16	18.42	18.95	18.45
C17:0	0.97	0.90	0.83	0.82	0.85	0.82
C18:0	4.29	4.01	4.11	4.14	4.46	4.46
C20:0	0.27	0.26	0.33	0.32	0.33	0.34
C21:0	0.91	0.71	0.63	0.56	0.87	0.75
C22:0	0.08	0.08	0.13	0.13	0.33	0.33
C24:0	0.29	0.27	0.26	0.27	0.30	0.31
Total SAFA	33.11	31.46	27.91	28.23	29.71	28.96
C16:1 *n-7*	4.80	4.39	3.88	3.98	3.92	3.79
C18:1 *n-9*	22.10	20.66	21.63	21.83	19.94	19.31
C20:1 *n-9*	0.83	0.76	3.17	3.11	2.58	3.06
C22:1 *n-9*	0.13	0.13	0.86	0.73	0.61	0.91
C24:1 *n-9*	0.59	0.52	1.01	1.00	0.83	0.98
Total MUFA	28.45	26.47	30.55	30.65	27.89	28.06
C18:2 *n-6*	6.80	6.36	6.09	6.15	6.36	6.19
C18:3 *n-3*	0.68	0.63	0.63	0.64	0.60	0.60
C18:3 *n-6*	0.15	0.14	0.10	0.14	0.35	0.34
C20:4 *n-6*	1.05	1.06	1.77	1.73	3.73	3.75
C20:5 *n-3*	10.16	10.43	12.21	12.55	11.44	12.21
C22:2 *n-6*	0.09	0.10	0.05	0.04	0.04	0.04
C22:6 *n-3*	16.23	16.04	19.11	19.40	17.89	18.87
Total PUFA	35.17	34.77	39.96	40.66	40.42	42.00
EPA/ARA	9.64	9.86	6.91	7.27	3.06	3.26

SAFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; EPA, eicosapentaenoic acid; ARA, arachidonic acid.

Table 3. Growth, feed, utilization and survival in Atlantic salmon (*Salmo salar* L.*)* fed the experimental diets

Parameters	**ARA1/ E1**	**ARA1/ E2**	**ARA2/ E1**	**ARA2/ E2**	**ARA3/ E1**	**ARA3/ E2**	***ARA***	***Vit E***	***ARAxVitE***
Final weight (g)	23.3 ± 0.9	22.8 ± 0.4	23.9 ± 1.4	23.8 ± 1.9	21.8 ± 0.6	22.4 ± 1.3	ns	ns	ns
Feed consumption (%BW day-^{1})[b]	1.33 ± 0.0	1.29 ± 0.0	1.30 ± 0.1	1.29 ± 0.1	1.38 ± 0.0	1.28 ± 0.0	ns	ns	ns
FCR[c]	1.07 ± 0.0	1.03 ± 0.1	1.02 ± 0.1	1.01 ± 0.1	1.13 ± 0.0	1.03 ± 0.1	ns	ns	ns
SGR (% day-^{1})[d]	1.34 ± 0.0	1.32 ± 0.0	1.37 ± 0.1	1.36 ± 0.1	1.27 ± 0.0	1.30 ± 0.1	ns	ns	ns
TGC (% day-^{1})[e]	1.03 ± 0.0	1.01 ± 0.0	1.06 ± 0.1	1.05 ± 0.1	0.97 ± 0.0	0.99 ± 0.1	ns	ns	ns
K[f]	1.10 ± 0.0	1.09 ± 0.1	1.12 ± 0.1	1.03 ± 0.0	1.09 ± 0.1	1.09 ± 0.1	ns	ns	ns
HSI[g]	1.04 ± 0.1	0.98 ± 0.1	0.95 ± 0.0	1.06 ± 0.2	1.02 ± 0.0	1.11 ± 0.1	ns	ns	ns
PER[h]	1.70 ± 0.0	1.76 ± 0.0	1.76 ± 0.2	1.78 ± 0.1	1.59 ± 0.0	1.74 ± 0.1	ns	ns	ns
Survival (%)	98.4 ± 2.7	98.8 ± 1.2	98.8 ± 1.2	99.2 ± 0.7	98.4 ± 1.8	99.6 ± 0.7	ns	ns	ns

Values are expressed as mean ±SD (n=3), ns : no significant effect (P> 0,05)

[a]Weight gain(%): [(final weight – initial weight) / initial weight]×100.

[b]Feed consumption (%Body weight/day): 100 × [(consumed feed / final weight/2 + initial weight/ 2) /days].

[c]FCR (feed convertion ratio): weight gain (g) / total feed consumed (g).

[d]SGR (Specific growth rate): [(Ln. final weight - Ln. initial weight) / days]×100.

[e]TGC (Thermal growth coefficient): [(final weight$^{1/3}$ – initial weight$^{1/3}$) × 1000] / ∑ (temperature × days).

[f]K (Condition factor): (final weight / final length ^{3}) × 100

[g]HSI (hepatosomatic index): (liver weight(g) / final weight) × 100

[h]PER (protein efficiency ratio): gain in body mass (g) / protein intake (g)

Table 4. Free alpha - tocopherol content (mg kg^{-1} of tissue) and fatty acid profile (% of total fatty acid identified) in liver of *Salmo salar* after being fed with the six experimental diets during 12 weeks.

	Experimental diets						p-value (two-way ANOVA)		
	ARA1/E1	**ARA1/E2**	**ARA2/E1**	**ARA2/E2**	**ARA3/E1**	**ARA3/E2**	**ARA**	**VitE**	**ARA*VitE**
α-tocopherol acetate	5.1 ± 0.1	224.0 ± 109.7[#]	*15.1 ± 8.2*	*209.9 ± 17.4[#]*	*10.7 ± 1.6*	*90.2 ± 9.5[#]*	*ns*	**	*ns*
Fatty acid composition									
C14:0	1.4 ± 0.0	1.4 ± 0.1	1.5 ± 0.0	1.5 ± 0.1	1.4 ± 0.1	1.5 ± 0.0	*	ns	ns
C15:0	0.5 ± 0.0	0.4 ± 0.1	0.4 ±0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	ns	ns	ns
C16:0	22.5 ± 0.5	20.6 ± 2.3	21.4 ± 0.0	21.2 ± 0.2	21.8 ± 0.2	21.5 ± 0.4	ns	ns	ns
C17:0	0.9 ±0.0	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.0	0.9 ± 0.0	0.9 ± 0.0	ns	ns	ns
C18:0	4.7 ± 0.0	4.5 ± 0.6	4.5 ± 0.2	4.9 ± 0.2	4.8 ± 0.1	5.1 ± 0.1	ns	ns	ns
C21:0	0.3 ± 0.0	0.3 ± 0.0	0.3± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	ns	ns	ns
C22:0	0.2 ± 0.0	0.2± 0.0	0.2± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3± 0.1	ns	ns	ns
Total SAFA	30.5 ± 0.69	28.2 ± 2.9	29.2 ± 0.2	29.3 ± 0.3	29.8 ± 0.2	30.1 ± 0.3	ns	ns	ns
C16:1 *n-7*	1.6 ± 0.0	1.8± 0.1	1.7 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	1.7 ± 0.1	ns	ns	ns
C18:1 *n-9*	11.3 ± 0.4[b]	12.5 ± 0.3[ab]	12.7 ± 0.7[a]	12.8 ± 0.8[a]	11.7 ± 0.1[b]	11.0 ± 0.8[b]	*	ns	ns
C20:1 *n-9*	0.5 ± 0.1	0.6 ± 0.1	0.7 ± 0.0	0.6 ± 0.1	0.6 ± 0.0	0.6 ± 0.1	ns	ns	ns
C24:1 *n-9*	0.6 ± 0.0	0.9 ± 0.3	0.9 ± 0.0	0.9 ± 0.2	0.7 ± 0.2	0.8 ± 0.1	ns	ns	ns
Total MUFA	14.0 ± 0.6[b]	15.8 ± 0.7[ab]	16.0 ± 0.8[a]	16.0 ± 1.0[a]	14.7 ± 0.0[b]	14.9 ± 1.0[b]	*	ns	ns
C18:2 *n-6*	2.6 ± 0.0	2.6 ± 0.0	2.6 ± 0.1	2.7 ± 0.1	2.8 ± 0.0	2.6 ± 0.0	ns	ns	ns
C18:3n-3	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ±0.0	ns	ns	ns
C20:2	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	ns	ns	ns
C20:4 *n-6*	3.3 ± 0.2[a]	3.3 ± 0.1[a]	2.9 ± 0.21[a]	3.3 ± 0.2[a]	6.2 ± 0.2[b]	5.7 ± 0.6[b]	**	ns	ns
C20:5 *n-3*	5.8 ± 0.1[a]	5.9 ± 0.1[a]	6.2 ± 0.1[a]	6.1 ± 0.1[a]	5.1 ± 0.1[b]	5.6 ± 0.6[ab]	*	ns	ns
C22:6 *n-3*	42.8 ± 0.7[a]	43.1 ± 2.7[a]	41.6 ± 1.3[b]	41.2 ± 0.8[b]	40.6 ± 0.6[b]	39.8 ± 1.0[b]	*	ns	ns
Total PUFA	55.0 ± 0.7	55.4 ± 2.8	54.0 ± 1.3	54.0 ± 0.6	55.2 ± 0.1	54.5 ± 1.0	ns	ns	ns
EPA/ARA	1.7 ± 0.1[b]	1.8 ± 0.0[b]	2.2 ± 0.2[a]	1.9 ± 0.1[ab]	0.8 ± 0.0[c]	1.0 ± 0.2[c]	*	ns	*

Values are expressed as mean ± SD (n= 3 replicates). ARA1/E1, low ARA/low vitamin E; ARA1/E2, low ARA/high vitamin E; ARA2/E1, medium ARA/low vitamin ; ARA2/E2, medium ARA/high vitamin E; ARA3/E1, high ARA/low Vitamin E; ARA3/E2, high ARA/high vitamin E. [#]Significantly different from liver of fish fed low dietary vitamin E based on a student t-test. Different superscript letters within an individual row denote significant statistical differences in fatty acid content according to Duncan's post-hoc test. Asterisks indicate significant differences as *P<0.05; **P<0.001; n.s. indicates non-significant differences. SAFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; EPA, eicosapentaenoic acid; ARA, arachidonic acid.

Table 5. Effects of the dietary ARA and vitamin E levels as well as their interaction on the studied immune parameters.

	ARA1/E1	**ARA1/E2**	**ARA2/E1**	**ARA2/E2**	**ARA3/E1**	**ARA3/E2**	***ARA***	***Vit E***	***ARA*VitE***
Respiratory burst	0.18±0.01[a]	0.18±0.01[a]	0.23±0.02[b]	0.23±0.02[b]	0.24±0.01[b]	0.24±0.02[b]	**	ns	ns
Phagocytic activity	19.7±4.5	22.7±5.2	18.6±4.5	16.1±4.9	17.5±4.7	24.0±6.5	ns	ns	ns
Lysozyme activity	0.07±0.03[a]	0.06±0.01[a]	0.12±0.03[b]	0.06±0.03[a]	0.06±0.02[a]	0.09±0.01[a]	ns	ns	**

Asterisks indicate significant differences as **P<0.001; n.s. indicates non-significant differences

Figure 1. Effect of dietary ARA/Vitamin E content on the cumulative mortality of Atlantic salmon (*Salmo salar*) juveniles after challenge test with *Piscirickettsia salmonis* (SRS) at a dose of 0.2 mL SRS per fish at a 10^{-2} dilution.