This is the peer reviewed version of the following article: Dantagnan, P., Gonzalez, K., Hevia, M., Betancor, M.B., Hernández, A.J., Borquez, A. and Montero, D. (2017), Effect of the arachidonic acid/vitamin E interaction on the immune response of juvenile Atlantic salmon (Salmo salar) challenged against Piscirickettsia salmonis. *Aquacult Nutr*, 23: 710–720, which has been published in final form at https://doi.org/10.1111/anu.12438. This article may be used for non-commercial purposes in accordance With Wiley Terms and Conditions for self-archiving.

1 EFFECT OF THE ARACHIDONIC ACID/VITAMIN E INTERACTION ON THE

2 IMMUNE RESPONSE OF JUVENILE ATLANTIC SALMON (*Salmo salar*)

3 CHALLENGED AGAINST *Piscirickettsia salmonis*

4

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24

25   **Abstract**

26

27   Atlantic salmon (*Salmo salar*) were fed 6 experimental diets containing three levels of

28   arachidonic acid (ARA) (0.18, 0.28 and 0.63 % for low, medium and high levels

29   respectively) and two levels of vitamin E (150 and 730 mg kg^{-1} for low and high levels

30   respectively). At the end of the experimental period, fatty acids in the liver and

31   immunity markers (lysozyme activity, respiratory burst and phagocytic activity) were

32   determined and fish subjected to a challenge test against the salmonid ricketsial

33   syndrome (SRS) pathogen. ARA, vitamin E or their interaction did not exert an effect

34   on fish performance, whereas ARA alone clearly increased the deposition of ARA.

35   Dietary vitamin E only enhanced liver vitamin E deposition, while the interaction of

36   ARA and vitamin E influenced lysozyme activity and EPA/ARA ratio pointing out the

37   effect of both nutrients on the fish immune system and metabolism. Only the medium

38   concentration contributed to reducing mortality when the fish were exposed to the SRS

39   pathogen. In conclusion, different levels of supplementation with ARA and vitamin E in

40   the diet had no effect on productivity, but did have effects on immune markers and

41   cumulative mortality when fish were exposed to the SRS pathogen.

42

43

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

68 ARA, produce pro-inflammatory effects derived from higher levels of PGE2, LT4 and

69 LX, whereas diets high in n-3 PUFAs, particularly EPA, produce anti-inflammatory

70 effects derived from PGE3 and LT5 (Lall 2000). Although overproduction of PGE2 due

71 to high dietary ARA can also have an immunosuppressant effect (Bell *et al*. 1996), this

72 effect can be mitigated depending on the concentrations of EPA and DHGLA given that

73 both fatty acids have an inhibitory effect on ARA. Because ARA is generally the

74 preferred substrate of lipoxygenases for LT synthesis (Bell *et al*. 1994) and products

75 derived from these enzymes produce stronger immune stimulatory effects than those

76 derived from cyclooxygenases (Hwang 1989), one would expect that incorporating

77 ARA into the diet would contribute to an improved immune response in fish and

78 therefore increased resistance against attack from pathogens. Thus, n-6 fatty acids play

79 an important role in inflammatory processes, stimulating the production of pro-

80 inflammatory cytokines such as the tumour necrosis factor-alpha (TNF-α) and

81 interleukins (IL) 1 and 6 and consequently leading to pro-inflammatory events such as

82 vasodilation and increased vascular permeability (Calder 2002).

83

84 Although anadromous teleost fish such as rainbow trout (*Oncorhynchus mykiss*) and

85 Atlantic salmon (*Salmo salar*) have the ability to elongate and desaturate short-chain

86 fatty acids to long chain PUFA (LC-PUFA) in their fresh water phase and their

87 requirements can be satisfied by fatty acids such as linoleic acid (LA; 18:2n-6) and α-

88 linolenic acid (LNA; 18:3n-3) (Bell & Sargent 2003), it is not clear whether these fatty

89 acids are sufficient for maintaining fish in adequate physiological condition. Thus,

90 supplementation with LC-PUFA, such as ARA, EPA and docosahexaenoic acid (DHA;

91 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.

115   Furthermore, given that ARA is a minor component of fish cell membranes and that is

116   required in small amounts compared to DHA and EPA, its importance has been

117   underestimated (Bell *et al*. 2003; Xu *et al*. 2010). However, the limited studies on ARA

118   have suggested an important contribution of this fatty acid fish growth, survival and

119   bone formation as well as its influence on modulating the immune response and disease

120   resistance in several teleost fish species (Khozing – Goldberg *et al*. 2006; Van Anholt *et*

121   *al*. 2004, 2012; Xu *et al*. 2010; Li *et al*. 2012; Boglino *et al*. 2014; Tian *et al*. 2014).

122   Nevertheless, there is not enough evidence linking dietary ARA and vitamin E levels in

123   freshwater fish species. Therefore, it is expected that appropriately balanced

124   incorporation of this fatty acid with vitamin E could improve processes in which ARA

125   is involved such as a favourable immune response.

126   Salmonid rickettsial syndrome (SRS), caused by *Piscirickettsia salmonis*, is considered

127   to be the most important sanitary problem in the Chilean salmon farming industry, with

128   economic losses up to \$100 million per year. Thus, the impact of SRS on salmon

129   farming in Chile could be mitigated by strengthening the immune activity in the fish

130   through an appropriate balance between dietary ARA and vitamin E. In the present

131   study, we aimed to elucidate the interaction between ARA and vitamin E on juvenile

132   Atlantic salmon immune response and their protective effect when fish were challenged

133   with the causative agent of SRS.

134

135   **Materials and methods**

136

137   *2.1. Experimental fish*

138

139   One thousand five hundred and thirty juvenile Atlantic salmon were obtained from a

140   local fish farm (Pesquera los Fiordos; commune of Curarrehue, region IX, Chile) and

141   transferred to the Quimey-Co de Sociedad Nalcahue Ltda. fish farm (commune of

142   Pucón, Caburga sector, region IX, Chile). The fish were acclimatised for two weeks and

143   fed a commercial diet (BIOMAR S.A., Chile). Prior to the start of the experimental

144   trial, the fish were distributed among eighteen 100 L tanks at a ratio of 85 fish tank^{-1}

145   with an average weight of 6.5 ± 1.1 g to reach an initial density of 5.5 kg m^{-3}. Tanks

146   had a continuous water supply at a rate of 1.5 L h^{-1} and fish were maintained under a 24

147   h light photoperiod, being the water the temperature in tanks 9.9 ± 0.2 ºC and dissolved

148   oxygen concentrations above 10 mg L^{-1}.

149

150   *2.2 Experimental diets and feeding*

151

152   Six experimental feeds were formulated and manufactured to contain three dietary

153   levels of ARA (1.05, 1.73 and 3.73 % for low, medium and high respectively) and two

154   levels of vitamin E as α-tocopherol acetate (0.12 - 0.19 and 0.72 - 0.76 g kg^{-1}, for low

155   and high respectively). Feeds were isolipidic and isoproteic, and levels of other fatty

156   acids held constant. A set of 5 different oils was employed to obtain the desired levels

157   of ARA altering minimally the levels of other PUFA. In this sense, an ARA-enriched

158   oil was employed in ARA3 diets, whereas a n-6-rich oil (Yardquim oil) was used in

159   ARA2 and ARA3 feeds. All the feeds contained n-3-rich oil (ROPUFA® n-3 INF oil) to

160   help maintain adequate levels on n-3 PUFA as well as olive oil to equalize the fat

161   content of all the feeds. Finally, the diets containing the lowest ARA levels were

162   supplemented with an oil rich in EPA (ROPUFA® n-3 EPA oil) to maintain similar

163   EPA levels to the other feeds. The diets were labelled as ARA1/E1 (low ARA/low

164   vitamin E), ARA1/E2 (low ARA/high vitamin E), ARA2/E1 (medium ARA/low

165   vitamin E), ARA2/E2 (medium ARA/high vitamin E), ARA3/E1 (high ARA/low

166   Vitamin E) and ARA3/E2 (high ARA/high vitamin E). The diets were formulated and

167   manufactured in the feed pilot plant of the School of Aquaculture of the Catholic

168   University of Temuco (Chile) using a CLEXTRAL experimental extruder, model B21

169   (Firminy Cedex, France), and oiled by a Dinissen vacuum oiler (Pegasus Menger, 400 l,

170   Sevenum, Holland). Each diet was tested in triplicate for 12 weeks. Fish were fed to

171   apparent visual satiety, ten times per day by hand, and uneaten feed was removed by

172   siphoning, dried and weighed to calculate fish feed intake. VEVODAR® (DSM

173   nutritional products S.A., Puerto Varas, Chile) was used as the source of ARA and

174   Rovimix E-50® for vitamin E (DSM nutritional products S.A., Puerto Varas, Chile).

175   The formulation, proximal composition, fatty acid profile and vitamin E concentration

176   of the diets were analysed in the fish nutrition laboratory of the School of Aquaculture

177   at the Catholic University of Temuco and are shown in Tables 1 and 2.

178

179   *2.3. Productive indicators*

180   The following productive variables were evaluated:

181   •   Increase in weight (%) = [(final weight − initial weight) / initial weight] × 100.

182   •   Feed conversion factor = increase in weight (g)/total food intake (g).

183   •   Specific growth rate (% day^{-1}) = [(ln final weight - ln initial weight) / days] ×

184   100.

185   •   TGC (thermal growth coefficient) = [(final weight$^{1/3}$ − initial weight$^{1/3}$) × 1000] /

186          $\sum$ (temperature × days)

187   •   Condition index = (final weight / final length^3) × 100

188   •   Hepatosomatic index = (liver weight (g) / final weight) × 100

189   •   Protein efficiency ratio = gain in body mass (g) / protein intake (g)

190   •   Percent survival (%) = (Nº final specimens/initial number of specimens) × 100

191

192 *2.4. Analysis and measurements*

193 2.4.1. Collection of blood and tissue samples for analysis.

194 At the end of the experimental period, fish were starved for 24 hours prior to the final

195 sampling. Six fish per tank (n =18 per treatment) were anesthetized with 20%

196 benzocaine (BZ-20; Veterquimica S.A., Santiago, Chile) and bled by puncturing the

197 caudal vein with 1 mL syringes. Blood samples were immediately placed in Eppendorf

198 tubes containing 5 µL of heparin to prevent blood clotting. The tubes containing blood

199 were centrifuged at 3500 rpm for 10 minutes at a constant temperature of 4 °C to

200 separate the plasma from the cells. Fresh plasma was frozen at -80 °C until lysozyme

201 activity was determined.

202 Then, fish were sacrificed in 10 L containers with an overdose of benzocaine together

203 with ice to maintain a temperature of 4 ℃ in order to obtain fresh samples. Liver

204 samples were collected for determination of fatty acids (6 fish tank^{-1}, n = 18 per

205 treatment), and samples stored at -80 ℃ until further analysis. Kidney samples were

206 also obtained for immune parameters analysis and quickly frozen at -80 °C until further

207 analysis.

208

209 *2.4.2 Total lipids and fatty acid analysis.*

210   Total lipids were extracted using a chloroform:methanol mixture (2:1) according to the

211   method by Folch *et al*. (1957). Fatty acids were methylated according to the method

212   proposed by Morrisony Smith (1964). Fatty acids were separated using a Hewlett

213   Packard 5890 series II Plus (Wilmington NC, USA) gas chromatograph with a capillary

214   column of 30*0.25*0.20 mm (SPTM 2380, SUPELCO, Bellafonte, PA USA). Helium

215   gas was used as a carrier. Fatty acids were identified by comparison to a SUPELCO 37

216   fatty acid standard (Sigma Aldrich, St. Louis, MO, USA). Fatty acids are expressed as

217   percentage of the total fatty acids identified.

218

219   *Vitamin E analysis*

220   The free α-tocopherol level of the diets and liver tissues were determined at the Quality

221   Control Laboratory, DSM Nutritional Products S.A. (Puerto Varas, Los Lagos Region,

222   Chile), according to the company analytical methods and procedures for vitamins and

223   carotenoids. Analysis equipment included an HPLC Perkin Elmer series 200 (Shelton,

224   CT, USA) with auto sampler, a UV-visible detector, a Link interface 600 series data

225   system recollection "Totalchrom Workstation" version 6.2.1., and a column Lichrosorb

226   Si 60.5 μm * 25 * 4 mm Alltech brand- Grom (Germany). Briefly, the HPLC conditions

227   used for the vitamin assessment were as follows: reflux extraction with potassium

228   hydroxide solution in methanol and continuation with a hexane:toluene (1:1) solution,

229   room temperature conditions, and a mobile phase with a hexane:dioxane (97:3) solution

230   injected (20 μL) through an automatic nozzle. The results are expressed as mg kg^{-1} of

231   free α-tocopherol.

232

233   *2.4.3. Immune parameters*

234

235 • *Extraction of leucocytes to determine phagocytosis and respiratory burst*

236

237 Leucocytes from the anterior portion of the kidney were used to determine phagocytic

238 activity and respiratory burst following the method proposed by Sakai *et al*. (1996).

239 Leucocytes were extracted from the same specimens used for blood sampling. Kidneys

240 were aseptically removed and placed in L-15 medium (Sigma, St. Louis, MO, USA)

241 supplemented with 10% foetal bovine serum, 0.2% heparin (Sigma, St. Louis, MO,

242 USA) and 0.1% penicillin/streptomycin (Sigma, St. Louis, MO, USA). Maceration was

243 conducted and the resulting cellular suspension was layered onto a 34/51% Percoll

244 gradient (Pharmacia, Uppsala, Sweden). After centrifugation, the interphase cells were

245 isolated, washed in Hanks medium and number adjusted to 10^7 cells mL^{-1}. Viable

246 phagocytic cells were quantified by trypan blue exclusion (viability > 95%).

247

248 • *Leucocytes respiratory burst activity*

249

250 To measure intracellular superoxide anion production, the method developed by Yin *et*

251 *al*. (2006) was used. The leucocyte suspension was distributed into 96-well plates and

252 the reduction of nitroblue tetrazolium (NBT) was evaluated. Untreated leucocytes were

253 used as a negative control and leucocytes treated with 0.5 mg mL^{-1} phorbol myristate

254 acetate (PMA) were used as a positive control. The cell culture was maintained at 18º C

255 for 18 h. The extract, PMA treatments and cell monolayer were washed twice with

256 Hank's saline solution (HBSS, Sigma). Then, 100 µL of NBT solution (1 mg mL^{-1} in

257 RPMI 1640) were added per well and incubated for 60 min at 18º C. After incubation,

258 the NBT solution was removed and the cells were fixed with methanol for 10 min. The

259 cells were air-dried and the formazan in each well was dissolved in 120 µL of 2 M

260 KOH and 140 µL of dimethyl sulphoxide (Sigma). Optical density was measured with a

261 Multiscan spectrophotometer (Spectra, Count, Pakard USA) at 630 nm.

262

263 • *Phagocytic activity*

264

265 The phagocytic activity of the leucocytes was determined by the direct count method,

266 which involves co-incubating leucocytes isolated from the anterior kidney with yeast

267 cells stained with Congo red in 96-well plates. The mixtures were incubated at 18 °C for

268 3 hours and the percentage of leucocytes with phagocytosed cells was counted and

269 considered a direct measure of the level of immune stimulation of the cells. The counts

270 involved a total of 15 optical fields in triplicate for each specimen tested.

271

272 • *Lysozyme activity*

273 The turbidimetric assay for measuring lysozyme (Parry *et al*. 1965) was performed in a

274 96-well plate. A volume of 200 µL of a suspension of *Micrococcus lisodeikticus* (0.2

275 mg mL^{-1} in 50nM sodium phosphate buffer) and 5, 10, 20 or 40 µL of blood plasma

276 were mixed in different wells, and then absorbance recorded at 520 nm at 1, 3, 6 and 9

277 min with a microplate reader (Elx808BioTex USA). One unit of activity was defined as

278 the amount of sample causing a 0.001 min^{-1} decrease in absorbance.

279

280 *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).

299
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

306   among the treatments at the 95% confidence level. If there were differences, Duncan's

307   test was then used to differentiate the means among the three different ARA levels,

308   revealing those treatments that exhibited significant differences (Sokal & Rohlf 1995).

309   For vitamin E levels, a student t-test was employed to denote significant differences

310   between the two vitamin levels. The arcsine transformation was applied to data

311   expressed as percentages to achieve homoscedasticity. The statistical software MS

312   STATISTICA 12 was used in the statistical analyses.

313

314   **Results**

315

316   *3.1. Growth and dietary utilisation.*

317   Fish growth in terms of final weight did not differ ($P>0.05$) among the different

318   experimental groups or their replicates. All fish groups increased their weight up to

319   three times relative to initial weight, reaching an average value between 21.8 and 23.9g.

320   The specific growth rate (SGR) varied between 1.27 and 1.37% per day. The

321   conversion factor rate (CF) was similar among the experimental diets, ranging from

322   1.01 to 1.13. Hepatosomatic index (HSI) values were within the normal range (0.95 to

323   1.11) without showing significant differences ($P>0.05$). None of the evaluated

324   performance parameters showed significant differences ($P>0.05$) when subjected to

325   statistical analysis (Table 3).

326

327   *3.2 Liver fatty acids and vitamin E contents*

328   Table 4 shows the fatty acid composition and measured vitamin E content in the liver of

329   juvenile Atlantic salmon fed the six dietary treatments. The levels of vitamin E

330   concentrations in the liver were directly proportional to its dietary incorporation, and

331   higher ($P<0.05$) in the high vitamin E group. Fish fed diets containing a high

332   concentration of ARA showed significantly higher ($P<0.05$) accumulation of this fatty

333   acid than fish fed low and medium levels of ARA, together with a reduction in the

334   EPA/ARA ratios (Table 4). Besides, there was no interaction between the two nutrients,

335   ARA concentration did not affect ($P>0.05$) the vitamin E concentration in liver tissue

336   and vitamin E dietary levels did not affect ($P>0.05$) the ARA content in the tissue. The

337   only interaction between both nutrients was found in the EPA/ARA ratios ($P<0.05$;

338   Table 4), whereas ARA levels did affect C14, total monounsaturated, EPA, DHA

339   ($P<0.005$), as well as ARA ($P<0.01$) percentages according to the two-way ANOVA

340   performed.

341

342   *3.3 Fish health: Immune parameters and challenge test.*

343

344   No significant differences ($P>0.05$) or interactions between ARA and vitamin E were

345   found for phagocytic activity in fish fed any of the dietary treatments (Table 5).

346   However, a significant effect ($P<0.05$) of vitamin E was found on lysozyme activity,

347   with the group fed ARA2/E1 displaying significantly ($P<0.05$) higher lysozyme

348   enzymatic activity (Table 5), although no differences were observed with fish fed

349   ARA3/E2. Besides, ARA levels significantly ($P<0.05$) affected leucocyte respiratory

350   burst activity with the lowest ($P<0.05$) activity corresponding to fish fed the lowest

351   ARA level (Table 5). Furthermore, an interaction between ARA and vitamin E levels

352   was observed in lysozyme activity ($P<0.01$; Table 5), whereas individual ARA did exert

353   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).

378

379 EPA, ARA and DHGLA are fatty acids precursors of eicosanoids *via* the actions of

380 lipoxygenases and cyclooxygenases. It is known that eicosanoids derived from ARA,

381 mainly LTB4 and PGE2, are pro-inflammatory, whereas those derived from EPA and

382 DHGLA have an anti-inflammatory action (Hwang 1989). Thus it is expected that

383 altering the EPA/ARA ratio in cell membranes may alter the production of eicosanoids

384 and modulate the inflammatory response (Bell *et al*. 1995). Some studies have shown

385 that ARA is the preferred precursor for the synthesis of eicosanoids in fish, competing

386 with EPA (Bell *et al*. 1994). In the present study, the changes in liver fatty acid

387 composition largely reflected that of the diets, showing variations in the ARA contents,

388 which were increased with increasing ARA dietary levels. Although in the present study

389 only fatty acids from total lipids were determined, a previous study in Atlantic salmon

390 showed that the ARA content of phospholipids fatty acids of several tissues was

391 correlated to the ARA dietary intake (Betancor *et al*. 2014). Thus, taking into account

392 that the percentage of neutral and polar lipids in Atlantic salmon fed fish oil-profile diet

393 is around 30 and 60% respectively (Torstensen *et al*. 2004), a considerable amount of

394 ARA will be available in the Atlantic salmon membranes for its bioconversion to

395 reactive eicosanoids.

396 Furthermore, dietary ARA influenced the deposition of other fatty acids such as oleic

397 acid (18:1n-9), EPA and DHA. It must be noted that liver is considered an important

398 site for LC-PUFA synthesis and lipid metabolism in Atlantic salmon (Monroig et al.

399 2010). Hepatic levels of ARA did not differ between fish fed low and medium ARA

400 levels which could be attributed to enhanced biosynthesis from 18:2n-6. Besides, as the

401 same $\Delta 6$ and $\Delta 5$ –desaturase and fatty acyl elongase enzymes participate in the synthesis

402 of both the n-3 and n-6 LC-PUFA (Zheng et al. 2005), the activation of one pathway

403 could also enhance the final and intermediate products of the other, what could account

404 for the increased EPA and DHA contents found in low ARA-fed fish. Enhanced

405 conversion from 18:2n-6 to 20:3n-6 was greater than for 18:4n-3 to 20:4n-3 in Atlantic

406 salmon fed diets devoid of n-3 LC-PUFA, which resulted in significantly higher

407 concentrations of ARA in liver (Miller et al. 2008).

408 Dietary ARA also elicited an effect in leucocytes respiratory activity, medium and high

409 levels leading to up-regulation of the respiratory burst activity. However ARA did not

410 trigger an effect on any of the other health-related parameters, which could indicate that

411 up-regulation of burst activity alone is not sufficient to enhance bactericidal activity

412 against SRS by Atlantic salmon leucocytes. Similarly, yeast β-glucans, known to have

413 an immune stimulatory effect in fish (Magnadóttir 2006) only elicited macrophages

414 respiratory burst, not eliciting any effect on bactericidal activity (Jørgensen and

415 Robertsen, 1995)

416 Low levels of ARA combined with high or low levels of vitamin E lead to low

417 concentrations of ARA in the liver, low stimulation of macrophages as well as

418 cumulative mortality between 56 and 65% from the SRS pathogen. Thus an increase in

419 vitamin E did not contribute to stimulate the immune response in the presence of low

420 levels of ARA, nor improved the response against the SRS pathogen as indicated by the

421 two-way ANOVA analysis. Blazer & Wolke (1984) found that T and B lymphocytes

422 function in rainbow trout was suppressed under vitamin E deficiency and that this

423 vitamin requirement may increase during immune stimulation. Furthermore, a

424 combination of medium levels of ARA with high or low levels of vitamin E not only

425 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

450 response to be evaluated. Curiously, the EPA/ARA ratio was also affected by the

451 interaction of ARA and vitamin E contents. A relationship between vitamin E and LC-

452 PUFA levels has been described in several fish species (Mourente et al., 2007; Lebold

453 et al., 2011), hypothesizing that tocopherols may influence the biosynthesis of PUFA,

454 especially n-3 PUFA, through alteration of cellular oxidation potential or peroxide tone.

455

456 In summary, inclusion of ARA in Atlantic salmon feeds improved some indicators of

457 non-specific immunity such as respiratory burst, being this effect independent of the

458 vitamin E concentration. ARA inclusion also seemed to elicit an effect on LC-PUFA

459 biosynthesis by enhancing EPA and DHA levels in fish fed low ARA feeds. Medium

460 inclusion of ARA combined with a high concentration of vitamin E was found to reduce

461 cumulative mortality by 65%. An interaction between ARA and vitamin E was observed

462 on lysozyme activity and EPA/ARA ratio probably indicating and effect of vitamin E on

463 LC-PUFA biosynthesis. To conclude, the interactive effect between vitamin E and ARA

464 was shown to be limited, although each nutrient, particularly ARA, proved to exert a

465 marked effect on Atlantic salmon immune response.

466

467 ACKNOWLEDGEMENTS

468

473

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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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719

720 Table 2. Chemical (g kg^{-1}) and fatty acids (% of total fatty acid identified) composition
721 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

722
723 SAFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA,
724 polyunsaturated fatty acids; EPA, eicosapentaenoic acid; ARA, arachidonic acid.
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727 Table 3. Growth, feed, utilization and survival in Atlantic salmon (*Salmo salar* L.*)* fed
728 the experimental diets
729

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

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731 Values are expressed as mean ±SD (n=3), ns : no significant effect (P> 0,05)
732 [a]Weight gain(%): [(final weight − initial weight) / initial weight]×100.
733 [b]Feed consumption (%Body weight/day): $100 \times$ [(consumed feed / final weight/2 +
734 initial weight/ 2) /days].
735 [c]FCR (feed convertion ratio): weight gain (g) / total feed consumed (g).
736 [d]SGR (Specific growth rate): [(Ln. final weight - Ln. initial weight) / days]×100.
737 [e]TGC (Thermal growth coefficient): [(final weight$^{1/3}$ – initial weight$^{1/3}$) $\times$ 1000] / $\sum$
738 (temperature $\times$ days).
739 [f]K (Condition factor): (final weight / final length ^{3}) $\times$ 100
740 [g]HSI (hepatosomatic index): (liver weight(g) / final weight) $\times$ 100
741 [h]PER (protein efficiency ratio): gain in body mass (g) / protein intake (g)
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744 Table 4. Free alpha - tocopherol content (mg kg^{-1} of tissue) and fatty acid profile (% of
745 total fatty acid identified) in liver of *Salmo salar* after being fed with the six
746 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	*

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749 Values are expressed as mean $\pm$ SD (n= 3 replicates). ARA1/E1, low ARA/low vitamin
750 E; ARA1/E2, low ARA/high vitamin E; ARA2/E1, medium ARA/low vitamin ;
751 ARA2/E2, medium ARA/high vitamin E; ARA3/E1, high ARA/low Vitamin E;
752 ARA3/E2, high ARA/high vitamin E. $^{\#}$Significantly different from liver of fish fed low
753 dietary vitamin E based on a student t-test. Different superscript letters within an
754 individual row denote significant statistical differences in fatty acid content according to
755 Duncan's post-hoc test. Asterisks indicate significant differences as *P<0.05;
756 **P<0.001; n.s. indicates non-significant differences. SAFA, saturated fatty acids;
757 MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; EPA,
758 eicosapentaenoic acid; ARA, arachidonic acid.
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761   Table 5. Effects of the dietary ARA and vitamin E levels as well as their interaction on
762   the studied immune parameters.
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|	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	**

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769   Asterisks indicate significant differences as **$P<0.001$; n.s. indicates non-significant
770   differences
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772 Figure 1. Effect of dietary ARA/Vitamin E content on the cumulative mortality of
773 Atlantic salmon (*Salmo salar*) juveniles after challenge test with *Piscirickettsia*
774 *salmonis* (SRS) at a dose of 0.2 mL SRS per fish at a 10^{-2} dilution.
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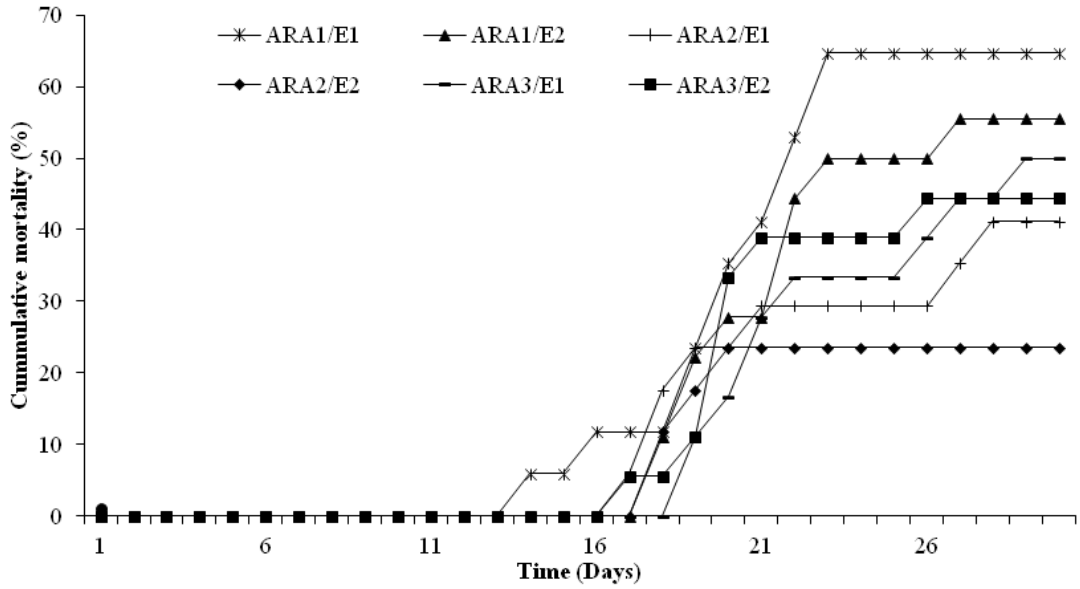
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