View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by IFAPA - Servifapa

1 **Antioxidant capacity and immunomodulatory effects of a chrysolaminarin-enriched**

2 **extract in Senegalese sole**

3 Carlos Carballo[a]; Evangelia G. Chronopoulou[b]; Sofia Letsiou[c]; Claudia Maya[d]; Nikolaos

4 E. Labrou[b]; Carlos Infante[d]; Deborah M. Power[e]; Manuel Manchado[a]*

5

6 [a]*IFAPA Centro El Toruño, Junta de Andalucía, Camino Tiro Pichón s/n, 11500 El Puerto de*
7 *Santa María, Spain*

8 [b]*Laboratory of Enzyme Technology, Department of Biotechnology, School of Food,*
9 *Biotechnology and Development, Agricultural University of Athens, Athens, Greece*

10 [c]*Laboratory of Biochemistry, Scientific Affairs Department, APIVITA S.A., Athens, Greece*

11 [d]*Fitoplanton Marino S.L. 11500 El Puerto de Santa María, Spain*

12 [e]*Comparative Molecular Endocrinology Group, Marine Science Center (CCMAR), Universidade*
13 *do Algarve, 8005-139 Faro, Portugal.*

14

15
16 *Corresponding author:
17 Manuel Manchado. IFAPA Centro *El Toruño*. Camino Tiro de Pichón s/n. 11500 El Puerto de
18 Santa María (Cádiz), Spain. Tel: +34 671532088. Fax: +34 856102033. Email:
19 manuel.manchado@juntadeandalucia.es[1]
20

21

22 **ABSTRACT**

23 The microalgae are an important source of bioactive molecules including β-glucans that can be
24 used as immunostimulants in aquaculture. In the present study, the antioxidant capacity,
25 cytotoxicity and immunomodulatory activity of a chrysolaminarin-enriched extract obtained from
26 the diatom *Phaeodactylum tricornutum* was evaluated. The extract showed a higher total
27 antioxidant activity as determined by ORAC and FRAP assays and a lower DPPH scavenging
28 activity than particulate yeast-β-glucan. The cytotoxicity test indicated that extract concentrations
29 higher than 0.01% w/v could impair cell viability of human dermal fibroblasts. To evaluate the
30 immunomodulatory activity, juvenile soles were intraperitoneally injected with the
31 chrysolaminarin-enriched extract suspended in coconut oil (1 mg/fish) followed by a reinjection
32 at 7 days. A sham group injected with the carrier solution was maintained as a negative control.
33 Cumulated mortality of fish injected with the chrysolaminarin-enriched extract was 29.4% after
34 six days and no mortality was recorded after extract reinjection. Expression analyses of fifteen
35 genes related to the innate immune system in kidney, spleen and intestine showed temporal and
36 organ-specific responses. A rapid (2 days post-injection; dpi) and strong induction of the pro-
37 inflammatory *il1b* and the antimicrobial peptide *hamp1* in the three immunological organs, the
38 *hsp90aa* in kidney and spleen, *irf3* in intestine and *c3* in spleen was observed indicating a potent
39 inflammatory response. The recovery of steady-state levels for all activated genes at 5 dpi, and
40 the down-regulation of c-lectin receptor as well as some interferon-related genes (*ifn1*, *irf1*, *irf3*,
41 *irf8*, *irf9* and *mx*) in kidney and *cxc10* in spleen indicated that the soles were able to activate a
42 homeostatic response against the β-glucan insult. The reinjection of the chrysolaminarin-enriched
43 extract did not activate a new inflammatory response but reduced the mRNA levels of *hsp90aa*
44 and *irf3* indicating that soles developed some resistance to β-glucans. Overall, these results reveal
45 this enriched extract as a novel and potent source of β-glucans with antioxidant and
46 immunomodulatory capacity suitable for immunostimulation in aquaculture.

47

48 **Keywords: Microalgae; glucans; antioxidant capacity; gene expression; Senegalese sole**

49 **1. Introduction**

50 The β-glucans are considered as potent immunosaccharides able to enhance the immune
51 response and prevent disease outbreaks [1, 2]. These bioactive molecules activate the
52 production of pro- and anti-inflammatory cytokines, which in turn modulate humoral and
53 cellular immunity through different pathways conferring antimicrobial, antitumor and
54 anti-proliferative properties [3-5]. Moreover, β-glucans exhibit antioxidant, anti-diabetic,
55 and anti-obesity effects [3, 6] and the broad spectrum of applications in biotechnology
56 and biomedicine fields makes them of high biotechnological and biomedical interest. In
57 aquaculture, β-glucans are mainly used as immunostimulants and prebiotics and are
58 administered by injection or as a dietary supplement able to enhance fish growth, survival
59 and the immune response and also improve resistance to several different pathogens [2,
60 7]. Moreover, recent findings that reported a role of β-glucans on trained immunity by
61 epigenetic reprogramming of monocyte-to-macrophage differentiation [8-10] have even
62 increased the interest on these polysaccharides.

63 The β-glucans are widely distributed in the nature and can be obtained from different
64 sources such as bacteria, algae, fungi and plants. They share a common basic structure
65 and are composed of glucose homopolymers bound together by glycoside bonds. The
66 position and distribution of the glycoside bonds, molecular size and type, solubility and
67 degree of branching, gives each β-glucan a unique structure and specific bioactive
68 properties [2, 11-13]. The antioxidant and anti-proliferative properties of β-glucans from
69 fungi and plants have been extensively studied for food and clinical uses [6, 14-16]. In a
70 similar way, β-(1,3;1,6)-glucans from yeast are the most used in aquaculture as
71 immunostimulants and to a lesser extent β-(1,3;1,6) from seaweeds (laminarin) or β-
72 (1,3;1,4) from cereal grains [2, 7]. However, little information is available about the

73 antioxidative properties and immunomodulatory actions of β-glucans from microalgae,
74 the chrysolaminarin (also known as leucosin).

75 Chrysolaminarin is a soluble β-glucan synthetized for energy storage by several marine
76 microalgae including haptophyte and stramenopiles (such as diatoms and
77 *Nannochloropsis*) [15, 17, 18]. The biosynthesis of this polysaccharide is highly dynamic
78 relying on light and nutrient levels and also shows growth-phase dependency [19-22].
79 Unlike glucans from fungi and plants, chrysolaminarin is low molecular weight (1-40
80 kDa) and contains only 20-30 linear residues with a low degree of branching [15, 18].
81 The interest in recent years in the production of biomass from the diatom *Phaeodactylum*
82 *tricornutum* has rapidly grown due to its biosynthetic capacity and high growth rates and
83 it is regarded as a valuable resource for novel food supplements, cosmeceuticals and
84 several other industrial applications [23, 24]. The chrysolaminarin cell content in this
85 diatom ranges between 4.9-17.1% of the dry mass and its isolation can be combined with
86 the purification of other bioproducts such as pigments and lipids [15, 21, 22, 25].
87 Although several species of microalgae are used as a feed source in fish hatcheries [26],
88 no information about the bioactivity of chrysolaminarin exists in fish.

89 The aquaculture production of the flatfish Senegalese sole is rapidly growing in Europe
90 with large investments in advanced recirculation facilities to transform production into a
91 highly intensive model [27]. However, this development needs to be accompanied by
92 disease control and prevention measures so as to reduce the risks of disease outbreaks and
93 minimize the use of chemicals. Currently the aquaculture of this species is mainly threated
94 by bacterial diseases such as *Vibriosis*, *Photobacterium damselae piscicida* and
95 *Tenacibaculum* spp. [28] and several prevention strategies such as inactivated vaccines
96 [29] or phage therapy [30] are under investigation. The use of immunostimulants such as

97 β-glucans suitable to be incorporated as vaccine adjuvants or feed supplements is another
98 strategy successfully used for several other species [1, 2, 7, 31]. The aims of the present
99 study were: i) characterize the antioxidant properties of a chrysolaminarin-enriched
100 extract from *P. tricornutum*; ii) study its *in vivo* and *in vitro* toxicity; iii) evaluate the
101 transcriptional response after intraperitoneal injection in juveniles. The results obtained
102 demonstrate the antioxidant and immunomodulatory actions of this microalgal extract
103 enriched in this unexplored β-glucan, still relatively little exploited by the aquaculture
104 industry.

105 **2. Materials and methods**

106 *2.1 Microalgae cell culture and β-glucan sources*

107 The *P. tricornutum* strain used in this study was obtained from the Microalgae Culture
108 Collection of Fitoplancton Marino, S.L. (CCFM). Microalgae were initially grown
109 indoors using autoclaved seawater (salinity 33 psu) enriched with filter-sterilized f/2
110 nutrients in 50 mL flasks bubbled filter-sterilized CO_2-enriched air (2%). Microalga
111 cultures were carried out in a temperature-controlled room (22ºC) under continuous
112 illuminating conditions using artificial daylight fluorescent light (150 µmol $\mu Em^{-2}s^{-1}$).
113 Once the cultures reached the early stationary phase they were used to inoculate in 1 L
114 culture flasks. This process of scaling up in volume was repeated until reach 50 L cultures,
115 which were then used to inoculate in the outdoor photobioreactors (PBRs). Light-dark
116 cycles and ambient temperature were influenced by seasonal environmental conditions
117 (10-11 h of light, temperatures ranging from 10-22 ºC) and pH was controlled by CO_2
118 injection when necessary. Cells were harvested by continuous-flow centrifugation 4 h
119 after sunrise, frozen at -20 ºC and freeze-dried for further use.

120 The chrysolaminarin-enriched crude extract was prepared at Fitoplancton Marino using
121 the warm-water extraction method [32] with slight modifications. Freeze-dried

122 microalgae were heated under alkaline pH conditions and the proteins were later
123 precipitated by a pH-shift approach after removing cell debris. The supernatant containing
124 the polysaccharides was precipitated by adding absolute ethanol (1 v:v) for 24h at 4ºC.
125 After collecting the precipitates by centrifugation, they were frozen at -80ºC and
126 preserved by freeze-drying. This chrysolaminarin-enriched crude extract was
127 characterized by the phenol-sulfuric acid method [33] indicating a 47.3% reducing sugar
128 with ~5% of total protein as determined by the Bradford assay. The particulate (1,3)-
129 (1,6)-β-glucan (Yestimun®) from brewers' yeast was purchased from Quimivita
130 (Barcelona, Spain; 42200P-030).

131 *2.2 Total antioxidant capacity assays*

132 The total antioxidant capacity (TAC) of the yeast β-glucan and the chrysolaminarin-
133 enriched extract (1% w/v) were measured using three methods: oxygen radical
134 absorbance capacity (ORAC) assay, ferric reducing antioxidant power (FRAP) and the
135 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay. The ORAC assay
136 was carried out using fluorescein as the probe as described by Gillespie et al. [34]. ORAC
137 values were calculated by subtracting the area under the blank curve from the area under
138 the sample curve (Net AUC) and expressed as Trolox equivalents (µmol Trolox/g). FRAP
139 assays were carried out according to Benzie and Strain [35] and the results were expressed
140 as µg ascorbic acid/mg of β-glucan dry mass. DPPH assays were performed according to
141 Molyneux [36]. DPPH activities were expressed as % free radical scavenging and the IC_{50}
142 values (the amounts of β-glucan (mg) required to scavenge 50% DPPH radicals) were
143 also estimated.

144 *2.3 Human skin cell culture and cytoxicity tests*

145 Primary Normal Human Dermal Fibroblasts (NHDF) isolated from normal human adult
146 skin were purchased from Lonza Clonetics™ (Lonza Walkersville, USA) and cultured as

147 previously described [37]. The cell proliferation test was assessed by using an MTT kit
148 (Vybrant MTT cell proliferation assay kit, Thermo Fisher Scientific) according to the
149 manufacture's protocol. The MTT assay is based on the reduction of the tetrazolium salt,
150 MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) by actively
151 growing cells to produce blue formazan product. NHDF were incubated with different β-
152 glucan concentrations (ranging 0.001-1% w/v in a final volume of 100 μL) for 24h and
153 optical density measured at 570 nm.

154 *2.4 Fish trials*

155 All procedures were authorized by the Bioethics and Animal Welfare Committee of
156 IFAPA and given the registration number 26–11–15-374 by the National authorities for
157 regulation of animal care and experimentation.
158 The chrysolaminarin-enriched extract was intraperitoneally (i.p.) injected using coconut
159 oil (Renuka Agri Organics LTD) as a vehicle for a slow and prolonged release of the
160 polysaccharide. The freeze-dried extract was firstly suspended in PBS, then added the
161 same volume (1:1) of coconut oil and vortexed to generate an emulsion. A control solution
162 mix using the same 1:1 PBS: coconut oil proportion but without extract was also prepared
163 and used for injection to the sham control. The experimental design is depicted in Figure
164 1. A total of 70 specimens of sole (average weight 19.3±3.3g) were supplied by Cupimar
165 S.A. (San Fernando, Cádiz, Spain). The animals were distributed into eight cylindrical
166 tanks (1 m^2 surface) in an open flow-through circuit. The animals were kept for one week
167 before starting the experiment and fed with commercial diets (Skretting; 1% biomass).
168 Before manipulating the animals, they were sedated with phenoxyethanol (100 ppm final
169 concentration). Four tanks (10 specimens each) were given intraperitoneal (i.p) injections
170 of 100 μL of the chrysolaminarin-containing solution (1 mg/fish). This dose of
171 chrysolaminarin by i.p. injection was selected based on a previous study [38]. Moreover,

172 three tanks (10 specimens each) were injected with the control solution (sham control
173 group). Mortality was monitored daily and six animals per treatment (two specimens from
174 each tank remaining the fourth tank in chrysolaminarin-injected fish untouched) were
175 sampled at 2 and 5 days post injection (dpi) and organs collected. Animals were
176 euthanized using an overdose of phenoxyethanol (250 ppm final concentration) and the
177 kidney, spleen and intestine dissected out. Samples for gene expression analysis were
178 fixed in RNA-later (Invitrogen) and stored at -80°C until use. In the trial, a temperature
179 of 22.3ºC±0.6, dissolved oxygen of 6.5±0.5 ppm, and salinity of 40.2±1 psu were
180 recorded. At 7 dpi, 27 surviving specimens were given a second i.p. injection of
181 chrysolaminarin-enriched extract (1 mg/fish, 14 specimens; group Chr_Chr) or the
182 control solution (13 specimens; group Chr_C) and distributed into four tanks. All fish
183 from sham control group were again injected only with the control solution (30
184 specimens; group C_C) and distributed into three tanks. In the trial, a temperature of
185 20.8ºC±0.7, dissolved oxygen of 6.0±0.7 ppm, and salinity of 38.5±0.6 ppt were recorded.
186 Mortality was monitored daily and two animals per tank were sampled at 2 dpi. Fish were
187 euthanized and samples collected as indicated above.

188

189 *2.5 RNA isolation and RT-qPCR analysis*

190 Total RNAs from the intestine (~40-50 mg) and spleen (~15 mg) was isolated using the
191 NucleoSpin RNA isolation Kit (Macherey-Nagel) following the manufacturer's protocol.
192 Samples were homogenized in the Fast-prep FG120 instrument (Bio101) using Lysing
193 Matrix D (Q-Bio-Gene) for 60 s at speed setting 6. In the case of kidney (~10-15 mg),
194 tissue homogenization was carried out as indicated above using 1 ml of TRI-Reagent
195 (Sigma-Aldrich). After adding chloroform (0.2 mL) and centrifugation, the aqueous

196 phase was transferred to columns of the NucleoSpin RNA isolation Kit (Macherey-Nagel)
197 to complete the RNA purification in a similar way to intestine and spleen. Isolated RNA
198 samples were treated twice on-column with DNase with DNase I using a NucleoSpin
199 RNA isolation Kit (Macherey-Nagel) for 30 min. RNA sample quality was checked by
200 agarose gel electrophoresis and quantification was determined spectrophotometrically
201 using the Nanodrop ND-8000 (Thermo Scientific). 1 µg of total RNA was reverse-
202 transcribed using the iScript^{TM} cDNA Synthesis kit (Bio-Rad) according to the
203 manufacturer's protocol.

204 To evaluate the immunomodulatory effects of chrysolaminarin the following gene panel
205 was selected: pro-inflammatory cytokines: interleukin 1b (*il1b*) and tumor necrosis factor
206 alpha (*tnfa*); cellular stress: heat shock protein 90 alpha (*hsp90aa*); antimicrobial peptide:
207 hepcidin (*hamp1*); bacterial defence: g-type lyzozyme (g-*lys*); chemokine: *cxc10*;
208 complement factor (*c3*); antiviral defence: interferon 1 (*ifn1*), interferon-induced Mx
209 protein (*mx*), interferon-related factors (*irf1*, *irf3*, *irf7*, *irf8* and *irf9*); glucan receptor c-
210 type lectin (*clec*). Primers for amplification of *hsp90aa*, *hamp1*, *mx*, *irf1* and *irf3* and the
211 reference genes *ubi* and *actb1* were previously published [39-43]. Primers used for
212 amplification of *il1b*, *tnfa*, *c3*, *clec*, *cxc10*, *ifn1*, *irf7*, *irf8* and *irf9* (Table 1) were designed
213 using the Oligo v7 software using specific sequences from SoleaDB [44]. The c-lectin
214 receptor was identified by blasting the salmon glucan receptor sequences for *sclrc* genes
215 [45].

216 Real-time analysis was carried out on a CFX96^{TM} Real-Time System (Bio-Rad). Real-
217 time reactions were performed in a 10 µL volume containing cDNA generated from 10
218 ng of original RNA template, 300 nM each of specific forward and reverse primers, and
219 5 µL of SYBR Premix Ex Taq (Takara, Clontech). The amplification protocol used was
220 as follows: initial 7 min denaturation and enzyme activation at 95ºC, 40 cycles of 30 s at

221 95ºC, 15 s at 68°C and 30 s at 72°C. Each PCR assay was performed in duplicate. The
222 PCR protocol to amplify the mx gene was described in Fernandez-Trujillo et al. [43].
223 Data were normalized using the geometric mean of ubiquitin (*ubi*) and β-actin (*actb1*)
224 [40] and relative mRNA expression calculated using the comparative Ct method [46].
225 PermutMatrix [47] was used to conduct a cluster analysis with the different experimental
226 conditions analysed using log 10 of fold change with parameters set as following:
227 Dissimilarity: Euclidean distance, Hierarchical: Complete Linkage Method, Seriation:
228 Multiple-fragment heuristic (MF).

229 *2.6 Statistical analysis*

230 All data were checked for normal distribution with the Kolmogorov–Smirnov test as well
231 as for homogeneity of variance with the Levene′s test and when necessary a log
232 transformation was applied. Survival rates were assessed by using one-way ANOVA test.
233 To test the existence of significant differences in gene expression in the injection trial, a
234 two-way ANOVA was performed using treatment and time as fixed factors and in case
235 of significance a Student's t-test was performed. For reinjection trial, a one-way ANOVA
236 followed by an LSD post hoc test was carried out. Differences with $p < 0.05$ were
237 considered to be statistically significant. Analyses were performed using SPSS v21
238 software (IBM Corp., Armonk, NY, USA) and Statistix 9 (Analytical Software,
239 Tallahassee, FL, USA).

240

241 **3. Results**

242 *3.1 Antioxidant activities*

243 The total antioxidant capacity (TAC) as determined by ORAC, FRAP and DPPH is
244 depicted in Figure 2. The chrysolaminarin-enriched extract had 14.2-folds higher TAC as
245 determined by ORAC than particulate yeast β-glucan (29.8 ± 7.3 vs 2.1 ± 0.7 µmol Trolox

246 g^{-1}). In a similar way, chrysolaminarin-enriched extract also showed higher reducing
247 antioxidant power than yeast β-glucan (1.0 ± 0.01 vs 0.2. ± 0.03 μg ascorbic acid/mg at
248 concentration10 mg/ml). In contrast, DPPH radical scavenging activity was higher in the
249 yeast β-glucan (estimated IC_{50} values were 1,896 ± 144 and 353 ± 28 μg/mL for the
250 chrysolaminarin-enriched extract and yeast β-glucan, respectively).

251 *3.2 Cell toxicity and fish survival*

252 The cytotoxic activity of the chrysolaminarin-enriched extract and yeast β-glucan
253 (ranging from 0.001 to 1% w/v final concentration) was assayed in the primary dermal
254 cell cultures NHDF using the MTT assay (Fig. 3A). Cell survival with the microalgal
255 crude extract was slightly higher (100%) than with yeast β-glucan (89.6%) at the lowest
256 concentration (0.001%) and impaired cell viability was observed at 0.01%. Mortality was
257 concentration-dependent in both β-glucans (only 39% survival at the highest
258 concentration 1%).
259 *In vivo* experiments using 0.005% w/w of chrysolaminarin-enriched extract resulted in
260 29.4% cumulated mortality at 6 dpi (Fig. 3B). No mortality was detected in the sham
261 control group (C_C). Moreover, reinjection of the chrysolaminarin-enriched extract or
262 control solution at 7 dpi to the fish previously injected with the extract (Chr_Chr or Chr_C
263 groups) did not provoke any additional mortality (Fig. 3B).

264 *3.3 Expression profiles in immunological organs*

265 To evaluate the immunomodulatory activity of the chrysolaminarin-enriched extract,
266 transcript abundance of genes related to the inflammatory response, cellular stress,
267 carbohydrate binding receptors and defence against bacteria and viruses was quantified
268 in three immunological organs: kidney, spleen and intestine. A cluster analysis clearly
269 separated the expression response observed in chrysolaminarin-injected soles from the
270 sham control at 2 dpi in the tree organs studied (Fig. 4) while it was hardly observable at

271 5 dpi (only in kidney appeared separated both experimental groups). The injection of the
272 microalgal extract rapidly (2 dpi) up-regulated the expression of *il1b*, *hsp90aa* and *hamp1*
273 (5.6-, 12.5- and 4.9-folds, respectively) and down-regulated that of *clec* and *ifn1* in the
274 kidney with respect to the sham control. Later at 5 dpi, the antiviral defence genes *irf1*,
275 *irf3*, *irf8*, *irf9* and *mx* significantly decreased their expression levels ($p < 0.05$) (Fig. 5).
276 The reinjection of the chrysolaminarin-enriched extract at 7 days (Chr_Chr group) only
277 reduced significantly the expression of *hsp90aa* and *irf3* with respect to both groups
278 reinjected with the control solution (C_C and Chr_C groups). No change in gene
279 expression was detected for *il1b*, *tnfa*, *c3*, *hamp1, clec, ifnc, irf7* or *mx* (data not shown).
280 In intestine and spleen a subset of ten genes was selected to evaluate the immune response
281 (indicated in Fig. 4). In intestine, the mRNA levels of *il1b*, *hamp1* and *irf3* were up-
282 regulated 16.6-, 43.7- and 10.5-folds, respectively at 2 dpi and the *c3* mRNA abundance
283 significantly increased 4.4-fold at 5 dpi (Fig. 6). In the spleen, expression of *il1b*,
284 *hsp90aa*, *c3* and *hamp1* increased 12.6-, 5.8, 5.4- and 10.0-folds, respectively at 2 dpi
285 while *cxc10* and *irf3* mRNA levels significantly decreased (3.9- and 3.2-folds,
286 respectively) at 5 dpi (Fig. 6). No change in gene expression was detected for *tnfa*, *clec*,
287 *irf7* and *g-lys* in intestine and spleen and for *hsp90aa* and *cxc10* in intestine (data not
288 shown).
289
290 **4. Discussion**

291 The advances in microalgae biomass production at an industrial scale have brought to the
292 market novel bioproducts that can be feasible exploited as it is the case of
293 chrysolaminarin. The antioxidant, prebiotic and immunostimulant activity of β-(1,3;1,6)-
294 glucans from different sources is well-recognized and hence, these polysaccharides have
295 been proposed as potential functional dietary supplements or as vaccine adjuvants to
296 enhance immune response in aquaculture [2, 9]. However, the antioxidant capacity and

297 immunomodulatory effects of these β-glucans in fish are still unexplored. In the present
298 study the antioxidant capacity, citotoxicity and immune response of a chrysolaminarin-
299 enriched extract from the diatom *P. tricornutum* have been determined using the flatfish
300 Senegalese sole as a model. These new data represent an initial study to develop new
301 applications to functional extracts from microalgal biomass.

302 Previous studies on β-glucans have demonstrated their antioxidant and reactive oxygen
303 species scavenger activity [20, 48-50]. In this study, our chrysolaminarin-enriched extract
304 from *P. tricornutum* also showed antioxidant activity with higher TAC than particulate
305 yeast β-glucan in the ORAC (~14-folds) and FRAP (~4-folds) assays but lower in the
306 DPPH scavenging method. We should note that although chrysolaminarin was a major
307 component in our crude extract, we cannot fully discard a contribution to TAC of other
308 minor cellular components. In fact, Kanmani et al. [51] reported that crude
309 exopolysaccharide extracts had higher reducing power and hydroxyl radical scavenging
310 activity than purified exopolysaccharide as consequence of secondary metabolites in the
311 extract. Anyway, the contrasting results in the three TAC assays between the microalgal
312 extract and yeast β-glucan could be explained by two main factors: a) the distinct
313 structure, composition and solubility of β-glucans [52] and b) the specific
314 thermodynamic, kinetic and chemical characteristics of each TAC assay and the oxidizing
315 power of each TAC reagent against a given antioxidant [53]. The chrysolaminarin has a
316 lower molecular weight (MW; ~10 kDa) and lower degree of polymerization (DP; 28-33)
317 than yeast β-glucan (branch-on-branch, high MW (>200 kDa) and high DP (>100) [11,
318 21, 54]. Such differences in size, branching and conformation play a key role in water-
319 solubility, a limiting factor in the FRAP assay [55] and hydroxyl radical scavenging
320 activity [14]. A previous study in the microalga *Odontella aurita* demonstrated that
321 chrysolaminarin had a stronger antioxidant activity for hydroxyl radical scavenging than

322 for DPPH radicals [20] indicating that structural differences are important for a better
323 neutralization the peroxyl radicals in ORAC and total reducing power in FRAP.
324 Moreover, those molecules with lower antioxidant values in the ORAC relative to the
325 DPPH assay as observed in yeast β-glucan have been proposed to act both as antioxidants
326 and powerful pro-oxidants and this makes chrysolaminarin β-glucan even more
327 interesting for the food and cosmetic industry [56].

328 Some β-glucans have been reported to exhibit anti-proliferative and antitumor activities.
329 For example, laminarin impairs the viability of human lung fibroblasts at concentrations
330 higher than 0.004% with high inhibition achieved at concentrations higher than 0.02%
331 w/v [48]. However, when used at an appropriate dose in *in vitro* cell assays, laminarin
332 has an antioxidant action that protects against reactive oxygen species [48]. Similarly, β-
333 glucans from cereals exert cytotoxic actions against cancerous cells at concentrations
334 lower than 0.02% w/v [57]. The data from the present study indicate that chrysolaminarin-
335 enriched extract at concentrations higher than 0.01% w/v affected cell viability of human
336 dermal fibroblasts as has previously been shown for other β-glucans. More research will
337 be necessary to investigate the specific cancer-protective actions of this microalgal
338 extract. Intriguingly, i.p. injection of this extract to soles induced a 29.4% mortality. This
339 was unexpected since the dose used (1 mg/fish; 0.052mg/g) was close to the range
340 previously used in other fish species (0.1-1mg/fish; 0.01-0.033 mg/g fish) [38, 58-60].
341 The absence of mortality after the second exposure to chrysolaminarin tends to preclude
342 the presence of adulterating toxic substances such as endotoxins. As the main differences
343 between the present and previous studies was the vehicle used for injection (coconut oil
344 instead of phosphate-buffered saline for a slow delivery), we hypothesize that the
345 prolonged release of chrysolaminarin triggered a sustained and potent inflammatory
346 response (see below), that in turn resulted in a non-adaptive stress response, a failure in

347 homeostatic response and mortality. The exact mechanisms by which *in vivo*
348 chrysolaminarin caused mortality will require further studies.

349 The β-glucans from different sources are well-known as enhancers of non-specific
350 immunity in fish through the activation of macrophages and the production of pro-
351 inflammatory cytokines. The i.p. injection of yeast β-glucan triggered the expression of
352 *il1b* in carp head kidney macrophages after 24 and 48 h [38]. Moreover, laminarin i.p.
353 injection activated the expression of *il1b* in salmonids using both *in vitro* (head kidney
354 macrophages) and *in vivo* (in kidney and spleen after 48 h) approaches [60, 61]. The
355 β(1,3) scleroglucan activated expression of *il1b* peritoneal cells of carp at 2 dpi due to
356 inflammatory leucocyte migration [58]. The polysaccharides from the herb *Astragalus*
357 containing β-glucans also increased *il1b* mRNA abundance in the head kidney of common
358 carp (*Cyprinus carpio*). [62]. Our study demonstrates that i.p. injection of the
359 chrysolaminarin-enriched extract in sole elicited a similar response to laminarin in other
360 fish and rapidly activated the expression of the pro-inflammatory cytokine *il1b* (at 2 dpi)
361 in the three organs analysed. This cytokine acts as a mediator of β-glucan actions and
362 triggers a generalized downstream response through the NF-κB and MAPK signalling
363 pathways to produce cytokines and activate the migration and phagocytic activities of
364 macrophages [63]. It should be noted that magnitude of the *il1b* activation was higher in
365 spleen and intestine than in kidney that also supports previous observations that reported
366 the spleen as a major target for β-glucan actions with larger and more intense responses
367 [60, 61, 64]. The lack of response of the pro-inflammatory cytokine *tnfa* could be due to
368 a dose or time-point effect. A previous study indicated that the activation of *tnfa* was
369 delayed with respect to *il1b* after β-glucan treatments in trout head kidney leukocytes [65]
370 and also showed dose-dependent effects as demonstrated by *in vitro* and *in vivo* trials
371 assays with organ-specific response [61, 62, 64, 65].

372 In addition to *il1b*, the microalgal crude extract also stimulated the expression of
373 chaperone *hsp90aa* in kidney and spleen and the antimicrobial peptide *hamp1* in the three
374 tissues although with a high variable response among specimens. The expression of *hsp90*
375 and *il1b* is up-regulated in a mutant zebrafish that lacks an adaptive immune response
376 [66]. The co-expression was associated with the role of Hsp90 in ensuring the correct
377 folding of several kinases and intermediates of the NF-κB signalling pathway and hence
378 modulating NF-κB action and pro-inflammatory cytokine production in macrophages
379 [67-69]. The modification of hepcidin expression in soles injected with the microalgal
380 extract may be related to its stimulation by pro-inflammatory cytokines such as IL-6 [70]
381 and to the inhibitory effect on cytokine production through interfering the NF-κB pathway
382 [71]. Interestingly, a similar activation pattern of pro-inflammatory cytokines (*il1b*, *il6*,
383 and *tnfa*) and *hamp1* was also observed by our group in kidney and intestine of juvenile
384 soles after infection with LCDV [42] indicating that this microalgal extract promotes the
385 up-regulation of genes representative of an acute innate response against a pathogen.

386 In contrast to the fast activation of the inflammatory response at 2 dpi, genes related with
387 the response to virus, *ifn1*, *irf1*, *irf3*, *irf8*, *irf9* and *mx*, and the chemokine *cxc10* were
388 down-regulated in the kidney and/or spleen at 5 dpi and the sugar receptor *clec* in kidney
389 (1.5-fold lower) at 2 dpi. The stabilization of mortality after 5-6 dpi observed in this study
390 also supports the acute response as the primary cause for the expression patterns observed.
391 In this regards, chrysolaminarin-enriched extract reinjection at 7 dpi did not induce further
392 mortality and did not activate a pro-inflammatory response. Instead, a decrease of
393 *hsp90aa* and *irf3* mRNA levels were measured as indicative of some resistance of sole to
394 consequent injections of chrysolaminarin. A previous study in *Labeo rohita* fingerlings
395 identified that only three out four injections of β-glucans and at a moderate concentration
396 specifically maintained enhanced phagocytic cell functions [59]. All these data suggest

397 that the β-glucan exposure rapidly activated an acute inflammatory response but in the
398 absence of a pathogen down-regulation of transcripts to restore the steady-state levels
399 occurred. Clearly it will be relevant in the future to assess how β-glucan supplementation
400 via diet changes the efficacy of the immune response to pathogens

401 **5. Conclusion**

402 This study demonstrates that crude extract from *P. tricornutum* enriched in
403 chrysolaminarin has a potent antioxidant capacity and impairs cell growth at
404 concentrations higher than 0.01%. *In vivo* testing of immunomodulatory actions in sole
405 demonstrated that they induce a fast (2 dpi) and transient activation of the pro-
406 inflammatory *il1b* and stress defence mechanisms in kidney, spleen and intestine that was
407 not clearly observable later at 5 dpi. Overall, the results support this microalgal extract
408 enriched in β-glucans as a novel product with a high potential to be used in aquaculture.
409 Further research will be required to identify all active metabolites in the extracts and to
410 establish optimal doses and delivery routes to enhance immune system and protection
411 against infection diseases.

412 **Conflict of interests**

413 Authors declare there is not conflict of interests.

414 **Acknowledgements**

415 This research has been funded by the project Algae4A&B from the UE H2020 research
416 and innovation programme under the Marie Skłodowska-Curie grant agreement No
417 691102.

418 **References**

419 [1] S.K. Song, B.R. Beck, D. Kim, J. Park, J. Kim, H.D. Kim, E. Ringo, Prebiotics as
420 immunostimulants in aquaculture: a review, Fish Shellfish Immunol 40(1) (2014) 40-8.
421 [2] D.K. Meena, P. Das, S. Kumar, S.C. Mandal, A.K. Prusty, S.K. Singh, M.S. Akhtar, B.K.
422 Behera, K. Kumar, A.K. Pal, S.C. Mukherjee, Beta-glucan: an ideal immunostimulant in
423 aquaculture (a review), Fish Physiol Biochem 39(3) (2013) 431-57.
424 [3] K.M.I. Bashir, J.S. Choi, Clinical and Physiological Perspectives of beta-Glucans: The Past,
425 Present, and Future, Int J Mol Sci 18(9) (2017).
426 [4] H. Stier, V. Ebbeskotte, J. Gruenwald, Immune-modulatory effects of dietary Yeast Beta-
427 1,3/1,6-D-glucan, Nutr J 13 (2014) 38.
428 [5] B. Du, C. Lin, Z. Bian, B. Xu, An insight into anti-inflammatory effects of fungal beta-glucans,
429 Trends Food Sci Technol 41 (2015) 49-59.
430 [6] L. Vannucci, J. Krizan, P. Sima, D. Stakheev, F. Caja, L. Rajsiglova, V. Horak, M. Saieh,
431 Immunostimulatory properties and antitumor activities of glucans (Review), Int J Oncol
432 43(2) (2013) 357-64.
433 [7] E. Ringø, R. Olsen, J. Vecino, S. Wadsworth, S. Song, Use of immunostimulants and
434 nucleotides in aquaculture: a review, J Marine Sci Res Development 2(1) (2012) 104-125.
435 [8] M.G. Netea, L.A. Joosten, E. Latz, K.H. Mills, G. Natoli, H.G. Stunnenberg, L.A. O'Neill,
436 R.J. Xavier, Trained immunity: A program of innate immune memory in health and disease,
437 Science 352(6284) (2016) aaf1098.
438 [9] J. Petit, G.F. Wiegertjes, Long-lived effects of administering beta-glucans: Indications for
439 trained immunity in fish, Dev Comp Immunol 64 (2016) 93-102.
440 [10] S. Saeed, J. Quintin, H.H. Kerstens, N.A. Rao, A. Aghajanirefah, F. Matarese, S.C. Cheng,
441 J. Ratter, K. Berentsen, M.A. van der Ent, N. Sharifi, E.M. Janssen-Megens, M. Ter Huurne,
442 A. Mandoli, T. van Schaik, A. Ng, F. Burden, K. Downes, M. Frontini, V. Kumar, E.J.
443 Giamarellos-Bourboulis, W.H. Ouwehand, J.W. van der Meer, L.A. Joosten, C. Wijmenga,
444 J.H. Martens, R.J. Xavier, C. Logie, M.G. Netea, H.G. Stunnenberg, Epigenetic
445 programming of monocyte-to-macrophage differentiation and trained innate immunity,
446 Science 345(6204) (2014) 1251086.
447 [11] L. Barsanti, V. Passarelli, V. Evangelista, A.M. Frassanito, P. Gualtieri, Chemistry, physico-
448 chemistry and applications linked to biological activities of beta-glucans, Nat Prod Rep 28(3)
449 (2011) 457-66.
450 [12] A. Synytsya, M. Novak, Structural analysis of glucans, Ann Transl Med 2(2) (2014) 17.
451 [13] I. Noss, G. Doekes, P.S. Thorne, D.J. Heederik, I.M. Wouters, Comparison of the potency
452 of a variety of beta-glucans to induce cytokine production in human whole blood, Innate
453 Immun 19(1) (2013) 10-9.

[14] K. Kofuji, A. Aoki, K. Tsubaki, M. Konishi, T. Isobe, Y. Murata, Antioxidant Activity of beta-Glucan, ISRN Pharm 2012 (2012) 125864.

[15] B. Gugi, T. Le Costaouec, C. Burel, P. Lerouge, W. Helbert, M. Bardor, Diatom-specific oligosaccharide and polysaccharide structures help to unravel biosynthetic capabilities in diatoms, Mar Drugs 13(9) (2015) 5993-6018.

[16] O. Fortin, B. Aguilar-Uscanga, K.D. Vu, S. Salmieri, M. Lacroix, Cancer chemopreventive, antiproliferative, and superoxide anion scavenging properties of *Kluyveromyces marxianus* and *Saccharomyces cerevisiae* var. *boulardii* cell wall components, Nutr Cancer 70(1) (2018) 83-96.

[17] I. Sadovskaya, A. Souissi, S. Souissi, T. Grard, P. Lencel, C.M. Greene, S. Duin, P.S. Dmitrenok, A.O. Chizhov, A.S. Shashkov, A.I. Usov, Chemical structure and biological activity of a highly branched (1 --> 3,1 --> 6)-beta-D-glucan from *Isochrysis galbana*, Carbohydr Polym 111 (2014) 139-48.

[18] A.H. Rojo-Cebreros, L. Ibarra-Castro, J.M. Martínez-Brown, G. Velasco-Blanco, M.A. Martínez-Téllez, M.A. Medina-Jasso, M. Nieves-Soto, D. Quintana-Zavala, Potential of *Nannochloropsis* in beta glucan production, in: M. Han, P. Kazik (Eds.), Nannochloropsis, Nova Science Publishers, Inc2017.

[19] S.M. Myklestad, E. Granum, Biology of (1,3)-beta-glucans and related glucans in protozoans and chromistans, in: A. Bacic, G.B. Fincher, B.A. Stone (Eds.), Chemistry, biochemistry, and biology of 1-3 beta glucans and related polysaccharides, Academic Press, San Diego, 2009.

[20] S. Xia, B. Gao, A. Li, J. Xiong, Z. Ao, C. Zhang, Preliminary characterization, antioxidant properties and production of chrysolaminarin from marine diatom *Odontella aurita*, Mar Drugs 12(9) (2014) 4883-97.

[21] M.A. Caballero, D. Jallet, L. Shi, C. Rithner, Y. Zhang, G. Peers, Quantification of chrysolaminarin from the model diatom *Phaeodactylum tricornutum*, Algal Res 20 (2016) 180-188.

[22] B. Gao, A. Chen, W. Zhang, A. Li, C. Zhang, Co-production of lipids, eicosapentaenoic acid, fucoxanthin, and chrysolaminarin by *Phaeodactylum tricornutum* cultured in a flat-plate photobioreactor under varying nitrogen conditions, J. Ocean Univ. China 16(5) (2017) 916-924.

[23] A. Bozarth, U.G. Maier, S. Zauner, Diatoms in biotechnology: modern tools and applications, Appl Microbiol Biotechnol 82(2) (2009) 195-201.

[24] F. Daboussi, S. Leduc, A. Marechal, G. Dubois, V. Guyot, C. Perez-Michaut, A. Amato, A. Falciatore, A. Juillerat, M. Beurdeley, D.F. Voytas, L. Cavarec, P. Duchateau, Genome engineering empowers the diatom *Phaeodactylum tricornutum* for biotechnology, Nat Commun 5 (2014) 3831.

491 [25] W. Zhang, F. Wang, B. Gao, L. Huang, C. Zhang, An integrated biorefinery process:
492 Stepwise extraction of fucoxanthin, eicosapentaenoic acid and chrysolaminarin from the
493 same *Phaeodactylum tricornutum* biomass, Algal Res 32 (2018) 193-200.
494 [26] S. Hemaiswarya, R. Raja, R. Ravi Kumar, V. Ganesan, C. Anbazhagan, Microalgae: a
495 sustainable feed source for aquaculture, World J Microbiol Biotechnol 27 (2011) 1737-1746.
496 [27] M. Manchado, J.V. Planas, X. Cousin, L. Rebordinos, M.G. Claros, Current status in other
497 finfish species: Description of current genomic resources for the gilthead seabream (*Sparus*
498 *aurata*) and soles (*Solea senegalensis* and *Solea solea*), in: S.A. MacKenzie, S. Jentoft
499 (Eds.), Genomics in aquaculture, Elsevier, Cambridge, MA, USA, 2016, pp. 195-221.
500 [28] S. Morais, C. Aragão, E. Cabrita, L.E.C. Conceição, M. Constenla, B. Costas, J. Dias, N.
501 Duncan, S. Engrola, A. Estevez, E. Gisbert, E. Mañanós, L.M.P. Valente, M. Yúfera, M.
502 Dinis, New developments and biological insights into the farming of *Solea senegalensis*
503 reinforcing its aquaculture potential, Rev. Aquacult. 6 (2014) 1-37.
504 [29] S. Arijo, R. Rico, M. Chabrillon, P. Diaz-Rosales, E. Martínez-Manzanares, M.C. Balebona,
505 B. Magariños, A.E. Toranzo, M.A. Moriñigo, Effectiveness of a divalent vaccine for sole,
506 *Solea senegalensis* (Kaup), against *Vibrio harveyi* and *Photobacterium damselae* subsp
507 *piscicida*, J. Fish Dis 28 (2005) 33–38.
508 [30] Y. Silva, C. Moreirinha, C. Pereira, L. Costa, R. Rocha, A. Cunha, N. Gomes, R. Calado, A.
509 Almeida, Biological control of *Aeromonas salmonicida* infection in juvenile Senegalese sole
510 (*Solea senegalensis*) with Phage AS-A, Aquaculture 450 (2016) 225-233.
511 [31] E. Vallejos-Vidal, F. Reyes-Lopez, M. Teles, S. MacKenzie, The response of fish to
512 immunostimulant diets, Fish Shellfish Immunol 56 (2016) 34-69.
513 [32] A. Chiovitti, P. Molino, S.A. Crawford, R. Teng, T. Spurck, R. Wetherbee, The glucans
514 extracted with warm water from diatoms are mainly derived from intracellular
515 chysolaminaran and not extracellular polysaccharides. , Eur J Phycol 39(2) (2004) 117-128.
516 [33] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, Colorimetric method for
517 determination of sugars and related substances, Anal. Chem. 28 (1956) 350-356.
518 [34] K.M. Gillespie, J.M. Chae, E.A. Ainsworth, Rapid measurement of total antioxidant capacity
519 in plants, Nat Protoc 2(4) (2007) 867-70.
520 [35] I.F. Benzie, J.J. Strain, The ferric reducing ability of plasma (FRAP) as a measure of
521 "antioxidant power": the FRAP assay, Anal Biochem 239(1) (1996) 70-6.
522 [36] P. Molyneux, The use of the stable free radical diphenylpicrylhydrazyl (DPPH) for
523 estimating antioxidant activity, Songklanakarin J. Sci. Technol. 26 (2004) 211-219.
524 [37] S. Letsiou, K. Kalliampakou, K. Gardikis, L. Mantecon, C. Infante, M. Chatzikonstantinou,
525 N.E. Labrou, E. Flemetakis, Skin protective effects of *Nannochloropsis gaditana* extract on
526 H2O2-stressed human dermal fibroblasts, Front. Mar. Sci. 4 (2017) 221.

527 [38] V. Selvaraj, K. Sampath, V. Sekar, Administration of yeast glucan enhances survival and
528 some non-specific and specific immune parameters in carp (*Cyprinus carpio*) infected with
529 *Aeromonas hydrophila*, Fish Shellfish Immunol 19(4) (2005) 293-306.
530 [39] M. Manchado, E. Salas-Leiton, C. Infante, M. Ponce, E. Asensio, A. Crespo, E. Zuasti, J.P.
531 Canavate, Molecular characterization, gene expression and transcriptional regulation of
532 cytosolic HSP90 genes in the flatfish Senegalese sole (Solea senegalensis Kaup), Gene
533 416(1-2) (2008) 77-84.
534 [40] C. Infante, M.P. Matsuoka, E. Asensio, J.P. Cañavate, M. Reith, M. Manchado, Selection of
535 housekeeping genes for gene expression studies in larvae from flatfish using real-time PCR,
536 BMC Mol Biol 9 (2008) 28.
537 [41] E. Salas-Leiton, V. Anguis, B. Martin-Antonio, D. Crespo, J.V. Planas, C. Infante, J.P.
538 Cañavate, M. Manchado, Effects of stocking density and feed ration on growth and gene
539 expression in the Senegalese sole (*Solea senegalensis*): potential effects on the immune
540 response, Fish Shellfish Immunol 28(2) (2010) 296-302.
541 [42] C. Carballo, D. Castro, J.J. Borrego, M. Manchado, Gene expression profiles associated with
542 lymphocystis disease virus (LCDV) in experimentally infected Senegalese sole (Solea
543 senegalensis), Fish Shellfish Immunol 66 (2017) 129-139.
544 [43] A. Fernandez-Trujillo, P. Ferro, E. Garcia-Rosado, C. Infante, M.C. Alonso, J. Bejar, J.J.
545 Borrego, M. Manchado, Poly I:C induces Mx transcription and promotes an antiviral state
546 against sole aquabirnavirus in the flatfish Senegalese sole (*Solea senegalensis* Kaup), Fish
547 Shellfish Immunol 24(3) (2008) 279-85.
548 [44] H. Benzekri, P. Armesto, X. Cousin, M. Rovira, D. Crespo, M.A. Merlo, D. Mazurais, R.
549 Bautista, D. Guerrero-Fernandez, N. Fernandez-Pozo, M. Ponce, C. Infante, J.L. Zambonino,
550 S. Nidelet, M. Gut, L. Rebordinos, J.V. Planas, M.L. Begout, M.G. Claros, M. Manchado,
551 De novo assembly, characterization and functional annotation of Senegalese sole (*Solea*
552 *senegalensis*) and common sole (*Solea solea*) transcriptomes: integration in a database and
553 design of a microarray, BMC Genomics 15 (2014) 952.
554 [45] V. Kiron, A. Kulkarni, D. Dahle, G. Vasanth, J. Lokesh, O. Elvebo, Recognition of purified
555 beta 1,3/1,6 glucan and molecular signalling in the intestine of Atlantic salmon, Dev Comp
556 Immunol 56 (2016) 57-66.
557 [46] T.D. Schmittgen, K.J. Livak, Analyzing real-time PCR data by the comparative C(T) method,
558 Nat Protoc 3(6) (2008) 1101-8.
559 [47] G. Caraux, S. Pinloche, PermutMatrix: a graphical environment to arrange gene expression
560 profiles in optimal linear order, Bioinformatics 21(7) (2005) 1280-1.
561 [48] X. Liu, H. Liu, Y. Zhai, Y. Li, X. Zhu, W. Zhang, Laminarin protects against hydrogen
562 peroxide-induced oxidative damage in MRC-5 cells possibly via regulating NRF2, PeerJ 5
563 (2017) e3642.

[49] E. Machová, S. Bystricky, Antioxidant capacities of mannans and glucans are related to their susceptibility of free radical degradation, Int J Biol Macromol 61 (2013) 308-11.

[50] B. Du, B. Xu, Oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) of β-glucans from different sources with various molecular weight, Bioact. Carbohydr. Dietary Fibre 3 (2014) 11-16.

[51] P. Kanmani, R. Satish kumar, N. Yuvaraj, K.A. Paari, V. Pattukumar, V. Arul, Production and purification of a novel exopolysaccharide from lactic acid bacterium Streptococcus phocae PI80 and its functional characteristics activity in vitro, Bioresour Technol 102(7) (2011) 4827-33.

[52] T.C.-T. Lo, C.A. Chang, K.-H. Chiuc, P.-K. Tsayd, J.-F. Jena, Correlation evaluation of antioxidant properties on the monosaccharide components and glycosyl linkages of polysaccharide with different measuring methods, Carbohydr. Polym. 86(1) (2011) 320-327.

[53] C.P. Rubio, J. Hernandez-Ruiz, S. Martinez-Subiela, A. Tvarijonaviciute, J.J. Ceron, Spectrophotometric assays for total antioxidant capacity (TAC) in dog serum: an update, BMC Vet Res 12(1) (2016) 166.

[54] D.B. Zekovic, S. Kwiatkowski, M.M. Vrvic, D. Jakovljevic, C.A. Moran, Natural and modified (1-->3)-beta-D-glucans in health promotion and disease alleviation, Crit Rev Biotechnol 25(4) (2005) 205-30.

[55] R. Apak, K. Guclu, B. Demirata, M. Ozyurek, S.E. Celik, B. Bektasoglu, K.I. Berker, D. Ozyurt, Comparative evaluation of various total antioxidant capacity assays applied to phenolic compounds with the CUPRAC assay, Molecules 12(7) (2007) 1496-547.

[56] M.K. Roy, M. Koide, T.P. Rao, T. Okubo, Y. Ogasawara, L.R. Juneja, ORAC and DPPH assay comparison to assess antioxidant capacity of tea infusions: relationship between total polyphenol and individual catechin content, Int J Food Sci Nutr 61(2) (2010) 109-24.

[57] A. Choromanska, J. Kulbacka, J. Harasym, R. Oledzki, A. Szewczyk, J. Saczko, High- and low-molecular weight oat beta-glucan reveals antitumor activity in human epithelial lung cancer, Pathol Oncol Res (2017).

[58] K. Fujiki, D.H. Shin, M. Nakao, T. Yano, Molecular cloning and expression analysis of carp (*Cyprinus carpio*) interleukin-1 beta, high affinity immunoglobulin E Fc receptor gamma subunit and serum amyloid A, Fish Shellfish Immunol 10(3) (2000) 229-42.

[59] C.K. Misra, B.K. Das, S.C. Mukherjee, P. Pattnaik, Effect of multiple injections of beta-glucan on non-specific immune response and disease resistance in *Labeo rohita* fingerlings, Fish Shellfish Immunol 20(3) (2006) 305-19.

[60] M. Lovoll, U. Fischer, G.S. Mathisen, J. Bogwald, M. Ototake, R.A. Dalmo, The C3 subtypes are differentially regulated after immunostimulation in rainbow trout, but head kidney macrophages do not contribute to C3 transcription, Vet Immunol Immunopathol 117(3-4) (2007) 284-95.

601 [61] B.N. Fredriksen, K. Saevareid, L. McAuley, M.E. Lane, J. Bogwald, R.A. Dalmo, Early
602 immune responses in Atlantic salmon (*Salmo salar* L.) after immunization with PLGA
603 nanoparticles loaded with a model antigen and beta-glucan, Vaccine 29(46) (2011) 8338-49.
604 [62] C. Yuan, X. Pan, Y. Gong, A. Xia, G. Wu, J. Tang, X. Han, Effects of Astragalus
605 polysaccharides (APS) on the expression of immune response genes in head kidney, gill and
606 spleen of the common carp, *Cyprinus carpio* L, Int Immunopharmacol 8(1) (2008) 51-8.
607 [63] J. Zou, C.J. Secombes, The Function of Fish Cytokines, Biology (Basel) 5(2) (2016).
608 [64] J. Douxfils, C. Fierro-Castro, S.N. Mandiki, W. Emile, L. Tort, P. Kestemont, Dietary beta-
609 glucans differentially modulate immune and stress-related gene expression in lymphoid
610 organs from healthy and *Aeromonas hydrophila*-infected rainbow trout (*Oncorhynchus*
611 *mykiss*), Fish Shellfish Immunol 63 (2017) 285-296.
612 [65] J.K. Chettri, M.K. Raida, L. Holten-Andersen, P.W. Kania, K. Buchmann, PAMP induced
613 expression of immune relevant genes in head kidney leukocytes of rainbow trout
614 (*Oncorhynchus mykiss*), Dev Comp Immunol 35(4) (2011) 476-82.
615 [66] P. Garcia-Valtanen, A. Martinez-Lopez, A. Lopez-Munoz, M. Bello-Perez, R.M. Medina-
616 Gali, M.D. Ortega-Villaizan, M. Varela, A. Figueras, V. Mulero, B. Novoa, A. Estepa, J.
617 Coll, Zebra Fish lacking adaptive immunity acquire an antiviral alert state characterized by
618 ppregulated gene expression of apoptosis, multigene families, and Interferon-telated genes,
619 Front Immunol 8 (2017) 121.
620 [67] A. Ambade, D. Catalano, A. Lim, P. Mandrekar, Inhibition of heat shock protein (molecular
621 weight 90 kDa) attenuates proinflammatory cytokines and prevents lipopolysaccharide-
622 induced liver injury in mice, Hepatology 55(5) (2012) 1585-95.
623 [68] A.L. Joly, G. Wettstein, G. Mignot, F. Ghiringhelli, C. Garrido, Dual role of heat shock
624 proteins as regulators of apoptosis and innate immunity, J Innate Immun 2(3) (2010) 238-47.
625 [69] G.S. Thangjam, C. Birmpas, N. Barabutis, B.W. Gregory, M.A. Clemens, J.R. Newton, D.
626 Fulton, J.D. Catravas, Hsp90 inhibition suppresses NF-kappaB transcriptional activation via
627 Sirt-2 in human lung microvascular endothelial cells, Am J Physiol Lung Cell Mol Physiol
628 310(10) (2016) L964-74.
629 [70] N.C. Andrews, Anemia of inflammation: the cytokine-hepcidin link, J Clin Invest 113(9)
630 (2004) 1251-3.
631 [71] V. Rajanbabu, C.Y. Pan, S.C. Lee, W.J. Lin, C.C. Lin, C.L. Li, J.Y. Chen, Tilapia hepcidin
632 2-3 peptide modulates lipopolysaccharide-induced cytokines and inhibits tumor necrosis
633 factor-alpha through cyclooxygenase-2 and phosphodiesterase 4D, J Biol Chem 285(40)
634 (2010) 30577-86.
635
636

Table 1

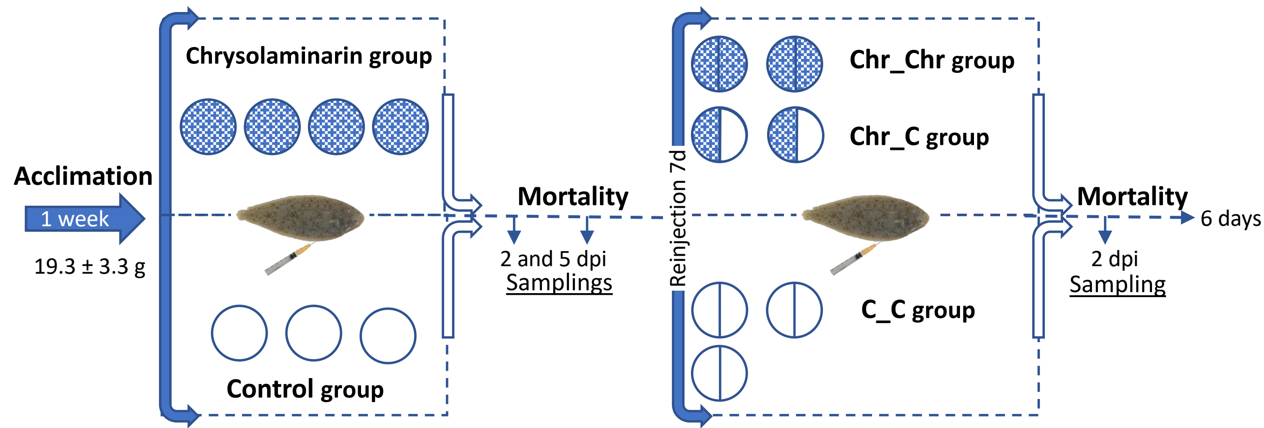
Primers used for RT-qPCR. Gene names, primer sequences, amplicon size and the target unigene from SoleaDB (Benzekri et al., 2014) are indicated.

Target	Primer	Sequence (5’→3’)	Amplicon (bp)	source
il1b	F	GCACCCTCATGTCTGCCCGCTTCCCT	105	unigene346347
	R	AGGTTCTGTAGCGTTGGGCCATCTCTGT		
tnfa	F	CCAAGCGTTTGCTCAGGGCGGCTTC	116	unigene26860
	R	GGCCTTCGCTGCAGGAGACTCTGAACGAC		
clec	F	CCCCCATCGTCTGCCCTCTGAACTGG	90	unigene416724
	R	ACTGACTCTCGTCCCACTCTCTGAAGACCC		
cxc10	F	CGCGCTCGACAACAGCCAGTCAAAGCAAC	121	unigene30191
	R	ACAGTGACAGTGCTGGTGAGGAACTCCTT		
irf7	F	GGCAAAATCAGCGAGTTCCCCAACGACA	93	unigene68007
	R	CACCATCTTGAAGCGCACGGACAGGTT		
irf8	F	AGCCTGCGACATGGAAGACAAGACTCCG	86	unigene17110
	R	GTCCAGCTGCGACCGTTCTCTCACCTCC		
irf9	F	TCACCGTGCAGACCGAGGAGCCCGTT	79	unigene2689_split_1
	R	TCCAGTCTGATCTCGTCCACCGCGTCTCT		
ifn1	F	AGCACAAGGAAAGCGCTGCCTGTGGT	94	unigene638812
	R	AAGCAGTTGTGGTGTTTGTGCAGGACGAT		

658
659
660
661

662

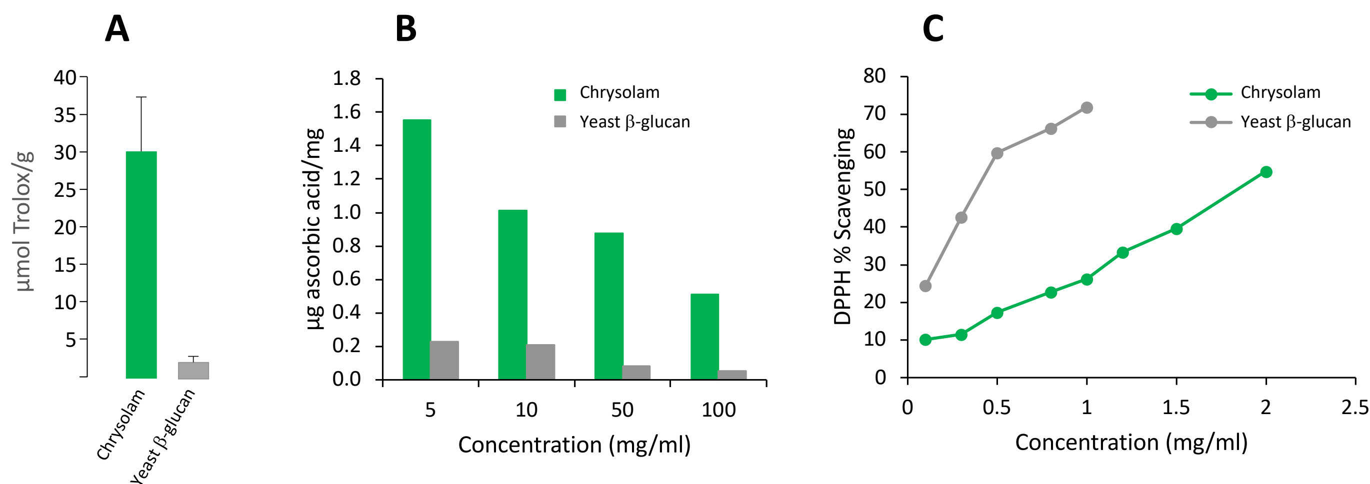
663 **Captions**



664

665 **Figure 1.** Experimental design. Soles (19.3 ± 3.3 g) were acclimated for 1 week before i.p. injection. Chrysolaminarin-enriched extract was
666 suspended in a coconut oil and PBS emulsion (1:1) and injected 1mg/fish (Chrysolaminarin group) and the sham control group just with the
667 emulsion. At 7 dpi (dash line), the chrysolaminarin-injected fish were reinjected with chrysolaminarin (Chr_Chr group) or the emulsion (Chr_C
668 group) whereas the sham control group was reinjected only with the emulsion (C_C group) Mortality was monitored for 13 days and samplings
669 were carried out at 2 and 5 dpi and 2 days after reinjection.

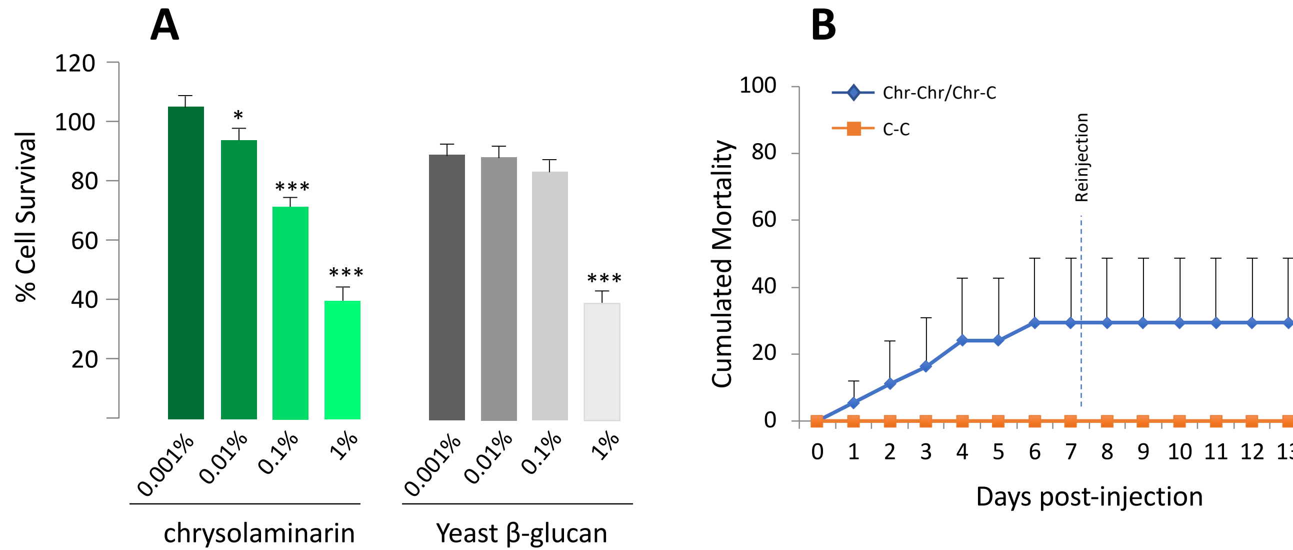
670



671

672 **Figure 2.** Total antioxidant capacity (TAC) of chrysolaminarin and yeast β-glucan. (A) the oxygen radical absorbance capacity (ORAC) assay.
673 (B) ferric reducing antioxidant power (FRAP). (C) the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay. ORAC values were
674 expressed as Trolox equivalents (µmol Trolox/g), FRAP as µg ascorbic acid/mg and DPPH as percentage of scavenging of free radical.

675



676

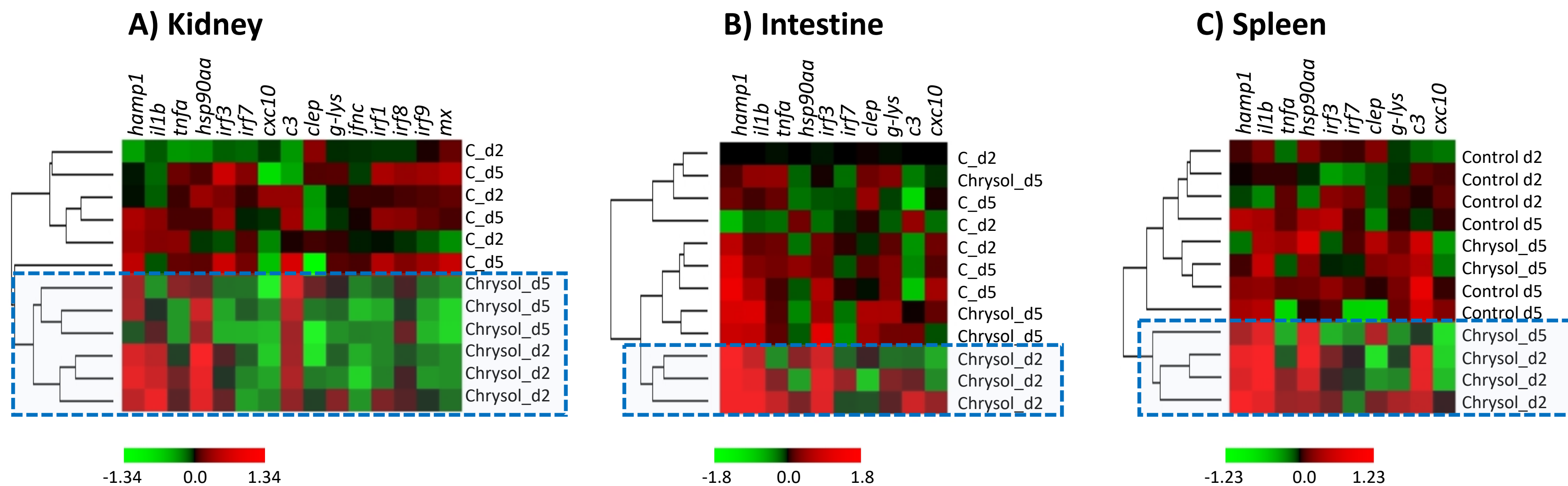
677 **Figure 3. (A)** Toxicity of chrysolaminarin-enriched extract and yeast glucan in human fibroblast cells as determined by MTT. Asterisks indicate

678 significant differences with respect to the lower concentration. **p*<0.05; ** *p*<0.01; *** *p*<0.001. (B) *In vivo* cumulative mortality in sole

679 intraperitoneally injected with chrysolaminarin-enriched extract. The experimental groups Chr_Chr, Chr_C and C_C are indicated

680

681



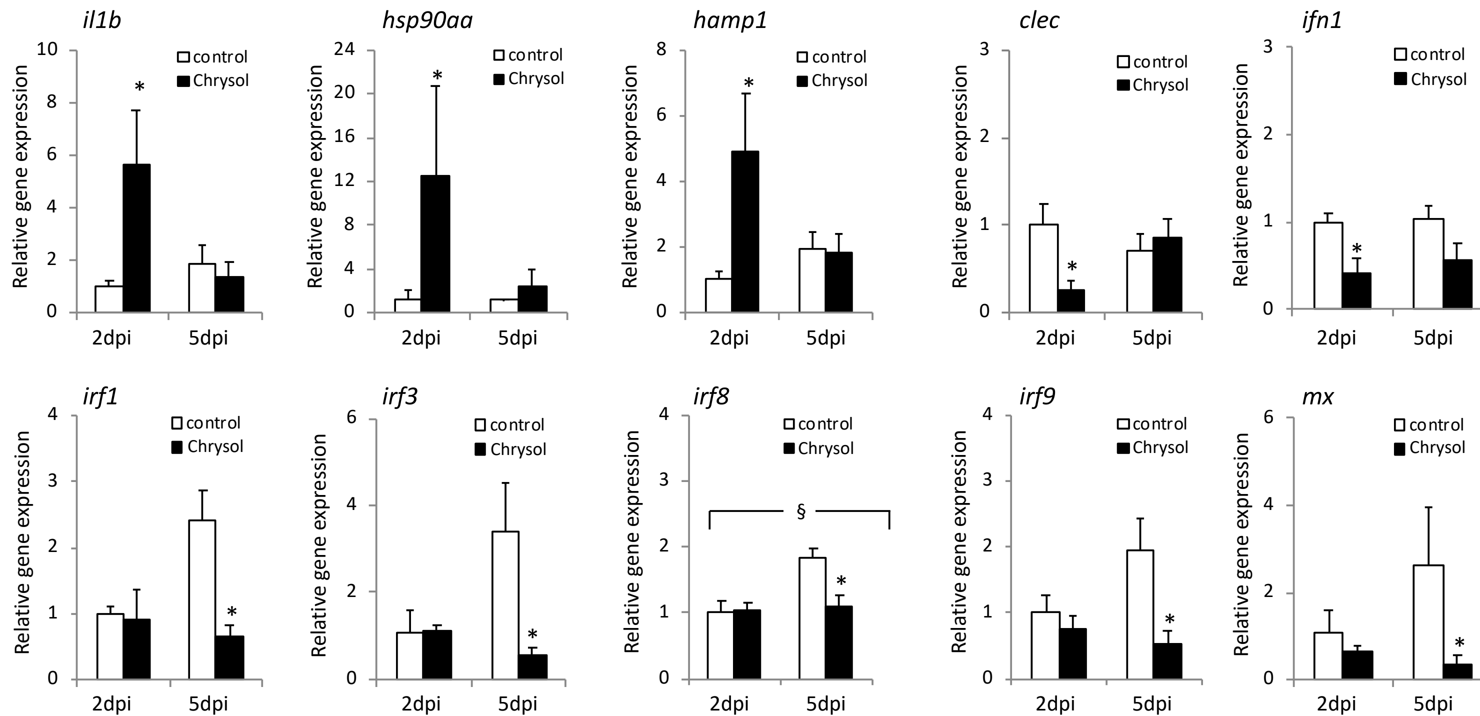
682 **Figure 4.** Hierarchical clustering analysis based on set of expressed transcripts in kidney (A), intestine (B) and spleen (C) as determined by

683 qPCR. Data were normalized and expressed as log10 of fold change. Green and red colours indicate low and high expression values according to

684 the scale shown. The samples are identified on the right (control and chrysolaminarin-enriched extract at 2 and 5 dpi) and the main clusters

685 grouping separating both experimental group samples are boxed in blue. Full-names for the transcripts shown on the top are indicated in M&M.

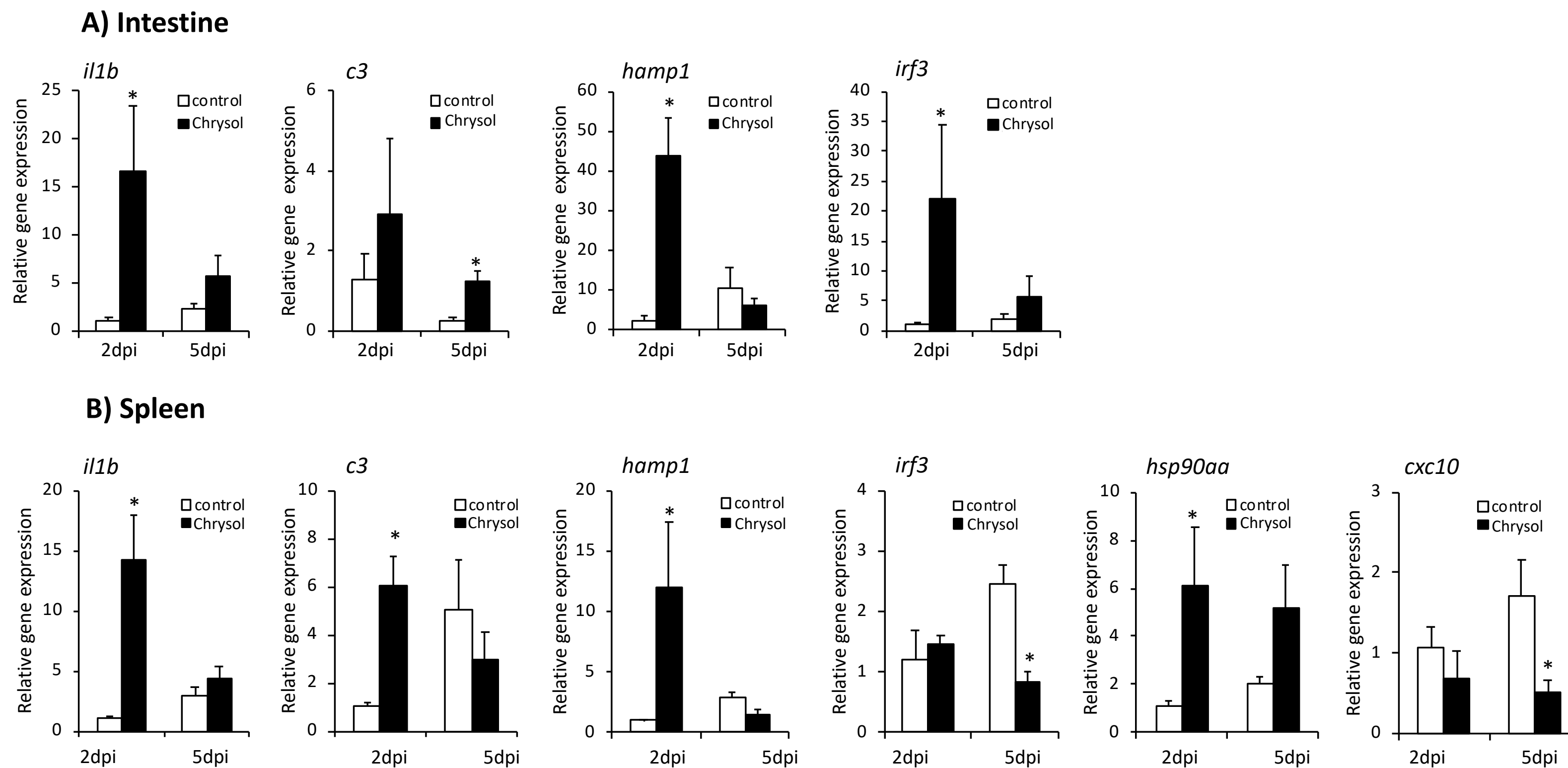
686



687

688 **Figure 5.** Differentially expressed genes in kidney after i.p. injection of the chrysolaminarin-enriched extract. Data were expressed as the mean
689 fold change (mean + SEM, n = 3) from the calibrator (control 2 dpi). A two-way ANOVA was used to determine statistical differences. Asterisks
690 denote significant differences between control (white) and chrysolaminarin-enriched extract (black) injected fish. § indicates significant differences
691 due to time.

692



693 **Figure 6.** Differentially expressed genes in intestine (A) and spleen (B) after i.p. injection of the chrysolaminarin-enriched extract. Data were
694 expressed as the mean fold change (mean + SEM, n = 3) from the calibrator (control 2 dpi). A two-way ANOVA was used to determine statistical
695 differences. Asterisks denote significant differences between control (white) and chrysolaminarin-enriched extract (black) injected fish.

696