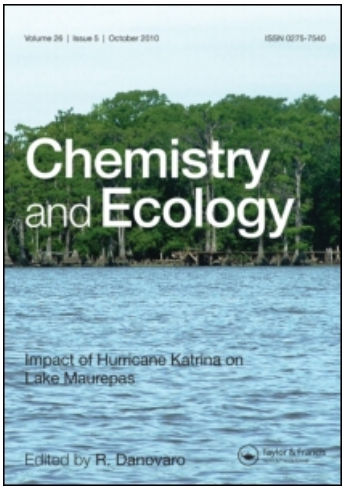

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Welfare improvement using alginic acid in rainbow trout (*Oncorhynchus mykiss*) juveniles

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The present study explored alternative strategies to improve the health status and growth metabolism in cultured juvenile fish. In this context the effects of alginic acid administration on the welfare of rainbow trout juveniles were investigated. Both the hepatic gene expression of heat shock protein 70 and the cortisol plasma levels were evaluated in order to estimate the possible beneficial role of alginic acid in improving animal well-being. At the same time the body weight was measured and was correlated with the hepatic expression of insulin-like growth factor I, myostatin, and two isoforms of the thyroid receptor. Finally, improvement of the immune system was investigated by monitoring the expression of innate immune-related genes, such as interleukin1β, interleukin8 and toll-like receptor3, in the spleen. The data obtained demonstrated an improvement in the welfare of rainbow trout juveniles, revealed by growth and innate immune response enhancement by alginic acid administration. These results may be of great importance for the further development of more sustainable aquaculture practices, and can be considered as a biotechnological application to produce a safer product for human health.

Keywords: welfare; growth; immune response; immunostimulant; alginic acid

1. Introduction

At present, natural fish stocks are declining and the elevated demand for fish food is yielding to a rapid and constant increase of production for the commercial aquaculture industry. This phenomenon has led to a deep and drastic remodelling of cultured fish life conditions, resulting in a decrease of the fish product quality due to more stressful conditions and disease occurrence [1]. Stress is closely associated with many different issues in fish biology and environmental research [2] and it is generally defined as the reaction to external forces and abnormal conditions that tend to disturb the organism's homeostasis. Animals that are exposed to alterations in their environment will respond with changes in their neuroendocrine systems [3]. Environmental changes, handling, crowding and confinement are all components affecting the physiological

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stress response in farmed fish [2,4,5,6]. It is well-established that this physiological stress has severe negative consequences on teleost performance with respect to growth and disease resistance [2,4,7], especially during the larval and juvenile stages [8].

When faced with disease, drugs and chemotherapeutic compounds are widely used and, in the last decades, the aquaculture industry has experienced widespread use of these substances. While in the case of disease the use of such products has an obvious benefit in treating infected animals, the use of antibacterial drugs has also been used either as a prophylactic or for growth enhancement.

This misuse of antimicrobial drugs has led to the emergence of antibiotic resistant bacteria, with the development of the associated negative effects for the fish but also for the surrounding environment and a risk to human health [9]. For such reasons, governments and organisations have introduced much tighter restrictions for antibiotic usage in animal production and, in 2006, the EU implemented a ban on the use of non-therapeutic anti-microbial drugs in animal production (World Health Organisation Antimicrobial Resistance Fact Sheet 194, http://www.who.int/inf-fs/en/fact194.html).

Good candidates for antimicrobial drug substitution are natural immunostimulants. Although prevention of infectious diseases is the most common reason for using immunomodulators, there are also other justifications, such as an improvement in stress-induced immunosuppression and general animal well-being [10].

In aquaculture, different kinds of substances are known to act as immunostimulants [11–14]. In this context, alginate is potentially a good candidate. This substance is a polysaccharide found in the cell walls of brown algae. Many studies have demonstrated the efficacy of this substance in increasing the non-specific defence response [13–18].

In this study, we assessed the effects of early life stressors (crowding and confinement) on general well-being parameters in rainbow trout (*Oncorhynchus mykiss*) juveniles following alginic acid dietary administration.

The results obtained in the present study consist of the use of modern biotechnology in the rearing processes to reduce chemical and drug employment in aquaculture and to enhance the production and quality of the aquatic species.

2. Materials and methods

2.1. *Animals and experimental design*

From a batch of 470,000 rainbow trout embryos from Spain, six groups of 10,000 specimens each were formed and distributed into six larval tanks of $6 \times 1 \times 0.8$ m, containing approximately 2400 litres with a water flow rate of 1500–2000 ml min^{-1}. Both alginic acid-fed and control groups were held in triplicate tanks. All experimental groups were fed twice a day, seven days a week, at a rate ranging between 2.5–3% body weight with a commercial diet (Microbaq, Dibaq, Spain). Three tanks received an alginic acid source diet (AquaVac Ergosan, Schering-Plough Aquaculture, UK) prepared according to the manufacturer's recommendations by mixing it in food at an incorporation rate of 0.5% (5 kg per ton). AquaVac Ergosan contains extracts of *Laminaria digitata* and *Ascophyllum nodosum* and its analytical composition is: crude protein 8.4%, crude fat 1.6%, crude fibre 4.5%, crude ash 35.7%, water 10.9%, NFE 38.9%, Ca 3.9%, Mg 0.94%, Na 2.5% and P 0.12%.

The first administration started 10 days post hatching. Alginic acid administration in the diet was carried out from August to November, in three cycles: (1st cycle: 30 days of treatment plus 15 days of normal feeding; 2nd cycle: 10 days of treatment plus 20 days of normal feeding; 3rd cycle: 10 days of treatment). The controls were only fed commercial diet (Microbaq, Dibaq, Spain).

Ten fish from each tank were sampled at the end of each treatment cycle and respectively on juveniles 40, 65 and 95 dph. Trout were anaesthetised with MS-222 (Sigma, 100 mg L^{-1}).

Blood was sampled in ammonium–heparin treated syringes by puncture of the caudal vessel and centrifuged at 6000 *g* for 10 min. Blood samples from animals of the same group were pooled to reach the volume required for the subsequent cortisol assay.

Plasma was transferred into a new tube, poured into liquid nitrogen and stored at −80 °C until a cortisol assay was performed.

Liver and spleen samples from all the experimental groups were removed and immediately poured into liquid nitrogen and stored at −80 °C until molecular biology analyses were performed.

2.2. *RNA extraction and cDNA synthesis*

Total RNA extraction from the liver and spleen was performed using the Minikit RNAeasy® (Qiagen) extraction kit following the manufacturer's protocol. Total RNA extracted was eluted in 50 μL of RNAse-free water. Final RNA concentrations were determined by spectrophotometer and the RNA integrity was verified by ethidium bromide staining of 28S and 18S ribosomal RNA bands on 1% agarose gel. RNA was stored at −80 °C until use.

Total RNA was treated with DNAse (10 UI at 37 °C for 10 min, MBI Fermentas); a total amount of 1 μg of RNA was used for cDNA synthesis, employing the iScript cDNA Synthesis Kit (Bio-Rad).

2.3. *Real time PCR*

Triplicate PCR reactions were carried out for each sample analysed. After real-time condition optimisation, PCRs were performed with SYBR green method in an iQ5 iCycler thermal cycler (Bio-Rad). The reactions were set on a 96-well plate by mixing, for each sample, 1 μL of diluted (1/20) cDNA, 5 μL of 2× concentrated iQ TM SYBR Green Supermix (Bio-Rad), containing SYBR Green as a fluorescent intercalating agent, 0.3 μM forward primer, and 0.3 μM of reverse primer. The primer sequences are shown in Table 1. The thermal profile for all reactions was 15 min at 95 °C and then 45 cycles of 20 s at 95 °C, 20 s at 60 °C and 20 s at 72 °C. Fluorescence monitoring occurred at the end of each cycle. Additional dissociation curve analysis was performed and showed in all cases one single peak.

The ARP was used as the house keeping gene in each sample in order to standardise the results by eliminating variation in mRNA and cDNA quantity and quality. No amplification product was observed in negative control and no primers-dimer formation was observed in the control templates. The data obtained were analysed using the iQ5 Optical System software version 2.0 (Bio-Rad).

Table 1. List of primers.

Gene	Forward primer	Reverse primer
IGFI	tggacacgctgcagtttgtgtgt	cactcgtccacaataccacggt
MYO	aaggatggacttatggaagaagat	ttggacgatggattcgggttcagt
TRα	gcacaacattccccacttct	agttcgttgggacactccac
TRβ	tcacct gtg aag gat gca ag	gac agc gat gca ctt ctt ga
TLR3	agccctttgctgccttacagag	gtcttcaggtcatttttggacacg
IL-1β	ggagaggttaaagggtggcga	tgccgactccaactccaaca
IL-8	cactgagatcattgccactctga	atgaccctcttgacccacgg
HSP70	ccctgggcatcgaaacc	ccctcgtagacctggatcatg
ARP	gaaaatcatccaattgctggatg	cttcccacgcaaggacaga

2.4. *Cortisol EIA assay*

Evaluation of cortisol levels in the plasma was performed using the Cortisol EIA kit (Cayman, Chemical) in accordance with the manufacturer's protocol. Preliminary tests were conducted to optimise the extraction protocol, the range of linearity and the sensitivity. A total of 500 μL of plasma was extracted with cold methylene chloride, then analysed using a standard curve (7.8–1000 pg mL^{-1}). The assay sensitivity and the inter- and intra-assay coefficients of variation were 2 pg/tube, 6.8% and 4.1%, respectively. To validate the cortisol assay, parallelism between the standard curve and serial dilution of the extracted solution was established.

2.5. *Statistical analysis*

The data obtained by real time PCR and EIA assay were analysed by two-way ANOVA to determine the effect of the treatment. One-way analysis of variance followed by Dunnett's multiple comparison test was used to determine differences among groups. The level for accepted statistical significance was $p < 0.05$.

3. Results

3.1. *Cortisol levels and HSP70 gene expression*

As shown in Figure 1(a), cortisol levels were significantly lower in treated fish compared with those of the control group at all the experimental times analysed.

HSP70 gene expression in the liver was significantly ($p < 0.05$) lower in fish fed on alginic acid with respect to the controls throughout the experimental period, indicating a better tolerance to farming conditions by these animals (Figure 1(b)).

3.2. *Body weight and growth-related gene expression*

At the end of the treatment (95 dph), the body weight of alginic acid-fed animals had significantly increased with respect to the controls. No significant differences were observed at the two previous sampling points (Figure 2). As shown in Figure 3(a), at the second and third sampling (65 and 95 dph), the IGFI expression was significantly higher in treated groups with respect to the controls, whilst no significant differences were evident at the first sampling (40 dph).

MYO gene expression levels in treated and control animals were similar at the first two sample points but significantly decreased in treated groups at 95 dph with respect to the control group (Figure 3(b)).

Finally, as shown in Figure 3(c), TRα mRNA levels were significantly ($p < 0.05$) higher in fish fed on an alginic acid source compared with the control fish throughout the experimental period. As shown in Figure 3(d), at the second and third sampling (65 and 95 dph), the TRβ expression was significantly higher in treated groups with respect to the controls, whilst a significant decrease in the levels of this gene were evident at the first sampling (40 dph) in the treated group.

3.3. *Immune-related gene expression*

As shown in Figure 4(a), (b) and (c), in the spleen, IL-1β, IL8 and TLR3 gene expression was significantly ($p < 0.05$) higher in fish fed on alginic acid throughout the experimental period.

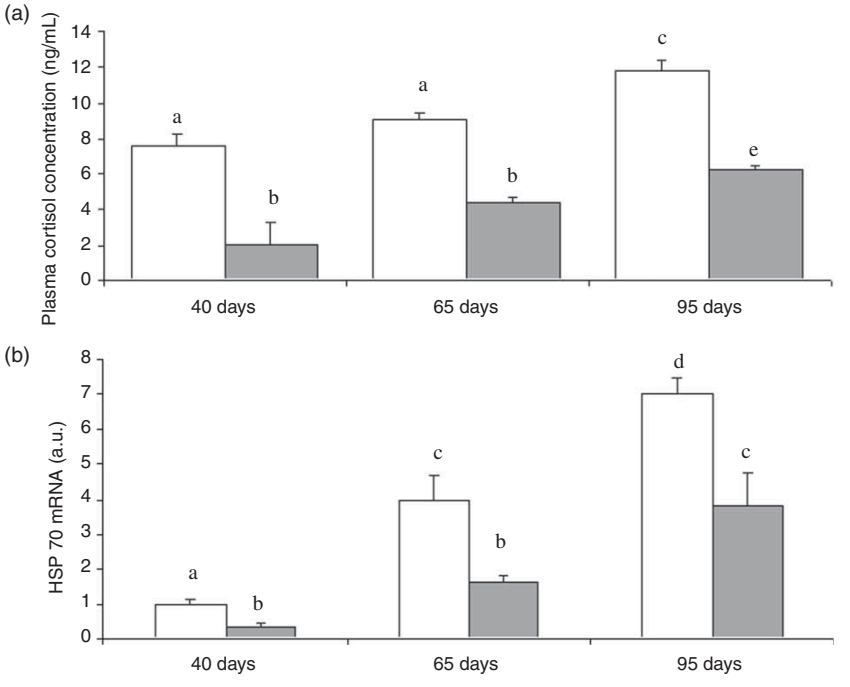

Figure 1. Plasma cortisol levels (a) and HSP70 mRNA quantification (b) in controls (white bars) and fish fed dietary alginic acid (grey bars) at 40 days, 65 days and 95 days from the beginning of the first solid feeding. Values with different letters indicate statistical significance ($p < 0.05$).

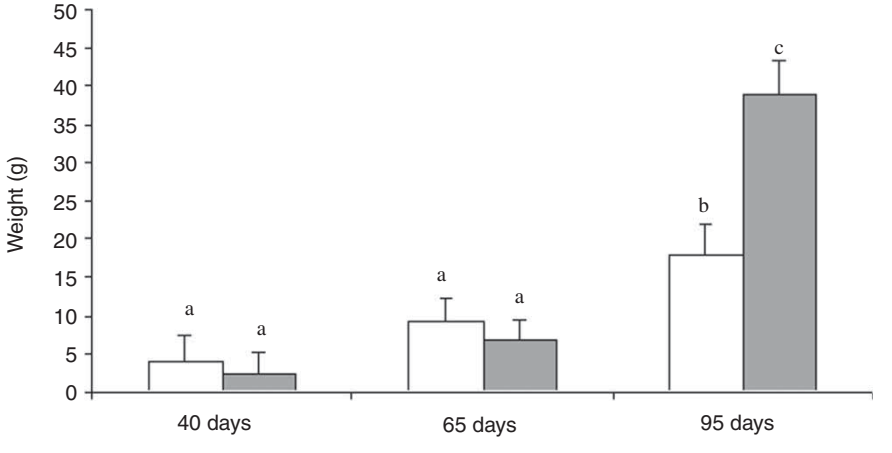

Figure 2. Total body weight (g) in controls (white bars) and fish fed on dietary alginic acid (grey bars) ($n = 20$) at 40 days, 65 days and 95 days from the beginning of the first solid feeding. Values with different letters indicate statistical significance ($p < 0.05$).

4. Discussion

In recent years the health and well-being of animals farmed in intensive culture systems has been the focus of significant public debate [19,20].

In aquaculture practice, high stocking densities, crowding and sub-optimal chemical and physical water parameters are the major causes of stress occurring in reared animals, which,

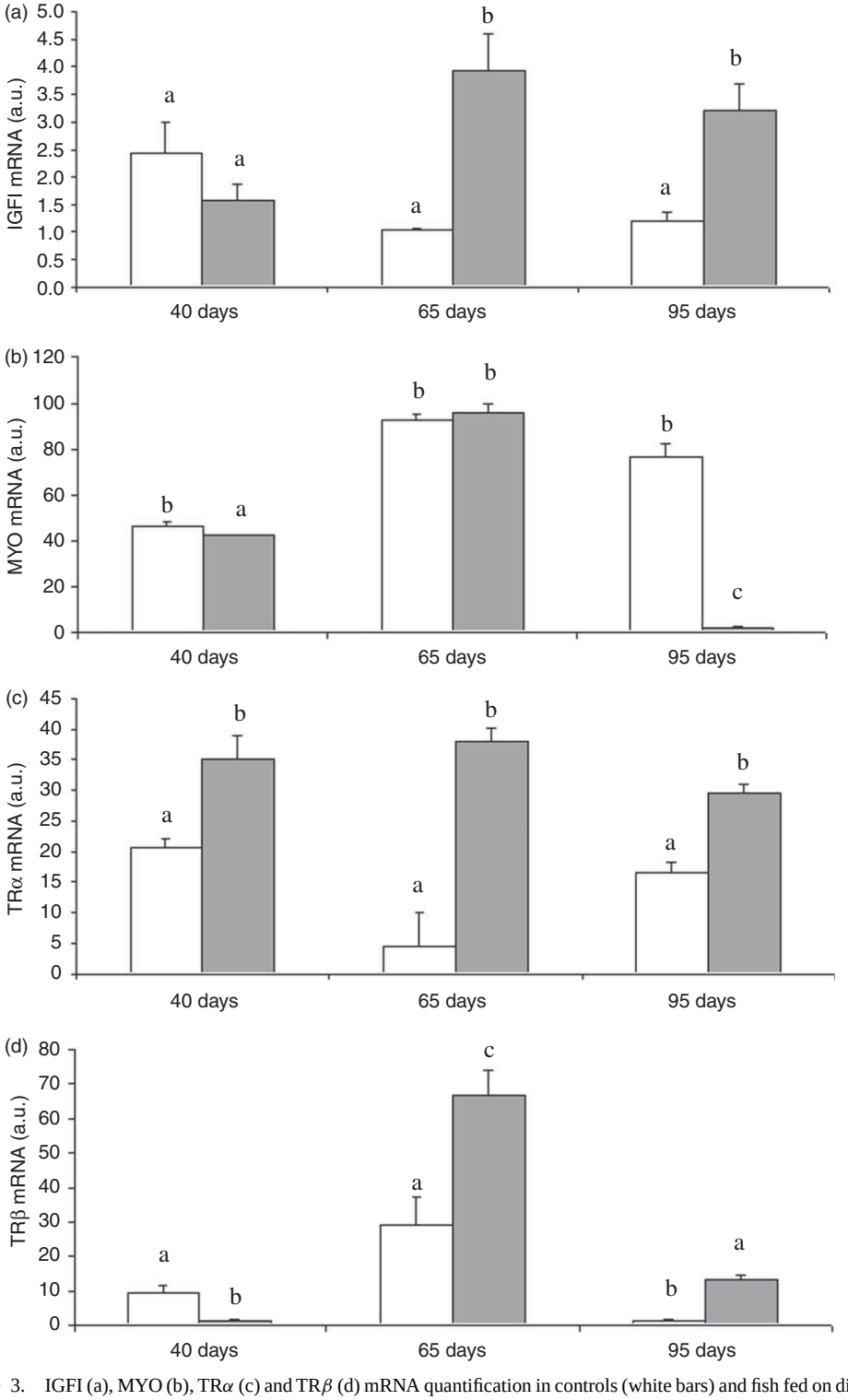

Figure 3. IGFI (a), MYO (b), TRα (c) and TRβ (d) mRNA quantification in controls (white bars) and fish fed on dietary alginic acid (grey bars) at 40 days, 65 days and 95 days from the beginning of the first solid feeding. Values with different letters indicate statistical significance ($p < 0.05$).

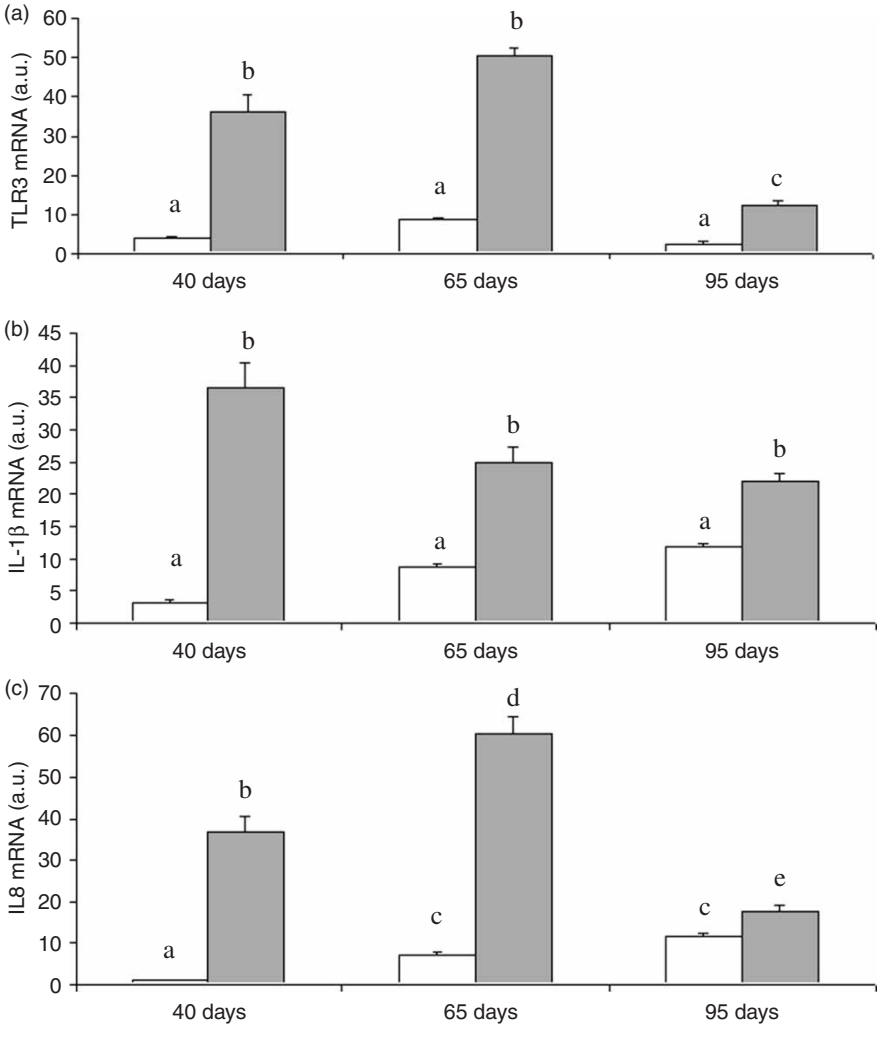

Figure 4. TLR3 (a), IL-1β (b) and IL8 (c) mRNA quantification in controls (white bars) and fish fed on dietary alginic acid (grey bars) at 40 days, 65 days and 95 days from the beginning of first solid feeding. Values with different letters indicate statistical significance ($p < 0.05$).

in turn, may result in an increase in the incidence of disease [21,22]. For this reason it was important to obtain basic information on the molecular mechanisms controlled by immunomodulators during the early life history in rainbow trout.

The data obtained in this work suggest that the daily administration of an alginic acid source can represent an alternative procedure to drug administration in order to improve the welfare of rainbow trout juveniles by enhancing growth performance and the innate immune system in addition to lowering the cortisol and HSP70 levels.

It is well-known that in vertebrates, chronic stress may activate the neuroendocrine pathway, including the hypothalamic–pituitary–interrenal axis, causing cortisol release into the blood [23]. In teleosts, cortisol is the principal corticosteroid hormone and the evaluation of plasma cortisol levels is a common practice in estimating their stress status [24,25]. High cortisol levels are generally considered deleterious to the immune system, inhibiting inflammatory response and antibody production [26]. In addition, as observed in mammals, glucocorticoid induces catabolic

and antianabolic effects [27] and delays somatic growth [28]. Another common signal of stress in fish, as well as in mammals, are the proteins belonging to the HSP family. These components play an important chaperoning role, allowing the cells to cope with acute stressor insults, especially those affecting protein machinery [29]. For this reason, HSPs are good indicators, at a molecular level, of sub-lethal cellular damage as a result of environmental stress [30].

The data obtained in this study showed the increase in both HSP70 gene expression and plasma cortisol levels at the experimental times tested in the control group, indicating that the farming conditions at which the fish were kept became increasingly stressful.

On the contrary, in the treated group, significantly lower levels of HSP70 gene expression, associated with lower plasma cortisol levels, were found at all experimental times, indicating a better tolerance to chronic stressful rearing conditions in fish fed on alginic acid.

Some components of the endocrine system are also known to directly or indirectly regulate different aspects of growth in fish. These include insulin-like growth factor-I (IGF-I), myostatin and thyroid hormones [31,32]. IGF-I in fish has been shown not only to stimulate DNA synthesis, cartilage sulphation and protein synthesis, but also to enhance salt-water adaptability, stimulate spermatogenesis and induce final oocyte maturation [33–35]. Furthermore, administration of recombinant IGF-I to salmon has resulted in growth stimulation [36] and, more recently, it has been demonstrated that circulating levels of IGF-I are positively correlated with growth rate [37–43] and could be used as an important growth index in fish. Moreover, it has also recently been shown that muscle growth is regulated by the interplay of positive and negative signals: among the negative signals, MYO has been identified. Myostatin is a member of the transforming growth factor-β (TGF-β) super family. It was first characterised in the mouse, where it is expressed during embryogenesis in developing somites [44] and in adult skeletal muscle. Reduction of myostatin gene expression in mice resulted in a highly muscled phenotype, with both muscle hypertrophy and hyperplasia [44]. In fish, studies on MYO gene regulation [45–49] have only recently been performed, confirming the negative role of this signal in muscle growth.

Several observations suggest that the thyroid hormones (THs) have a role in the early development of fish embryos. For example, post-hatched fish embryos immersed in solutions of THs exhibit accelerated patterns of development [50], indicating the presence of TH receptors (TRs) at that developmental stage. In addition, mRNA transcripts encoding for the two known TR isoforms, TRα and TRβ, have been found in oocytes and early embryo stages of several fish species [32,51–54], suggesting a possible TR role in the development of fish embryos and juveniles.

The expression levels of all genes controlling the improvement of growth (IGFI, TRα and TRβ) were found to be significantly increased at all the experimental times in fish fed on alginic acid. In addition, from data obtained here, the myostatin gene seems to play a crucial part in controlling growth at this stage of the lifecycle. The significant decrease in its mRNA levels in treated animals found at 95 days post-hatching is concomitant with the significant increase in body weight found at the same time.

Finally, the constant presence of different stressors (crowding, handling, environmental changes, etc.) can negatively affect fish immune systems [12,21,22]. Thus, the up-regulation of immunity immediately prior to or during a stressful period should protect fish from pathogenic infections. Innate immune mechanisms act as a first line of defence against infection by inducing inflammation as an early immune response. As remarkable progress has been achieved in isolating and characterising cytokine genes from fish in recent years [55], many researchers have used the mRNA expression of cytokine genes as a tool for measuring immune responses [56–59]. In this study we focused our attention on the expression of TLR3, IL1-β and IL8 genes in the spleen, one of the most important haematopoietic organs in fish.

Toll-like receptors (TLRs) are a family of transmembrane proteins that recognise conserved pathogen structures in order to induce innate immune effector molecules [60]. TLR3 is involved in the recognition of double-stranded RNA (dsRNA) which is produced during the replication

of many viruses [61,62]. TLR3 activation leads to cytokine secretion [62,63]. IL-1β is a key mediator in response to microbial invasion and tissue injury, and can stimulate immune responses by activating lymphocytes or by inducing the release of other cytokines that are able to activate macrophages, NK cells and lymphocytes [64,65]. The chemokine IL8 is a small secreted cytokine involved in the control of the traffic of immune cells [66].

The data obtained in this study confirmed the immunomodulatory role of an alginic acid source by enhancing the expression of the three innate immune-related genes investigated here throughout the experimental period, evidencing for the first time the involvement of alginic acid in the modulation of TLR3 transcription.

The usage in aquaculture of a natural immunomodulator to enhance animal welfare can be considered a biotechnological tool for further development of a sustainable and environmentally friendly aquaculture.

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