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Effect of yeast (*Xanthophyllomyces dendrorhous*) and plant (Saint John's wort, lemon balm, and rosemary) extract based functional diets on antioxidant and immune status of Atlantic salmon (*Salmo salar*) subjected to crowding stress

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ABSTRACT

Salmon farming may face stress due to the intensive culture conditions with negative impacts on overall performance. In this aspect, functional feed improves not only the basic nutritional requirements but also the health status and fish growth. However, to date no studies have been carried out to evaluate the effect of functional diets in salmon subjected to crowding stress. Thus, the aim of this study was to evaluate the effect of yeast extract (*Xanthophyllomyces dendrorhous*; diet A) and the combination of plant extracts (common Saint John's wort, lemon balm, and rosemary; diet B) on the antioxidant and immune status of Atlantic salmon grown under normal cultured conditions and then subjected to crowding stress. Fish were fed with functional diets during 30 days (12 kg/m^3) and then subjected to crowding stress (20 kg/m^3) for 10 days. The lipid peroxidation in gut showed that both diets induced a marked decrease on oxidative damage when fish were subjected to crowding stress. The protein carbonylation in muscle displayed at day 30 a marked decrease in both functional diets that was more marked on the stress condition. The expression of immune markers (IFNγ, CD4, IL-10, TGF-β, IgM$_{mb}$, IgM$_{sec}$, T-Bet, and GATA-3) indicated the upregulation of those associated to humoral-like response (CD4, IL-10, GATA-3) when fish were subjected to crowding stress. These results were confirmed with the expression of secreted IgM. Altogether, these functional diets improved the antioxidant status and increased the expression of genes related to Th2-like response suggesting a protective role on fish subjected to crowding stress.

1. Introduction

Aquaculture is the fastest growing animal food-producing industry, outpacing population growth. In this context, Atlantic salmon (*Salmo salar*) production has increased strongly in recent decades thanks to the expansion in the northern Europe and in North and South America with Norway and Chile as the mains world producers [1]. This fast and continued growing in the salmon industry has side effects. Under intensive culture conditions, fish may be exposed to several environmental and husbandry related stimuli that may have a potential noxious or stressful effect. All these factors have negative impacts on fish welfare and overall performance. Also, a higher susceptibility to disease

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has been observed since reduced immune response allows pathogens to act with greater efficiency [2] with an impact in the industry causing sanitary crisis and economic losses [3].

It is widely accepted that fish nutrition has major health implications and therefore diets should be formulated according to the fish industry health requirements. Moreover, feeds probably represent the single largest expense to the industry and hence must be fully integrated into health management strategies for aquaculture [4]. One strategy to potentially reduce this harmful effect in the fish health is through the application of functional feeds. A functional feed is a diet which contains components that provide specific benefits to the host beyond the basic nutritional requirement [5]. These diets may improve fish health and welfare. There is an increasing interest in the use of functional feeds as a preventive strategy against environmental threats. The functional diet compounds include a broad range of products such as pre and probiotics, vitamins, minerals, plant and algae extracts, most of them with several biological functions such as antioxidants and immunostimulants properties [4].

There is an increasing interest on natural stimulants for the characteristics that their constituent compounds possess in conferring fish protection. On the last fifteen years several studies using yeast extracts administered by diet in different species have been evaluated [6]. Despite the wide range of studies performed in this area, functional diets have been general and typically analyzed from the evaluation of growth, stress, and immune response-associated markers (mainly from the analysis of non-specific markers). From the point of view of functional diets evaluation, the studies have typically focused on the exposure to pathogens [6]. Therefore, the study of the capacity to improve the health status of fish fed with functional diets is mainly based on the higher accumulated survival against the exposure to a pathogen of interest. Therefore, the potential effect on the health status of fish fed with functional diets to the exposure of stressors currently present in the aquaculture industry has been less described. In fact the effect of supplemented diets has been only evaluated in fish subjected to salinity [7], anoxia [8] and hypoxia [9], ammonia [10], thermal [11], starvation [12], pH [13] and handling [14]. The effect of functional diets in fish subjected to acute crowding stress has been evaluated in hapuku (*Polyprion oxygeneios*) [15] and tilapia (*Oreochromis niloticus*) [16], meanwhile to chronic crowding stress has been analyzed only in sea bream [17], rainbow trout [18], and carp [19]. However, and despite the economic interest that Atlantic salmon represents and considering the high stocking density at industrial rearing conditions, as far as we know no studies have carried out to evaluate the effect of functional diets in salmon subjected to crowding stress. For this reason, it has turned fundamental to expand the knowledge about the effect of functional diets to other matters of interest for the aquaculture industry, particularly on the beneficial effect of these strategies on the stress and its relation with immune related markers to improve the fish farmed welfare status.

The use of yeast extract as supplement diet has been classified as pathogen-associated molecular pattern (PAMP) immunostimulant diet due to the presence of structurally conserved pathogenic components [6]. Thus, it is not surprising that β-glucan be one of the most used yeast PAMPs with immunostimulant effect in fish. However, the utilization of other yeast compounds has been used to exploit the yeast stimulants properties. Among them, astaxanthin (3,3′-dihydroxy-b,b-carotene-4-4′-dione) is a high-value carotenoid which has concentrated special interest. In rainbow trout (*Oncorhynchus mykiss*, Walbaum) it was observed its immunostimulant role, increasing the alternative complement activity and phagocityc rate associated with dietary intake of astaxanthin from red yeast (*Phaffia rhodozyma*) [20]. Astaxanthin stands out for its antioxidant properties, whose effect have been reported to be greater than other classical antioxidant carotenoid compounds such as β-carotene and α-tocopherol [21]. Accordingly, it has been reported that astaxanthin rich red yeast has a reducing effect on oxidative stress in rainbow trout decreasing the lipid peroxides level [22].

Since the increased attention in the last years on the utilization of specific compounds with beneficial properties for aquaculture, one of the most important strategies is the use of dietary antioxidants. Diverse efforts have been made in order to evaluate the effects of plant extracts in various fish species as dietary supplement [6]. In this matter, the utilization of promising new sources of natural antioxidants as bioactive compounds opens the possibility to the development of new functional feeds. *Hypericum perforatum* (also called common Saint John's wort, Family Hypericaceae) is a flowering plant commonly used as medicine herb. Hypericin has been identified as one principal constituent of St. John's wort. Its properties include its use as medicine in the treatment of depression but also have long been known to possess antibacterial and antiviral activity [23]. St. John's wort also contains tannins and flavonoids which could be involved in the significant antioxidant activity inhibiting the oxidative damage [24].

Melissa officinalis (also named lemon balm, Family Lamiaceae) is a perennial herbaceous and commonly used medicinal plant. Pharmacological reports revealed that *M. officinalis* possess sedative, carminative, antispasmodic, antibacterial, antiviral, anti-inflammatory and neuroprotective therapeutic properties [25,26]. Particularly, lemon balm has a high antioxidant capacity mainly due to the presence of phenolic compounds as rosmarinic acid [27]. Another member of the Family Lamiaceae is *Rosmarinus officinalis* (commonly known as rosemary or “dew of the sea”) is a perennial herb commonly used for culinary and medicinal purposes, mainly due to its aromatic properties and health benefits [28]. The biological effect of this plant includes antibacterial and antifungal activity, and anti-inflammatory properties among others, and those effects are mainly related to the phenolic and volatile constituents [29]. The antioxidant activity of rosemary has been also reported and it is hypothesized the role of phenolic [30] and volatile constituents [31]. Therefore, the utilization of common Saint John's wort (*Hypericum perforatum*), lemon balm (*Melissa officinalis*), and rosemary (*Melissa officinalis*) opens the possibility to develop a functional feed for fish which may provide specific benefits both for antioxidant and immunological activity. Thus, the aim of this study was to evaluate the effect of yeast extract (*Xanthophyllomyces dendrorhous*) and the combination of plant extracts with previously reported antioxidant properties (common Saint John's wort, lemon balm, and rosemary) on the antioxidant and immune status of Atlantic salmon grown on normal cultured conditions and then subjected to crowding stress in order to evaluate their utilization as functional feed supplement. Antioxidant parameters (lipid peroxidation and protein carbonylation) and the expression of genes associated to immune response (IFNγ, TBet, IL-10, GATA3, TGF-β, CD4) were evaluated during 30 days under normal cultured conditions. After this period, the effect of either candidate functional diet was evaluated in fish subjected to crowding stress during 10 days.

2. Materials and methods

2.1. Yeast extract

Xanthophyllomyces dendrorhous (sexual stage *Phaffia rhodozyma*) was grown at 22 °C with constant agitation in YM medium (1% glucose, 0.3% yeast extract, 0.3% malt extract and 0.5% peptone) and cells were harvested by centrifugation at 4,000*g* for 5 min. The cell pellet was washed, suspended in sterile distilled water and disrupted using glass beads (0.5 mm diameter, Sigma). The samples were shaken for 30 s in a mini beadbeater-16 homogenizer (Bio Spec, Bartlesville, USA), and subsequently cooled on ice for 1 min four times. The supernatant was harvested and lyophilized to obtain a dried extract.

2.2. Plant extract

The dry plant extract of common Saint John's wort (*Hypericum*

Table 1
Formulation and composition of experimental basal diet for Atlantic salmon fry (*Salmo salar*).

Ingredients	Reference diet (%)
Fishmeal[a]	61.99
Feather meal[b]	4.00
Corn gluten[b]	4.00
Wheat flour[c]	15.00
Fish oil[a]	13.00
Vitamin pre-mix[d]	1.00
Mineral pre-mix[d]	1.00
Inert marker (Y_2O_3)[e]	0.01
TOTAL	**100**
Estimating proximate analysis (%)[f]	
Total protein	55
Ethereal extract	18
Total ash	12
Nitrogen free extract	15
Gross energy (MJ/Kg)	20
Total phosphorus	1.5

[a] Fishery Itata S.A., Talcahuano, Chile.
[b] Biomar Chile, Pto Montt, Chile.
[c] Mills Gorbea, Gorbea, Chile.
[d] VETERQUIMICA, Puerto Montt, Chile.
[e] SIGMA, Saint Louis MO, USA.
[f] Results expressed on a dry basis.

perforatum; series #08.736.1), lemon balm (*Melissa officinalis;* series #08.735.1), and rosemary (*Rosmarinus officinalis;* series #01.452.9) were purchased from Laboratorios Ximena Polanco (San Miguel, Chile). Saint John's wort, lemon balm, and rosemary were mixed at a ratio 3:2:1 and used as dietary supplement.

2.3. Experimental diets

Two functional diets (diet A and diet B) were prepared based on the experimental basal diet for Atlantic salmon fry (Table 1). Fish and feather meal were used as dietary protein sources. Fish oil was used as lipid source. Contents of total protein, ethereal extract and ash were 55%, 18% and 12%, respectively. The yeast extract (diet A) and the plant extract (diet B) were used to separately supplement the basal diet (control diet). In the case of diet A, 0.5 g Kg^{-1} of yeast extract and 1.0 g Kg^{-1} of vitamin C were used to supplement the basal diet. By contrast, for diet B, 6 g Kg^{-1} of plant extract was used to supplement the control diet. The experimental functional feeds (diet A and diet B) were adequately prepared to enable efficient extrusion process of pellets on selects calibers (1, 2 and 3 mm). The pellets were carefully ground in an ultracentrifuge mill (Retsch ZM200 Haan, Germany) to obtain a final particle size of 150–300 μm. The homogenized mixture of ingredients was extruded in a twin-screw extruder (Clextral BC21, Firminy, France) and the pellets were dried at a constant temperature of 60 °C in a vertical dryer (COMIND, Santiago, Chile). The yeast extract (in the case of diet A) and plant extract (for diet B) was added to the feed together with the pellet coating with fish oil in a vacuum oiler system (Dinnissen VC10 Horsterweg, Netherlands).

2.4. Fish and experimental design

The Atlantic salmon (*Salmo salar*) fry (4.83 ± 0.2 g) were obtained from a local farm. During two weeks, the fish were acclimated in optimal density culture conditions (12 kg/m^3) and controlled conditions of pH, oxygen and nitrogen compounds (nitrite, nitrate, ammonia) levels. The experiment was carried out in a recirculating aquaculture system (water flow rate 0.5–1.0 L/min). During this process fish were fed with Golden Optima (Biomar, Puerto Montt, Chile). Six groups of 70 fish each were stocked in tanks (100 L) in triplicates at 15 °C and continuous air support. Fish were fed with diet A, diet B or control diet in optimal density culture conditions (12 kg/m^3). This time-point was considered the begins of the experiment (day 0). Atlantic salmon were fed thrice daily at a rate of 1% wet body weight for 40 days. Fish were slowly hand-fed to prevent the waste pellet accumulation. At day 30, fish were subjected to crowding stress (20 kg/m^3) reducing the level of water in tanks. During 10 days, the fish were maintained under this stress condition and then the fish samples were obtained immediately. The possible improvements on antioxidants and immune status were analyzed and compared in fish fed with the functional diets (which contain a plant or yeast extract as supplement), in order to determine if they have a beneficial effect on fish subjected to crowding stress in regard to fish fed with control diet that contain the background nutrients used in the functional diets and their basal effect when subjected to same crowding stress.

2.5. Sample collection

The first fish samples groups were obtained after 20 days that the fish were fed with control diet or some of two candidate functional diets. The second fish samples group were obtained after 30 days that the fish began to be fed with some evaluated diet (equivalent to day 0 that the fish were subjected to stress condition). The third fish sample group were obtained after 40 days of feeding with some evaluated diet (equivalent to day 10 that the fish were subjected to stress condition). In all sample time-points, nine fish (three fish from each triplicate tank) per dietary treatment were sacrificed by an overdose of MS-222 (Sigma-Aldrich). Gut and muscle were taken to determine oxidative stress parameter, while the spleen was used to determine the immune response-related gene modulation by real time-PCR. The organs were collected and immediately frozen in liquid nitrogen and stored at −80 °C until use.

If some of the two candidate functional diets had benefits on oxidative or immune parameters was analyzed independently for each sample time, comparing the results obtained for the diet A, diet B and the control diet.

All experiments were carried out in accordance with the ethical standards of the Institutional Ethics Committee of Universidad de Santiago de Chile and the legislation in force.

2.6. Assays of oxidative stress

For the detection of thiobarbituric acid reactive substances, the gut (n = 9) was macerated adding phosphate buffer (NaCl 0.14 M, KCl 2.7 mM, Na2HPO4·2H2O 8 mM and KH2PO4 1.76 mM). Then, trichloroacetic acid (TCA) 10% w/v (Sigma, St Louis, MO, USA) was added and centrifuged at 13000*g* for 15 min. The supernatant was mixed with thiobarbituric acid (TBA) (Sigma) in a ratio 1:1 and incubated for 1 h at 90 °C. The absorbance at 535 nm was measured in triplicated in spectrophotometer Infinitive 200Pro (TECAN Austria GmbH, Grodig, Austria). The results were obtained from malondialdehyde (MDA) standard curve according to protocol describes by Esterbauer et al. [32]. The results are expressed in moles of MDA/mg of tissue. The modulation on lipid peroxidation level between fish fed with each candidate functional diet and control diet on each time-point evaluated was expressed as the percentage of increase or decrease of MDA/mg of tissue in fish fed with experimental diet versus fish fed with control diet according to the formula (100-(diet*100/control)) and represented as ΔFunctional diet (A or B)/Control.

Determination of carbonyl groups was performed by dividing the muscle in 3 parts, which were each one macerated with phosphate buffer (NaCl 0.14 M, KCl 2.7 mM, $Na_2HPO_4{\cdot}2H_2O$ 8 mM and KH_2PO_4 1.76 mM). Then, TCA was added to 50% w/v (Sigma). The mixture was centrifuged at 13.000 g for 15 min, the supernatant was removed and the pellet retained. One vol of 0.3% w/v 2,4- dinitrophenylhydrazine (Sigma) was added to the pellet. The samples were incubated for 1 h.

The pellet was then washed 3 times with absolute ethanol (Merck) and ethyl acetate (Merck) in ratio 1:1 for 10 min. The washed pellet was dried at 37 °C and subsequently incubated with urea 6 M for 1 h. Protein content was then quantified by the method of Bradford. The carbonyl content was measured by spectrophotometer Infinitive 200Pro (TECAN Austria GmbH) at 370 nm [33]. The results are expressed in nmoles of carbonyls/mg of proteins. The modulation on protein carbonylation level between fish fed with each candidate functional diet and control diet on each time-point evaluated was expressed as the percentage of increase or decrease of carbonyls/mg of proteins in fish fed with experimental diet versus fish fed with control diet according to the formula (100-(diet*100/control)) and represented as ΔFunctional diet (A or B)/Control.

2.7. RNA extraction and cDNA synthesis

Spleen was homogenized in 1 mL of TRIsure (Bioline, London, UK) according to the manufacturer's instructions and using tissue master cell disruptor (Omni, Kennesaw, GA, U.S.A.). Total RNA pellet was resuspended in RNase-free water and quantified spectrophotometrically in Infinitive 200Pro (TECAN Austria GmbH). RNA (1.5 μg) was treated with RQ1 RNase free DNase (Promega) and cDNA synthesis was performed using reverse transcriptase M-MLV (Promega) and Oligo dT (Promega) following the manufacturer's recommendations. The cDNA samples were stored until use at −20 °C.

2.8. Real time PCR

The relative quantification of selected immune response gene markers was performed by qPCR. The genes evaluated were interferon gamma (IFNγ), T-Bet, Interleukin 10 (IL-10), GATA3, transforming growth factor beta (TGF-β), CD4, membrane and secreted immunoglobulin M (IgM) isoforms. Primer sequences for the different genes are listed in Table 2. The qPCR reaction was developed in 20 μL with SensiMix™SYBR Kit (Bioline, Taunton, MA, U.S.A.) containing 5 μM forward primers and 5 μM reverse primers and 2 μL cDNA. Reactions were run in triplicates in a thermalcycler Mx Pro3000P (Stratagene, U.S.A.). qPCR parameters were 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s, 60 °C for 15 s and 72 °C for 15 s. The gene expression levels are shown normalized to fish fed with control diet and relative on the basis of the quantification of eF1α expression in all cases (normalized relative expression, NRE).

2.9. Statistical analysis

Data of oxidative parameters and cytokine gene expression levels were compared by Two-way ANOVA test with Bonferroni multiple comparisons post-test. GraphPad software v5.0 for Windows (GraphPad Software) was used to calculate the mean and standard error of the mean (SEM) and to perform statistical tests. P value less than 0.05 was considered statistically significant.

3. Results

3.1. Determination of oxidative stress parameters in fish under crowding stress and fed with functional diets

To evaluate the antioxidant effect of functional diets, the lipid peroxidation was determined by the TBA reaction in the gut of Atlantic salmon fry collected after 20, 30 and 40 days of feeding with a basal diet (control) supplemented with yeast (diet A) and plant extract (diet B). The results showed that after 20 days of feeding none of the diets produced statistical significant changes on lipid peroxidation in regard to the fish fed with basal diet (control diet) (Fig. 1A). However, at day 30 of feeding the level of lipid peroxidation decreased a 39% in diet A compared to basal diet (Fig. 1A). Importantly, a lower lipid peroxidation was observed in fish subjected to crowding stress during 10 days (day 40 of feeding) fed with either functional diet (52% and 44% decrease in diet A and diet B compared to control, respectively) (Fig. 1A). Thus, diets A and B induced a marked decrease on lipid peroxidation compared to control diet in fish fed with functional diets at 30 days of feeding and also when those fish were subjected to crowding stress through 10 days (Fig. 1A).

To evaluate whether the administration of functional feeds supplemented with yeast and plant extract diminished the level of other oxidative stress parameters, the protein carbonylation levels was determined in muscle of Atlantic salmon fry collected after 20, 30 and 40 days of feeding with functional candidate or control diets in order to determine the protein damage. The results showed that after 20 days of feeding the protein damage increase in fish fed with diet A (42%) while in diet B no differences in regard to the control diet were observed (Fig. 1B). At day 30 a marked decrease (58% diet A; 69% diet B) was observed in either functional diet. This decrease was even more marked on day 40, when the protein carbonylation dropped 94% in diet A and 95% in diet B, thus almost reaching the basal protein carbonylation level in stressed fish fed with functional diets but not in the control diet, whose value remained constant during the experiment (Fig. 1B).

Taking together these results indicate that functional diets based on

Table 2
Sequence of primer used in real-time PCR analysis.

Gene	Sequence (Fw/Rv) 5′→3′	Amplicon size (pb)	Amplification efficiency (%)	GenBank acc. number
eF1a	GGGTGAGTTTGAGGCTGGTA TTCTGGATCTCCTCAAACCG	156	105,6	NM_001141909
IFNγ	CCGTACACCGATTGAGGACT GCGGCATTACTCCATCCTAA	133	108,2	AY795563
CD4	TCCTCATCGAAGGCACCAATGCTA TCTGACAGGGCCACAGAGTTGAA	157	103,3	NM_001146408
IL-10	GAGGCTAATGACGAGCTGGAG GTCAAACGGTTTCTTACAGGAG	255	98,2	EF165028
TGF-β	AGCTCTCGGAAGAAACGACA AGTAGCCAGTGGGTTCATGG	136	104,5	EU082211
$_{mb}$IgM	GAGGACTGGAGCAATGGGAC CTCCAACGCCATACAGCAGAG	217	93,3	Y12457
$_{sec}$IgM	TCCACAGCGTCCATCTGTCT GTTCTCTCCACCGGCTCATC	144	90,6	AY870259
T-Bet	TACCAACGGGAAGCGAAGGT TCCGCCCCTGTTTCGTTATG	225	98,8	GU979861
GATA-3	ATGGACCCCTCGCAGTATCC TGCAGTGAGCTCTTCTGGAAA	257	98,1	NM_001171800

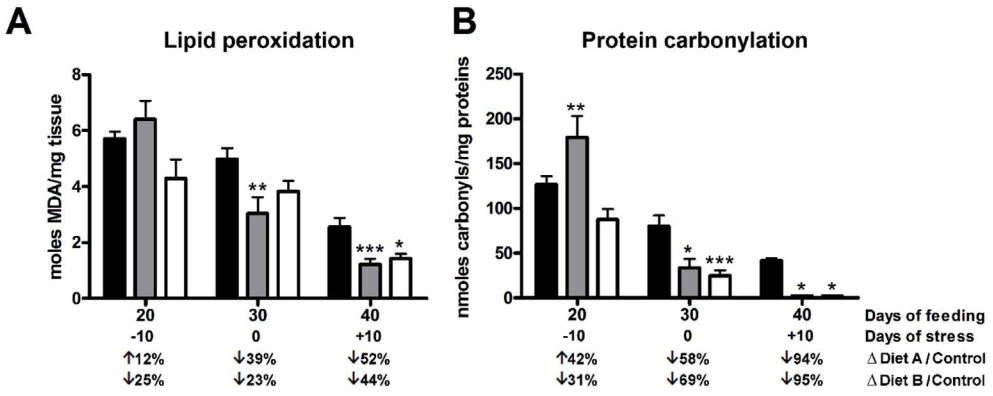

Fig. 1. Antioxidant effects of functional diets on antioxidant status in Atlantic salmon. Fish were fed with functional diets supplemented with yeast (*Xanthophyllomyces dendrorhous*, diet A) or plant (Saint John's wort, lemon balm, and rosemary, diet B) extract. At day 30 of feeding, fish were subjected to crowding stress (day zero of stress). The stress condition was maintained during 10 days while fish were fed with the same preceding feeding regimen. Statistically significant differences on the antioxidant effect of diet A (grey bars) and B (white bars) compared to fish fed with control diet (black bars) were determined by two-way Anova with Bonferroni post-test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). (A) Lipid peroxidation. Two-way ANOVA test shows a significant effect of time [$F(2, 133) = 44.39$; p-value $<$ 0.0001], diet [$F(2, 133) = 5.489$; p-value = 0.0051], and also the interaction between these two variables [$F(4, 133) = 3.508$; p-value = 0.0093]. (B) Protein carbonylation. Two-way ANOVA test shows a significant effect of time [$F(2, 115) = 86.54$; p-value $<$ 0.0001], diet [$F(2, 115) = 13.77$; p-value $<$ 0.0001], and also the interaction between these two variables [$F(4, 115) = 7.652$; p-value $<$ 0.0001]. ΔDiet A/Control: indicate the increase (↑) or decrease (↓) of diet A compared to basal diet in each antioxidant parameter tested. ΔDiet B/Control: indicate the increase (↑) or decrease (↓) of diet B compared to basal diet in each antioxidant parameter tested.

yeast (*Xanthophyllomyces dendrorhous*) and the mixing of plant extract (common Saint John's wort, lemon balm, and rosemary) with previously reported antioxidant properties, produced a lower long-term oxidative damage effect in Atlantic salmon.

3.2. Effect of functional diets on immune gene expression profile in Salmo salar

To evaluate the effect of functional diets on fish immune status, the gene expression level of molecules associated with pro- and anti-inflammatory immune response were determined by qRT-PCR in spleen of Atlantic salmon fry. The expression of genes related to Th1 response was evaluated. The expression of TBet (known as the major transcription factor responsible for the expression of IFNγ) did not vary at 20 nor 30 days of feeding with both diets but it did after 10 days of crowding stress although only in the case of diet A (2.3 fold; Fig. 2A). However, no correlation was observed for IFNγ whose expression increased after 20 days (2.3- and 3.6-fold in fish fed with diet A and diet B, respectively) but not at 30 days of feeding in fish fed with both diets (Fig. 2B). No changes were either noted in the expression of IFNγ in fish subjected to crowding stress.

The expression of genes associated to Th2 immune response was also analyzed. The expression of GATA-3 (T cell specific transcription factor essential for the development of the T-cell lineage and Th2 differentiation) showed that either functional diet induced the expression of GATA-3 on day 30 of feeding (5.1 fold on diet A and 5.6 fold on diet B compared to control diet) and also at 10 days of crowding stress (7.9 fold on diet A and 4.6 fold on diet B) (Fig. 2C). The expression of IL-10 was upregulated only in those fish fed with diet A after 10 days of crowding stress (3.8 fold; Fig. 2D). The expression of other anti-inflammatory cytokine as TGF-β showed an increase on its expression at 20 days of feeding in fish fed with diet A (3.1-fold) and diet B (2.3-fold) compared to control diet (Fig. 2E).

The expression of CD4 (surface T cell marker) was upregulated with either functional diet (2.4 fold on either diet; Fig. 2F) after 10 days of crowding stress.

Thus, the results indicate that a controlled pro-inflammatory response could take place in fish fed with functional feed under normal cultured conditions. The feeding with functional diets during 30 days could help to promote the expression of immune markers associated to humoral-like response (CD4, IL-10, GATA-3) when fish were subjected to crowding stress.

3.3. Effect of functional diets on humoral response in Salmo salar at gene expression level

The effect of diets on humoral immune status was evaluated by gene expression analysis of membrane and secretory IgM isoforms. At day 20 of feeding the expression of membrane IgM increased in either diet (2.2 fold with diet A and 3.2 fold with diet B) and then decreased on days 30 and 40 of feeding with no differences comparable to control diet (Fig. 3A). Conversely, secreted IgM showed an inverse effect than the observed for membrane IgM, noting at day 20 and 30 of feeding a significant increase only in diet B (3.6 and 3.4 fold, respectively). At 10 days of crowding stress (day 40 of feeding) this augment was observed in either functional diet in which the gene expression increased reaching even 3.4 fold with diet A and 5.5 fold with diet B (Fig. 3B).

Altogether, these results indicate that either functional diet increase the expression of membrane IgM, while the expression of secreted IgM is upregulated later and in an increasing manner in those fish subjected to crowding stress.

4. Discussion

The aim of this study was to evaluate the effect of functional diets based on yeast (*Xanthophyllomyces dendrorhous*; diet A) and the combination of plant extracts with previously reported antioxidant properties (obtained from mixing of common Saint John's wort, lemon balm, and rosemary; diet B) on the antioxidant and immune status in Atlantic salmon grown to normal cultured conditions and then subjected to crowding stress. The utilization of diets containing antioxidants in fish subjected to stress reduced the oxidative damage and modified the expression of key cytokines in the regulation of the immune response, conferring protection to fish subjected to crowding stress. Thus, the use of these compounds as supplement can improve the fish health status and therefore can be considered as an interesting alternative to formulate new functional feeds.

A common practice in the evaluation of new functional diets is the impact of these on the growth performance of fish, basically based on the diet-based potential growth performance improvements [6]. In our study, the effects of two functional diets were evaluated. No significant differences were observed among these functional diets and control diet in all the growth performance parameters tested (Supplementary Table). Despite these results, the nutritional approaches may have other effects at physiological level that could influence to keep animal welfare and contribute to mitigate or alleviate threats in farmed aquatic animals such as stressors. To respond against a stressor, several mechanisms are activated in the host to cope with the allostatic load produced by the stressor and recover the balance throughout

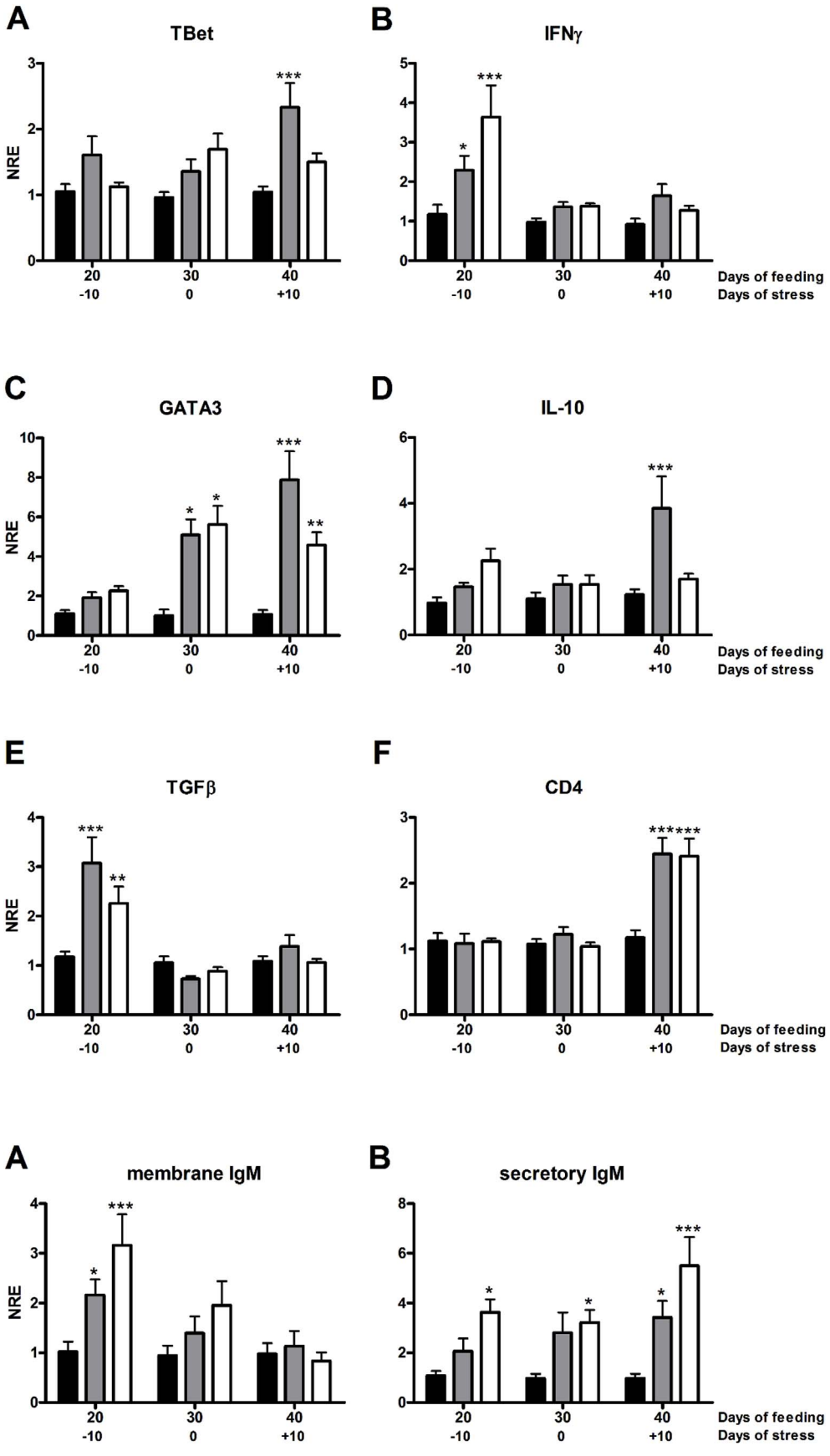

Fig. 2. Effect of functional diets on immune gene expression profile in Atlantic salmon. Fish were fed with functional diets supplemented with yeast (*Xanthophyllomyces dendrorhous*, diet A) or plant (Saint John's wort, lemon balm, and rosemary, diet B) extract. At day 30 of feeding, fish were subjected to crowding stress (day zero of stress). The stress condition was maintained during 10 days while fish were fed with the same preceding feeding regimen. Data is shown as the mean normalized relative expression (NRE) ± SE. Statistically significant differences on the antioxidant effect of diet A (grey bars) and B (white bars) compared to fish fed with control diet (black bars) were determined by two-way Anova with Bonferroni post-test (*p < 0.05; **p < 0.01; ***p < 0.001). (A) TBet. Two-way ANOVA test shows no significant effect of time [F(2, 190) = 2.612; p-value = 0.0760], but diet [F(2, 190) = 8.734; p-value = 0.0002], and also the interaction between these two variables [F(4, 190) = 2.79; p-value = 0.0277]. (B) IFNγ. Two-way ANOVA test shows a significant effect of time [F(2, 180) = 8.092; p-value = 0.0004], diet [F(2, 180) = 5.429; p-value = 0.0051], and also the interaction between these two variables [F(4, 180) = 2.837; p-value = 0.0258]. (C) GATA3. Two-way ANOVA test shows a significant effect of time [F(2, 175) = 6.709; p-value = 0.0016], diet [F(2, 175) = 9.383; p-value = 0.0001], and also the interaction between these two variables [F(4, 175) = 3.225; p-value = 0.0139]. (D) IL-10. Two-way ANOVA test shows a significant effect of time [F(2, 206) = 3.516; p-value = 0.0315], diet [F(2, 206) = 5.352; p-value = 0.0054], and also the interaction between these two variables [F(4, 206) = 3.974; p-value = 0.0040]. (E) TGFβ. Two-way ANOVA test shows a significant effect of time [F(2, 216) = 24.25; p-value < 0.0001], diet [F(2, 216) = 5.449; p-value = 0.0049], and also the interaction between these two variables [F(4, 216) = 5.882; p-value = 0.0002]. (F) CD4. Two-way ANOVA test shows a significant effect of time [F(2, 234) = 34.86; p-value < 0.0001], diet [F(2, 234) = 8.061; p-value = 0.004], and also the interaction between these two variables [F(4, 234) = 7.501; p-value < 0.0001].

Fig. 3. Effect of functional diets on humoral response markers at gene expression level in Atlantic salmon. Fish were fed with functional diets supplemented with yeast (*Xanthophyllomyces dendrorhous*, diet A) or plant (Saint John's wort, lemon balm, and rosemary, diet B) extract. At day 30 of feeding, fish were subjected to crowding stress (day zero of stress). The stress condition was maintained during 10 days while fish were fed with the same preceding feeding regimen. Data is shown as the mean normalized relative expression (NRE) ± SE. Statistically significant differences on the antioxidant effect of diet A (grey bars) and B (white bars) compared to fish fed with control diet (black bars) were determined by two-way Anova with Bonferroni post-test (*p < 0.05; **p < 0.01; ***p < 0.001). (A) Membrane IgM. Two-way ANOVA test shows a significant effect of time [F(2, 182) = 7.147; p-value = 0.0010], diet [F(2, 182) = 4.8; p-value = 0.0093], but not the interaction between these two variables [F(4, 182) = 2.395; p-value = 0.0521]. (B) Secretory IgM. Two-way ANOVA test shows a significant effect of diet [F(2, 192) = 17.74; p-value < 0.0001], but not in time [F(2, 192) = 2.516; p-value = 0.0834], nor the interaction between these two variables [F(4, 192) = 1.114; p-value = 0.3373].

physiological systems in order to regain homeostasis [2]. In this study, the effect of functional diets on the susceptibility of *Salmo salar* to lipid peroxidation and protein carbonylation were evaluated both in an appropriated culture condition (12 kg/m3) as also in *Salmo salar* subjected to crowding stress. These levels of lipid peroxidation and protein carbonylation were compared to oxidation levels in fish in both conditions but fed with control diet. Lower oxidation rates were registered in fish subjected to crowding stress during 10 days and fed with functional diets. Thus, diet A and diet B contribute to improve the physiological antioxidant status. Aquatic animals are likely to suffer oxidative stress when cultured under high stocking densities and the endogenous antioxidant system plays a crucial role in protecting against oxidative stress [34,35]. However, the high stock density may block the activity of metabolic and antioxidant enzymes causing considerable oxidative stress and undesirable results for fish welfare by disrupting the physiological balance [36].

Oxidative stress occurs when the balance between antioxidants and ROS are disrupted due to depletion of antioxidants or excessive accumulation of ROS, or both. When oxidative stress occurs, cells function to counteract the oxidant effects and to restore the redox balance by activation or silencing of genes encoding defensive enzymes, transcription factors, and structural proteins [37–39]. To minimize the damaging effects of ROS, aerobic organisms evolved both non-enzymatic defenses that include compounds of intrinsic antioxidant properties, such as vitamins C and E, glutathione, and β-carotene and enzymatic antioxidant defenses, such as superoxide dismutases (SOD) catalases (CAT) and peroxidases, that protect by directly scavenging superoxide radicals and hydrogen peroxide, converting them to less reactive species [38].

The overproduction of reactive species results in oxidative stress might result in the attack of cellular components, as damage in lipids, proteins and deoxyribonucleic acid (DNA). Lipid peroxidation, mediated by free oxygen radicals causes an important destruction and damage to cell membranes since polyunsaturated fatty acids of cellular membranes are degraded with the consequent disruption of membrane integrity, changes in membrane fluidity, permeability and protein degradation, resulting in cell lysis [40,41]. The reaction of ROS with lipids is considered one of the most prevalent mechanisms of cell damage [42,43]. Thus, the functional diets A and B reduced the lipid peroxidation provoked by oxidative stress when fish were subjected to short-term chronic crowding condition. On the other hand, oxidative stress has been also linked to accelerated rates of proteolysis and protein modifications. Among them, protein carbonylation is one of the most harmful irreversible oxidative protein modifications, and considered as a major hallmark of oxidative stress-related disorders. Protein carbonylation is generally defined as an irreversible post-translational modification that yields a reactive carbonyl moiety in a protein, such as an aldehyde, ketone, or lactam [44], causing conformational changes affecting the protein activity or inducing proteasome degradation [45]. This effect is directly linked to the result observed for protein carbonylation in muscle, noting even a more marked reduction after functional feed treatment compared to control diet.

These antioxidant activities observed in either functional diet may be explained by the presence of biological active compounds of *X. dendrorhous*, common Saint John's wort, lemon balm, and rosemary, and whose antioxidant activity has been reported. Hence, it is not surprising that our results show less lipid peroxide level in either functional diet compared to control diet. In the case of diet A the yeast *X. dendrorhous* was used as supplement. The yeast extract was mainly composed of a high content of astaxanthin as its main carotenoid but also a lower concentration of other carotenoids such as phoenicoxanthin, keto-γ-carotene, HO-keto-torulene, β-carotene, licopene, neurosporene, γ-carotene, echinenone and HO-echinenone [46–49] and whose antioxidant activity from lipid peroxidation has also been reported [50,51]. Carotenoids are the precursors for vitamin A and it has proposed that they prevent oxidative damage to cells due to their capability to eliminate excess energy, acting as free radical scavenger [52,53]. According to our results, the administration of dietary astaxanthin derived from *X. dendrorhous* suppresses oxidative damage by reduction of the lipid peroxide generation and serum transaminase levels [22,54]. Unfortunately, most of the studies on dietary astaxanthin in fish have been focused on muscle pigmentation but not on its potential antioxidant properties. In addition to carotenoids, the antioxidant effect of β-glucan (the major structural components of yeast and fungal cell walls [6]) inhibiting the levels of MDA, the end product of lipid peroxidation, suggests a protective role of β-glucan against oxidative damage preserving the cellular integrity [41]. Thus, in the antioxidant activity of diet A astaxanthin and β-glucan may be participating, as both components are present in the yeast extract. On the other hand, diet B contains tannins and flavonoids which could be involved in the significant antioxidant activity inhibiting free radical generation and lipid peroxidation [24,55,56]. In fish, flavonoids have been demonstrated to have a clear antioxidant effect in fish oil oxidation when assessed from formation of peroxides or TBA reactive substances [57]. Similarly, the presence of phenolic compounds in lemon balm and rosemary has been also reported as the responsible for their high antioxidant capacity [27,30]. Taking together, diet B showed an antioxidant effect which is directly related to the presence of flavonoids and phenolic structures protecting against reactive oxygen species by an additive effect to the endogenous scavenging compounds and can interfere with the radical producing systems [58].

The evaluation of the immune response showed at day 20 of trial the up-regulation of IFNγ in fish fed with either functional diet. However, at the same day no significant differences were observed on TBet, a transcription factor that controls the expression of IFNγ. Conversely, the up-regulation of TBet but not IFNγ was observed on day 10 of crowding stress. This apparent discordance may reside in an expression earlier than 20 days of feeding in the case of TBet, and in the same manner, a later on expression of IFNγ at 10 days of crowding stress. IFNγ is a cytokine that in higher vertebrates promotes Th1 differentiation and defines cell-mediated immunity either Th1-type adaptive immune response [59] or innate immune response produced by Natural Killer (NK) cells [60]. In fish, its turn on is an indicator of cell-mediated immunity activation which is essential in the defense against intracellular pathogens [61] but also Th1 effector cells may activate macrophages and induce B cells to produce opsonising antibodies (cell-mediated immunity) [62]. The up-regulation of IFNγ has been reported in response to dietary supplements, i.e. probiotics, as an indicator of immune protection [63]. In the adaptive cell-mediated immune response, IFNγ is produced by $CD4^+$ T cells (Th1) and $CD8^+$ T lymphocytes (CTL) and plays a major role in response to MHC antigen presentation [64]. In several fish species, the expression analysis of CD4 genes has suggested that teleost $CD4^+$ cells may function as helper T cells similar to mammalian $CD4^+$ cells [65]. In our study, the level of CD4 transcript was maintained unchanged after 20 days of feeding with diet A and diet B compared to CD4 transcript levels observed in fish fed with control diet under the same density of culture. This no significant expression of CD4 was expected because the expression of CD4 is antigen recognition dependent. Also, the IFNγ transcript levels expression could be explained by the activation of innate immune response, maybe mediated by NK cells and not mediated directly by actors directly involved on adaptive immune response. In mammals, a crosstalk between NK cells and adaptive immune cells has been reported. When the immune response is triggered, the NK cells are able to respond to a challenge quickly with a cytotoxic potential to release large amounts of cytokines, especially IFNγ. Previous evidence showed that NK cells can enhance or restrict the adaptive immune response with factors influencing the final outcome such as the stimulus-triggered nature, the tissue microenvironment where the response takes place, and subset of NK cell involved. In sum, they can provide help to promote the initiation of an immune response, but can also curb the activity of immune effectors and thereby prevent host immune mediated damage [66].

After 20 days of feeding, the expression of membrane IgM was induced by either functional feeding while the secretory isoform was only upregulated in fish fed with diet B. In salmonids, IgM is the predominant isotype antibody at basal level [67]. In general, the diet containing pre- and probiotics induced effects on the systemic humoral response reflected by increased levels of IgM [68,69]. In our study, the up-regulation of membrane and also secreted IgM in fish feeding with functional diets, suggest that functional feeds possibly are able to induce to B lymphocytes to produce opsonizing antibodies as a consequence of a crosstalk with the activated cellular-immunity, reflected by an IFNγ-enriched milieu [62]. The no variation in CD4 could be explained by an immunomodulatory effect of the anti-inflammatory cytokine TGF-β that in our study was upregulated after 20 days of feeding with diets. In mammals it has been reported that TGF-β inhibits the proliferation of $CD4^+$ T-cells [70] and inhibits IFNγ expression in $CD4^+$ T-cells [71]. In salmonids the up-regulation of TGF-β has been related to suppression of immune response [72,73].

The transcript profile analyzed at 30 days of feeding was marked by the gene expression induction in GATA-3 and the up-regulation of secreted IgM. Recently, in salmonids GATA-3 was described as a T cell specific transcription factor essential for the development of the T-cell lineage and differentiation of mammalian Th2 cells [74]. In salmonids, Th2 response has not been clearly shown, but a high expression of GATA-3 in concert with IL-4/13A suggests a similar role of this transcription factor in the differentiation of salmonid Th2 cells [75]. Importantly, the main difference with day 20 of feeding was the absence of up-regulation for IFNγ and TGF-β, suggesting that after 30 days of feeding salmon fed with either functional diets developed a humoral-like immune response. This humoral-like response was more evident when we analyzed the transcripts profile at 40 days of feeding (that corresponds also to 10 days of crowding stress) in which up-regulation of CD4, GATA-3 and secreted IgM in fish fed with either functional diets was observed. On the other hand, the expression of IL-10 was only observed in fish fed with diet A. In mammals an increased expression of CD4 and GATA-3 correspond to immune response dominated by Th2 cells [76], but in fish the existence of the paradigm Th1/Th2 is under debate, but the cloning in the last years of key cellular markers and cytokine genes involved in mammalian Th1/Th2 cell development suggest that these T helper cells may also be present in fish [77]. In mammals, IL-10 has a dual role because it is a potent anti-inflammatory cytokine that regulates and inhibits the expression of pro-inflammatory cytokines, contributing to the normal resolution of infection and reducing tissue damage caused by inflammation [78], but also induces tissue inflammation when IL-10 is expressed in a context of Th2 differentiation acting as a potent growth and differentiation factor for activated B lymphocytes that secrete large amounts of IgG, IgA and IgM [79]. Hence, the up-regulation of CD4, GATA-3, IL-10 and secreted IgM suggests that a humoral-like immune response take place when fish were fed with either functional diet.

There is evidence that strongly indicates a close interaction between endocrine and immune system based on their messengers belonging to the same family of molecules. In this matter, the head kidney plays a central role in organizing the stress response involving close communication between regulatory systems but also plays a key role mounting the immune response [2]. Thus, it has been reported, when the stressor is acute and short-term, that the response pattern is stimulatory and the fish immune response shows an activating phase that especially enhances innate responses. In contrast, if the stressor is chronic, the generation or accumulation of ROS can interfere with immune response [80]. This is because the excessive ROS production indicates an imbalance in antioxidant status triggering a limited immunity probably in an attempt to avoid over-production of ROS, indicating the occurrence of a physiological trade-off between the immune and antioxidant system [81,82]. However, the main consequence is that the immune response shows suppressive effects and therefore the chances of an infection may be enhanced [2]. In our study, a reduction in the lipoperoxidation and protein carbonylation was accompanied by the up-regulation of CD4, IL-10, GATA-3 and secreted IgM indicates a balance between the oxidative stress and the activation of the immune response in fish subjected to crowding stress fed with functional diets. This reinforces the hypothesis that the utilization of diets containing antioxidants is able to modify the expression of key cytokines in the regulation of the immune response in fish subjected to stress, thus probably reducing the health side effects provoked by chronic stress at immune response level. This is in agreement with the antecedents indicating that the regulation between redox status and cytokine production seems to be reciprocal, because it has been observed in previous reports that the increase or decrease of oxidative-related markers such as GSH/GSSG can be regulated by cytokines such as IFNγ and IL-4 respectively [83,84].

In our study, after 20 days of evaluation of functional diets in fish under optimal culture conditions, we suggest that experimental diets possibly favor a Th1 profile immune response, these could be explained because when fish are not subjected to stress conditions could accumulate high levels of antioxidants molecules in immunoregulatory cells, such as was suggested by Murata et al. that the accumulation of a reductive thiol redox status may be an indicative of the presence of a subpopulation of macrophages to produce IL-12, that drives a polarization to Th1 immune response [85]. On the contrary, induction of macrophages with a consumption of intracellular redox molecules, suggested by depletion on GSH, could skew to macrophages to produce IL-4 and a consequent polarization of Th2 immune response [85]. In our results, fish subjected to crowding stress increased the levels of transcripts associated to Th2-like response, but the possible damage produced by the oxidative stress induced by crowding and associated to mammals Th2 response was not observed, suggesting a protective role supported by either antioxidant enriched diet preventing cell damage generation under stress. More assays are necessary to confirm the presence of a Th2 response in fish fed with these functional feeds and their effects on fish health status and its role to confer protection in fish subjected to stress.

5. Conclusions

In summary, Atlantic salmon fed with functional diets based on yeast (*Xanthophyllomyces dendrorhous*) and plant (Saint John's wort, lemon balm, and rosemary) extract improve the antioxidant status and increase the expression of genes related to Th2-like response suggesting a protective role preventing cell damage in fish subjected to crowding stress.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2017.12.061.

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