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Department of Animal and Aquacultural Sciences
Faculty of Biosciences

Novel functional ingredients containing yeast cell wall components modulate molecular biomarkers in Atlantic salmon (*Salmo salar*) when exposed to acute hypoxic stress.

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Master of Science in Aquaculture

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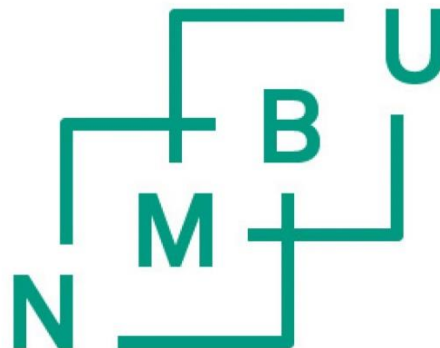

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For my master's thesis, I wanted to work in a project that improved sustainability and fish welfare. Hence the stress part of the resilient salmon trial was ideal for me.

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Abstract

Aquaculture is a continuously expanding global industry, however, some of the major problems associated with intensive fish farming are acute and chronic stressful conditions such as handling, sub-optimal nutrition, transport, diseases, and environmental problems. Stressful conditions can lead to decreased growth and immune response, resulting in compromised fish welfare. In addition, this situation can increase the susceptibility to diseases, causing economic losses in aquaculture. To face this problem, nutrition and the use of functional ingredients could be an alternative to regulate the fish's response to stress conditions. In this study, the short-term stress response (1 min hypoxia) was evaluated in plasma samples of Atlantic salmon fed for nine weeks with a commercial-like diet and two diets with 0.1% of different novel functional ingredients (L4 and L6) derived from non-saccharomyces yeast cell wall. The results showed that cortisol levels were similar between fish from control and L6 group, both groups having a cortisol peak 1h post-stress compared to initial control without stress (NS). A different pattern was seen in the L4 group, with no peak 1h post-stress. Moreover, glucose levels showed significant reduction only in the L4 group at 24h post-stress. There was also a reduction in glutathione peroxidase (Gpx) activity 12h post- stress in the L4 group, which could be due to the absence of increased cortisol in response to stress. Regarding immunological markers, there were no significant differences in the TNF-α levels at different time points among the diets. Nevertheless, compared to NS, an increase of TNF-α was detected at 3, 6 and 24h post-stress, which would suggest an attempt to control immunosuppressive profiles. On the other hand, IL-10 levels were significantly higher in all time points in L6 group. This phenomenon could either be beneficial or detrimental and hence future pathogen challenge trials must be done to validate its effects. A different pattern was detected in L4 group, with no increase in IL-10 compared with the control group. The data from this study propose that fish from L6 group maintained a similar pattern of cortisol response, compared to the fish from control group. On the contrary, the fish from L4 group could prevent the effects related to acute stress, avoiding the increase of cortisol. However, future studies need to be done to identify optimal feeding regime to plan its use in the window of action prior to stressful handling. In conclusion, this study demonstrated that the functional feed containing the L4 non-saccharomyces cell wall component could potentially be used before processes that require the handling of salmon and the feed containing the L6 component gave an indication for improved anti-inflammatory response.

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Acronyms

3β-HSD	3β-hydroxysteroid dehydrogenase
ACTH	Adrenocorticotropic hormone
CNS	Central Nervous system
CRH	Corticotropin Releasing hormone
GI	Gastrointestinal tract
Gpx	Glutathione Peroxidase
GR	Glutathione reductase
HPI	Hypothalamus-pituitary-interrenal axis
HRP	Horseradish Peroxidase
HSC	Hypothalamus-sympathetic-chromaffin axis
IL-10	Interleukin 10
MAMPs	Microbe associated molecular patterns
MI	Microbial ingredients
MOS	Mannan Oligosaccharides
NS	No stress group
PRRs	Pattern recognition receptors
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SNS	Sympathetic Nervous system
TEER	Transepithelial Electric resistance
TMB	3,3’,5,5’-tetramethylbenzidine
TNF-α	Tumor Necrosis Factor alpha

1. Introduction

1.1. Importance of aquaculture and current challenges

The human population is estimated to increase by two billion in the next thirty years (1). This will put a severe constraint on the current food sources and hence new sustainable solutions must be explored. In this global context, aquaculture is an attractive candidate to fill this gap, because fish can efficiently convert feeds that don't compete with human food into high quality protein source for human consumption (2–5). In addition, aquaculture is the fastest growing food production sector in the world and is expected to increase by thirty two percent in 2030 compared to 2018 (3,6).

In aquaculture, salmonid farming is one of the most important economic activities and Norway is the largest global producer of salmonids with an estimated annual production of USD 12 billion in 2018 (6). The Norwegian aquaculture is projected to increase from 1.2 million tons to 5 million tons by 2050 (3). However, the rapid growth and the subsequent intensification in this sector can cause increased stress incidences and the risk of infectious diseases in farmed fish, leading to large economic losses (6–8).

Different processes related to salmonid farming such as transportation, grading, vaccination, and everyday handling involve situations where fish is taken out of the water or where fish is exposed to low dissolved oxygen concentrations. These conditions can cause stress and impact fish welfare (9–11), and are considered as one of the main threats to the sustainable development of aquaculture. In farm conditions, these stressors co-exist, commonly referred to as multi-stressors, and can have negative synergistic effects (10,12,13). In salmonids, stress reduces growth and increases the fish's susceptibility to diseases by the suppression of the immune response (14). Traditionally, infectious diseases have been managed with different strategies such as the use of antibiotics and preventive vaccination plans (2). Nevertheless, the use of antibiotics leads to a myriad of problems including the development of antibiotic resistant strains (7,15–17), which have led to strict regulation of antibiotics in many countries, including Norway (18,19). On the other hand, vaccinations can be stressful for the fish, costly to administer, labour intensive and not fully efficient (20,21).

In farmed fish, the relationship between nutrition and immune system has been recognized as an important part of the production process because energy and nutrients provided by the feed are essential to maintain the homeostasis of the animal, along with optimal immune function (22). In Atlantic salmon, nutrition is ideally placed to tackle both stress and immune suppression as certain feed ingredients can reduce the risk of diseases and improve health and overall fish (23,24). Among many functional ingredients, microbial ingredients (MI) such as yeast or its cell-wall components are gaining increasing interest as ingredients in feeds for

salmonids (18,25,26). These ingredients have additional properties beyond their nutritional values such as modulators of fish's immune response through components that can be detected as microbial-associated molecular patterns (MAMPs) by pattern recognition receptors (PRRs) in the fish (27–29).

1.2. **Stress in aquaculture**

In aquaculture, stress can be caused by unsuitable temperature, salinity, pH and dissolved oxygen levels. These multiple conditions are continually present in daily practices, making them a major concern during fish farming (30). In addition, handling activities such as grading, sorting, tagging, vaccination and harvesting require the fish to be taken out of water and can cause acute hypoxic stress among other adverse effects. These activities, along with environmental factors, have been described to play an important role in the higher mortality of salmonids (31). Handling activities are unavoidable and therefore novel alternative ways need to be found to reduce stress on the fish (21). A good fish welfare principle is to make sure that the fish grown have a good quality of life and suffer as little as possible. Hence welfare is a growing concern (9).

1.2.1. Stress Response in Fish

The response to stress is largely conserved from fish to higher vertebrates such as birds and mammals (11). Stress includes behavioural, physiological, and immunological changes when an organism is encountered with different conditions which the organism perceives as a threat. During the initial stages of growth, stress exposure leads to long-term effects due to developmental plasticity (32).

A stress condition can be classified into two types, namely acute stress and chronic stress (14). Acute stress is caused by short term stressors such as handling and asphyxiation (33) and the effects are exerted by cortisol and catecholamines and lead to immune modulation. On the other hand, chronic stress is a persistent condition, with a longer duration and its effects are exerted mainly maladaptation, which indicates that the regulatory mechanisms have not been able to compensate the effects of the stressor (34).

In fish, the stress response starts with the perception of a stressor by higher brain centres. Once a stressor is detected, there are two pathways involved. One is the hypothalamus-pituitary-interrenal (HPI) axis that secretes cortisol and the other is the hypothalamus-sympathetic-chromaffin (HSC) axis that secretes adrenalin (35,36). If the stressors persist, the hypothalamus releases corticotropin releasing factor (CRF). CRF stimulates the pituitary to secrete adrenocorticotropic hormone (ACTH) into the blood stream. Once ACTH is secreted, it acts on melanocortin-2 receptors which are present on target cells where the cortisol is

produced and released (35,36). A simplified version of this pathway is shown in Figure 1. Although ACTH is the most predominant secretagogue of cortisol, several other molecules have been found to regulate cortisol levels, e.g. angiotensin II, catecholamines, prostaglandin E1 and extracellular calcium (37). This suggests a complex environment-endocrine interaction in the secretion of cortisol.

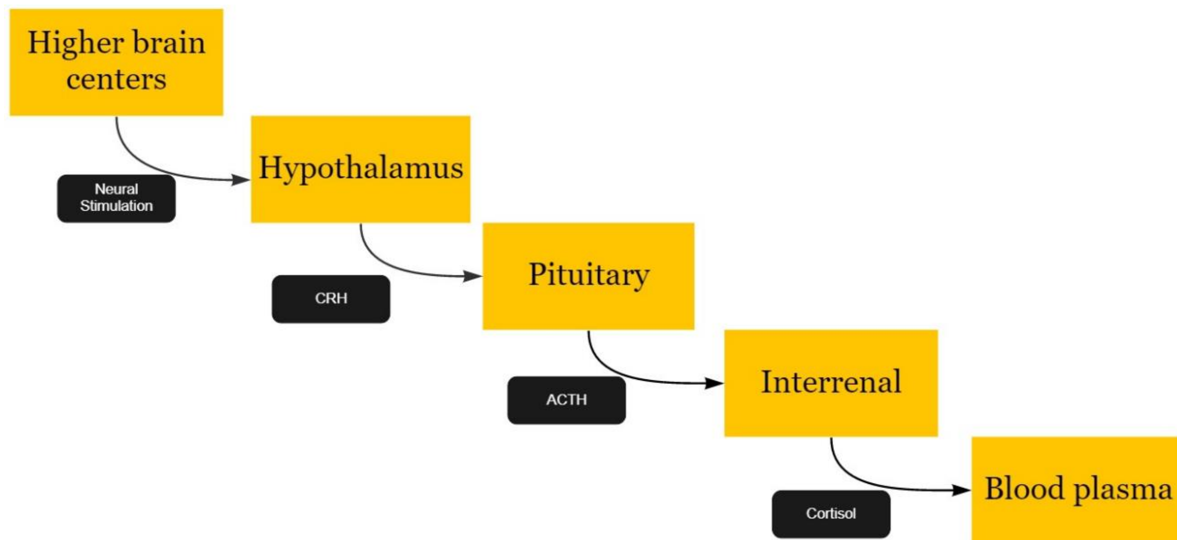

Figure 1. The stress response pathway in fish. Simplified version of Hypothalamus-pituitary-interrenal axis. CRH: Corticotropin releasing hormone. ACTH: adrenocorticotropin (adapted from 38).

In aquaculture, stress is considered counter-productive for several reasons. A stressor condition is manifested when the organism perceives a threat to its life. This means that providing energy short term to eliminate the stressor is prioritised over housekeeping activities such as growth, immunity, and digestion. This leads to reduced growth and subsequently to increased growth periods and production costs (39). Stress also renders the fish more susceptible to diseases in the long run (14). For example, stress can excessively increase the sloughing off mucus in the gut. This leads to a decrease in the adherent microflora, which allows other bacteria (including the pathogenic ones) to colonize the intestine (40).

Measuring the stress response cannot be done directly as it is not possible to read the emotions of the fish. Hence it must be measured in terms of behavioural or biochemical parameters (41). During stress conditions, cortisol is one of the major hormones that is increased in the blood stream, which makes this molecule commonly used to measure stress in salmon and determine fish welfare (36,39). Cortisol has multiple effects and to understand how stress and nutrition regulate cortisol, it is important to understand the biosynthesis, transportation, mechanism of action and clearance of cortisol.

Cortisol is synthesized from cholesterol (Figure 2). The synthesis takes place in two compartments namely the mitochondrial compartment (the enclosed area) and the cytosolic compartment. Cholesterol is converted into pregnenolone with the P450 side chain cleavage enzyme P450scc, which is present in the mitochondrial compartment. The pregnenolone is hydroxylated by the catalysis of the enzyme P450c17 to 17 OH-pregnenolone. This reaction takes place in the cytosolic compartment. After, 17-OH-pregnenolone is converted into 17-OH-progesterone by the catalysis of the enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD). 17-OH-progesterone gets converted into 11-deoxycortisol by the catalysis of P450c21, and finally then into cortisol by the catalysis of cytochrome P450c11 (37).

Once cortisol is synthesized it is transported through the bloodstream. This is available in two forms, namely free plasma cortisol, which is responsible for the physiological effects, and protein bound plasma cortisol. In previous literature, it has been shown that stress increases not only the levels of total plasma concentration but also the percentage of free plasma cortisol thereby having a double effect on the stress response (42,43). The free cortisol in the bloodstream is believed to diffuse passively through the cell wall because of their lipophilic nature; although in some cases carrier protein mediated transport has been observed (37,44).

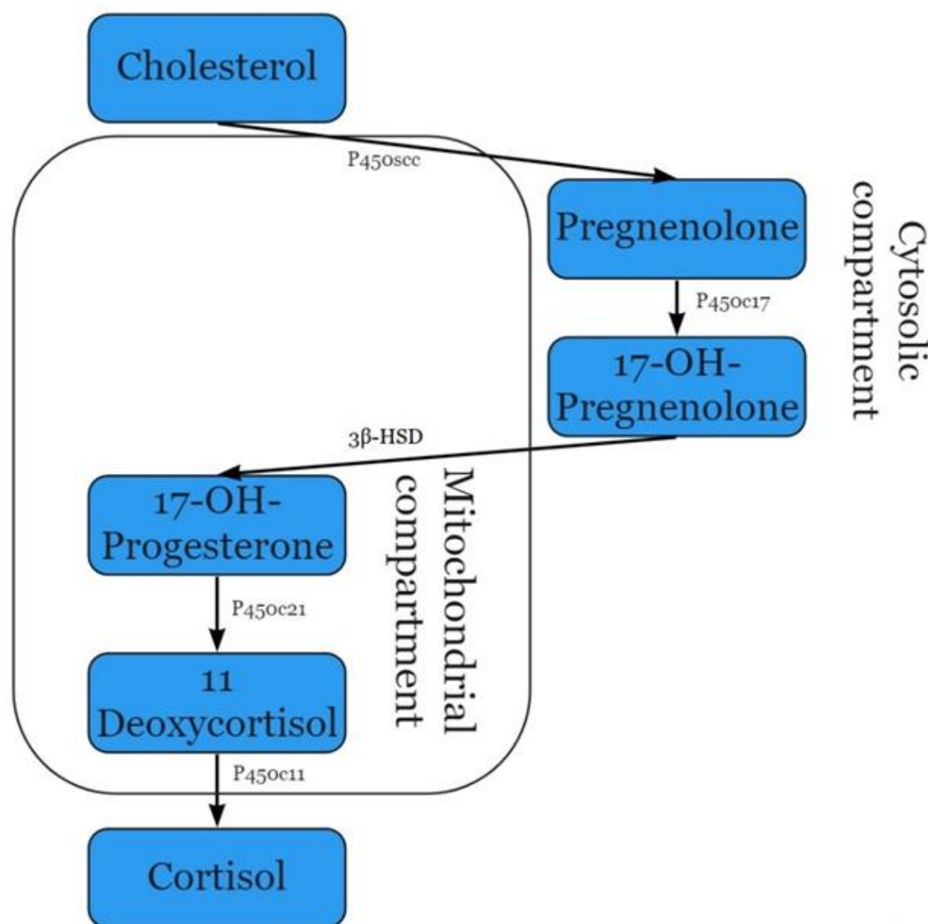

Figure 2: Pathway of biosynthesis of cortisol from cholesterol in fish (adapted from 37). 3β-hydroxysteroid dehydrogenase.

Cortisol can induce pleiotropic effects in the cell (45) through two types of receptors, mineralocorticoid receptors (MR) and glucocorticoid receptors (GR) (38). In response to acute stress, cortisol has been described as capable of reducing the number of leukocytes (46,47), induce loss of phagocytic functions in neutrophils (48) and control the gene expression of cytokines, adhesion molecules and enzymes (49). This, coupled with a systematic immunosuppression (14,50), could lead to increased disease susceptibility in fish (51).

Moreover, cortisol can induce effects in fish through a non-genomic pathway, which exerts a rapid response because it does not involve transcription and translation. These mechanisms are mediated by the release of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which can cause oxidative stress (52,53). A stressor condition can increase free radicals in the system in two ways (as shown in Figure 3). One involves glucocorticoid receptors that bind to cortisol and then reduce glucose uptake, increasing free radical synthesis. While, the other mechanism is through glutamate secreted in response to stress that binds to the NMDA receptor and induces secondary signalling of Ca^{2+} ions, also increasing the synthesis of free radicals (52,53).

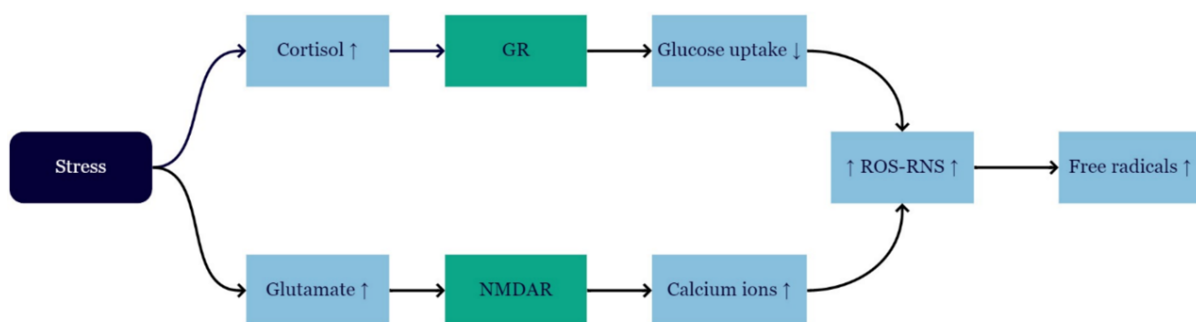

Figure 3: The figure describes the pathway from stress to the generation of free radicals. External factors are mentioned in black; biomolecules are in blue and receptors are in green. GR – Glucose receptor, NMDAR – NMDA receptor (adapted from 54).

This oxidative stress can be detected indirectly by measuring the activity of antioxidant defence enzymes such as glutathione peroxidase (Gpx) (55–57). Gpx is the general name of an enzyme family with peroxidase activity whose main biological role is to protect the organism from oxidative stress caused by ROS, which is known to cause damage to DNA, RNA, and proteins. ROS is usually synthesized as a by-product of aerobic respiration and are neutralised by the antioxidant defence mechanisms of the body (39). However, oxidative stress can occur when there is an imbalance between pro-oxidants and antioxidants. This imbalance increases the hydrogen peroxide which is converted into water by the activity of the enzyme Gpx. The activity of this enzyme is regulated by the hydrogen peroxide (Figure 4) and hence is an indirect measure of the degree of oxidative stress. In the previous studies, it was observed that Gpx activity increases 12h after acute stress (58). Also, Gpx activity is a

classical stress biomarker as it is relatively stable and hence can be analysed reliably (59). In addition, Gpx has been previously used in the study of redox reactions in response to stress in fish (60).

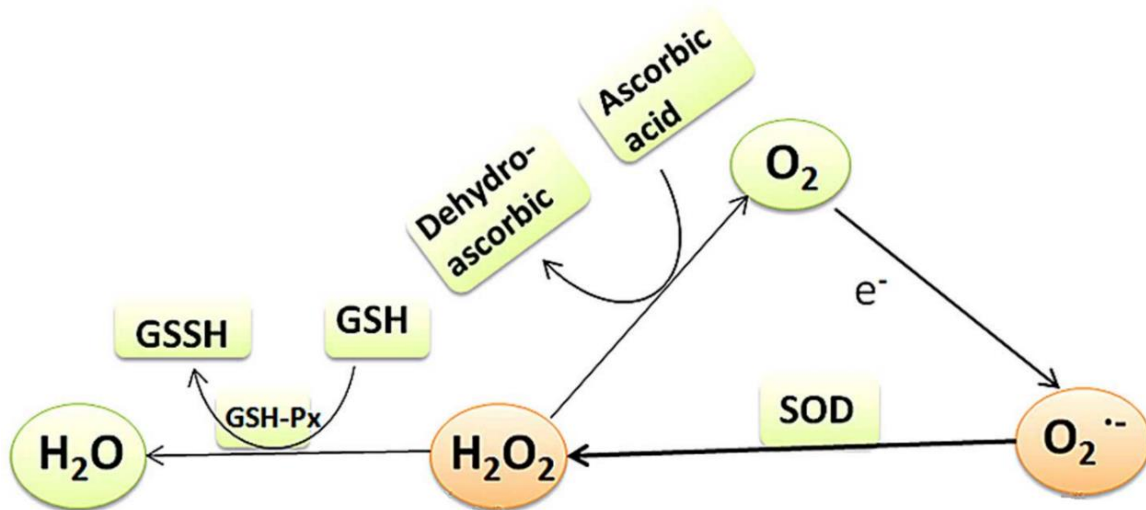

Figure 4. Schematic representation of antioxidant defence mechanism. The free oxygen radical is represented by O_2. This free oxygen radical is converted to hydrogen peroxide, catalysed by superoxide dismutase. The hydrogen peroxide is converted into water by Glutathione peroxidase (GSH-Px) (adapted from 61).

Regarding other parameters that can be modulated by stressful conditions through cortisol, it is interesting to highlight glucose concentrations (Figure 5). Cortisol increases the glycogen deposition in the liver, increases gluconeogenesis and increases insulin resistance i.e. decreases glucose uptake by the cells. It also prevents the glycogenesis in muscles (38).

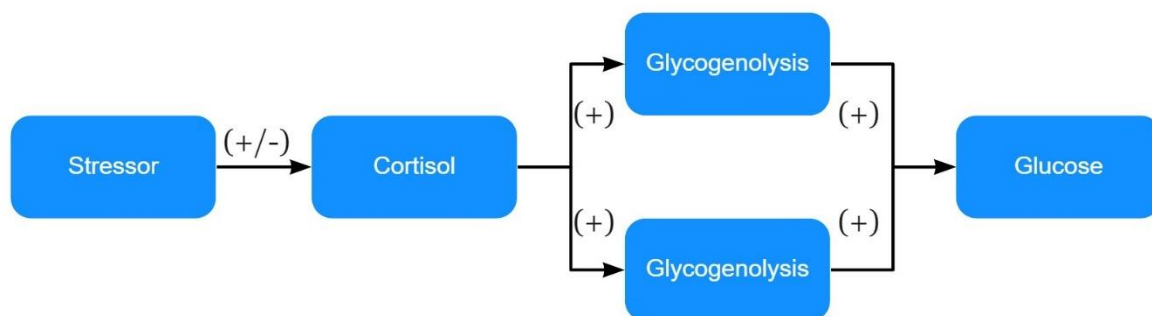

Figure 5. Mechanism of how cortisol regulates glucose. (+): positive modulation, (-): negative modulation (adapted from 62).

1.2.2. Stress and fish immunological biomarkers

The immune system of an organism protects against non-self-patterns. In fish, similar to higher vertebrates, this system can be broadly classified as innate and adaptive immunity, both with molecular and cellular components, that act in a coordinated manner (63). The innate immunity is the first and a fast line of defence against foreign agents and includes epithelial barriers (skin and mucosal surfaces), phagocytes and complement proteins, among others (63). On the other hand, adaptive immunity identifies specific antigens and creates a targeted response, by coordinating antigen-presenting cells and lymphocytes, which could induce cell-mediated immunity, antibody production and regulatory profiles (63,64).

The immune response are coordinated by molecules known as cytokines (e.g. interferons, interleukins, and chemokines) (64). During stressor conditions, these molecules can be modulated by the action of cortisol, which regulate their production and secretion (14,51). With this background, immunological biomarkers are a good strategy to assess the down-stream effects of cortisol and stress in fish (46,51).

For the characterization of the fish's immune response during stressful conditions, an interesting biomarker is interleukin 10 (IL-10). IL-10 is a central regulator for anti-inflammatory responses and hence it is a good candidate to study in stress models (65). This molecule can regulate the inflammation and also induces immunosuppression (66), inhibiting the activity of phagocytes, controlling the oxygen and nitrogen radical production and reducing the expression of proinflammatory molecules (67,68). Additionally, it has reported in cyprinids that the regulatory activities of IL-10 would not only be associated with immunosuppression but also be related to the maintenance of memory cells over time (67). However, further studies must be conducted to better understand this relationship. In Atlantic salmon it has been described that after a short-term hypoxia, IL-10 levels increased significantly 1-hour post-stress. This increase also showed a proportional relationship with plasma cortisol values (58).

Another important molecule to characterize the immune response of the fish is Tumour Necrosis factor-α (TNF-α). TNF-α is a pro-inflammatory molecule involved in the acute phase of inflammatory response to stress (69). TNF-α is found in two forms, a membrane bound and a soluble form (70), and it acts as a co-stimulator molecule for macrophages, natural killer cells and lymphocytes. TNF-α has been proven to promote the survival of macrophages (71). Furthermore, in rainbow trout, this molecule has been shown to regulate leucocyte proliferation and migration (72).

The detection of specific biomarkers related to the immune response can provide information on how the fish ultimately interacts with multiple stress conditions, since the immune system is a crucial part of maintaining the homeostasis of the organism.

1.3. **Functional feeds**

The term functional feed refers to ingredients added in small quantities to the feed that benefit the organism beyond its plain nutritive value (73,74). In salmonids, some functional feeds include microbial ingredients (MI) such as yeasts and its cell wall components (mannan oligosaccharides and β-glucans), which have been shown to be able to reduce the risk of diseases and improve overall health in fish (23,24).

1.3.1 Yeast as a functional ingredient

Several studies have used yeast as an alternative protein source (2,75,76). Yeast production has one of the least impacts on the environment even when grown in scale (77). They can be grown on substrates that don't compete with resources used for human consumption such as sugars derived from wood (3,25). These properties make yeast an ideal replacement for protein source in fish feed. However, downstream processing of yeast is necessary to break down its cell wall structure (26-32% dry weight) (25) and release entrapped nutrients. Hence, roughly 25% of the yeast biomass would be a by-product not suitable as a protein source. Therefore, economically speaking, the use of yeast as a major protein source and functional feed in aquaculture depends on the ability to use yeast by-products efficiently, to keep the feed cost at the minimum (3). The nutritional value and health beneficial effects of yeast cell-wall components largely depend on the species and strain, the substrate used in the fermentation media, as well as downstream processing methods used after harvest (25,78,79). Hence, the ideal yeast candidate, for use as a major protein source, will be the best combination of a protein fraction and a functional by-products fraction obtained from the same candidate. Thus, to identify the best candidate, it is important to understand the benefits of its by-products such as cell wall components.

The yeast cell wall comprises several different molecules, such as glucans, glycoproteins, mannan, and chitin (80), which are known to induce health benefits in animals. In fish, *in-vitro* studies have shown that β-glucans can increase Trans epithelial electric resistance (TEER) (80,81). This is important because a leaky gut is more likely to let in pathogens (82). Moreover, β-glucans are used in feeds for their immunostimulatory properties, because they act as MAMPs (Microbial-Associated Molecular Patterns) and act on pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) to activate the immune cells (64).

β-glucans can modulate both humoral and cell immunity, improving fish response to challenges (69). *Candida utilis*, when used as a protein source, has been found to counteract soybean induced enteritis (76). It has also shown to counteract the immunosuppressive responses to sea water transfer, to decrease cytokines secreted in the distal intestine and to

improve growth performance and feeding behaviour (2). Furthermore, several studies have also shown that addition of yeast cell wall components to diets have reduced the sea lice infestation in Atlantic salmon (83,84) and prebiotics are preferred to probiotics to improve beneficial gut microbiota as live microorganisms have low viability during feed manufacture and storage (85).

Regarding stressor conditions, β-glucans and mannan oligosaccharides, based on their source, can alter plasma cortisol levels in response to stress, improving the immune response and fish health (86,87). In zebrafish, mannan oligosaccharide has been found to make fish resistant to starvation stress (71). The decrease in stress response to acute stress can be attributed to the synergistic effect of both β 1-3/1-6 glucan and MOS (19,88).

To describe the effect of yeast cell wall components in functional feeds on the modulation of the fish's response to a hypoxia condition, this work evaluates and compares the plasma parameters cortisol, glucose, Gpx and immunological marker at the phenotypic level. This is to increase the knowledge about the stress and immune response in Atlantic salmon fed MI during short-term stressful conditions, which is important to improve fish welfare during aquaculture production processes.

2. Hypothesis

Novel functional feeds ingredients containing yeast cell wall components modulate immune and stress responses in Atlantic salmon when exposed to acute hypoxic stress.

3. Objectives of the study

Primary objective:

- The main objective of the study is to evaluate the effect of functional feeds containing yeast products on stress responses *in-vivo* in Atlantic salmon exposed to acute hypoxia stress.

Secondary objectives:

- To evaluate the effect of functional feed containing yeast products on molecular biomarkers at different time points in Atlantic salmon exposed to acute hypoxia stress.
- To evaluate the correlation between all measured molecular biomarkers in plasma at different time points in Atlantic salmon exposed to acute hypoxia stress.

4. Materials and Methods

4.1. Diets

In this study, three experimental diets were used. A commercial-like diet (diet 1: control diet) and two different experimental diets contained 0.1% of non-saccharomyces yeast cell wall components (diet 2: L4 and diet 3: L6) along with the base commercial-like diet. The feed was produced using extrusion technology at the centre for Feed Technology (FôrTek – NMBU). The feed was 3 mm pellets and contained the composition marked in Table 1. Yttrium Oxide (0.08 mg g^{-1}) was added to measure the digestibility.

Table 1: Composition of the base diet.

Ingredient	%
Fish meal[a]	39.00
Soy protein concentrate 67[b]	24.67
wheat gluten[c]	3.34
Wheat[d]	11.80
Fish oil (28% EPA+DHA)[e]	8.09
Rapeseed oil[f]	9.00
Premix[g]	2.85

[a]LT Fish meal: Norsildmel AS, Bergen, Norway

[b]Soy protein concentrate: Tradkon SPC HC-200, Sojaprotein, Becej, Serbia

[c]Wheat gluten: Amilina AB, Panevezys, Lithuania

[d]Wheat: Norgesmøllene, Bergen, Norway

[e]Fish oil: (28 % EPA + DHA), Nordsilmel AS, Bergen, Norway

[f]Rapeseed oil: AAK, Karlshamn, Sweden

[g]Premix: (vitamineral-p-AA-kolin), BioMar AS, Norway

[h]Yttrium oxide (Y_2O_3): Metal Rare Earth Limited, Shenzen, China

4.2. **Experimental design**

The experiment was performed according to the Norwegian Animal Research Authority's established guidelines at the fish laboratory at Norwegian University of Life Sciences (NMBU, Ås, Norway).

Atlantic salmon eggs were obtained from AquaGen and hatched at 5 °C. A timeline of the events is shown in Figure 6. Fish were fed till satiation and kept at 24 hours light until the body weight was 35 g. Then, all fish were vaccinated and 540 fish were transferred to nine experimental tanks (60 fish in each tank). Thereafter, they were kept at 8h/16h L/D (winter light) for a period of 5 weeks. Later, fish were placed at 24h light for 210 day degrees (~2 weeks at 16 °C).

Fish were fed using a roller feeder. Uneaten feed was collected once a day and were weighed to have an estimate on the actual intake of feed per tank. Unhealthy fish were taken out and euthanised. For each dietary group, 180 Atlantic salmon (belonging to the same family) were fed for a period of 7 weeks. The physical parameters of the experimental fish were measured(data not included) as a part of a bigger experiment and they did not show any significant differences between the diets. After the feeding period, the fish were pooled together and then divided into twenty-one tanks (one tank per time point per diet) with 6 fish per tank.

Fish were acclimatized and continued to be fed for approximately two weeks until exposure to stress test. Water temperature was controlled around 15°C and flow rate was adjusted to maintain the oxygen saturation at 80% and dissolved oxygen concentration at around 7.5 - 8 mg L^{-1}.

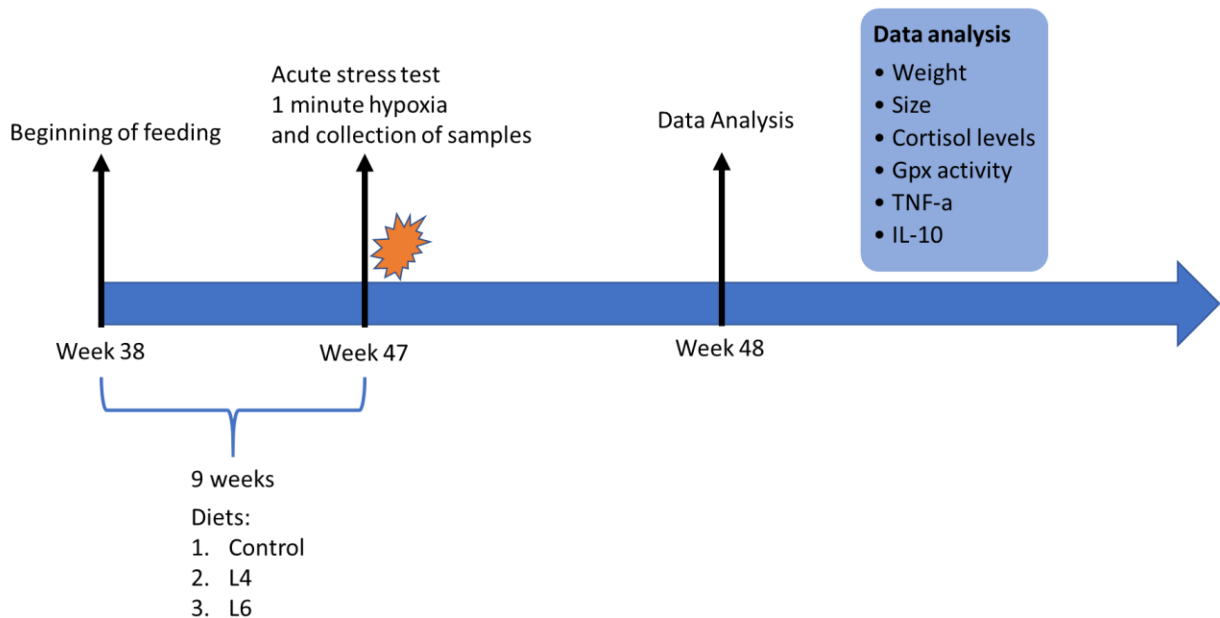

Figure 6: Experimental setup (time-line of 2020). Week 38: fish started on the trial feeds and were fed for 9 weeks. On week 47 weeks, stress test (1 minute hypoxia) was carried out. The parameters that were analysed are included in the blue box.

4.3. Acute stress test

Fish were starved 48 hours prior to the hypoxia stress test. The acute hypoxia stress was to net the fish out of water for exactly 1 minute and place them back in their respective tanks. For each diet, the fish were sampled at 0h (immediately after stress), 1h, 3h, 6h, 12h and 24h post-stress (Figure 7). The NS group was sampled without stress exposure.

Figure 7: The experimental design for the stress test, showing the diet groups and their corresponding time-points. Each dark blue tile is a tank with 6 fishes.

4.4. Fish sampling

At each sampling time, 6 fish (per diet) were taken out, sedated using 15 mg L^{-1} of tricaine methanosulfonate (MS222) and sampled for blood. The fish length and weight were recorded. The period between sedation in the bath and the sampling of blood did not exceed 7 min. Roughly 1 mL of blood was taken from the caudal vein using heparin syringes (2 mL). The plasma was isolated by centrifuging the blood samples at 3000 x g for 7 min. The supernatant was recovered into a sterile Eppendorf tube and stored at -80°C until further use.

4.5. Physical parameters of the experimental fish

The length and the weight of each fish was measured before the sampling was done. They were on the experimental diets for 9 weeks.

4.6. Plasma parameters

4.6.1. Cortisol levels

Cortisol was measured using a cortisol ELISA kit from Abcam (ab108665) following the supplier's manual. Briefly, 96-well plate was coated with anti-cortisol IgG. Then, plasma samples and cortisol-HRP conjugate were added to the wells. After 1 hour of incubation at 37 °C (followed by a washing step which removes all the unbound sample and cortisol-HRP conjugate), TMB substrate was added and incubated for 15 min to room temperature. Finally, the reaction was stopped, and the plate was measured at 450 nm (SpectraMax® M2e Multimode Microplate Reader). Cortisol concentration of each sample was calculated following the kit instructions.

4.6.2. Glucose levels

Glucose measurements were done using Abcam Glucose assay kit (ab65333) following the provider's protocol. First, the glucose is oxidised to gluconolactone by the enzyme glucose oxidase. This reaction generates hydrogen peroxide as a by-product. This hydrogen peroxide reacts with the probe to produce a coloured reagent which can be measured using a plate reader (SpectraMax® M2e Multimode Microplate Reader), at 570 nm. The intensity of the colour is directly proportional to the glucose levels.

4.6.3. Glutathione peroxidase activity

Glutathione peroxidase activity was determined using ab102530 colorimetric assay following the supplier's manual. Gpx catalyses the reduction of cumene hydroperoxide by oxidising GSH to GSSG. This oxidised GSSG is converted back to GSH by the enzyme Glutathione reductase

(GR). The decrease of NADPH is directly proportional to the Gpx activity. For each sample, Gpx activity was measured following the kit instructions.

4.6.4. Immune markers

Plasma total proteins were quantified using Pierce BCA Protein Assay kit (cat no: 23225). Then, the samples were diluted in carbonate buffer (60 mM $NaHCO_3$ pH 9.6) at 50 ng μL^{-1} and seeded (in duplicated) in a Nunc MaxiSorp™ 96-well plate. All samples were incubated overnight at 4 °C. To the next day, 200 µL of blocking solution was added and the plate was incubated at 37 °C for two hours. After, 100 µL of the primary antibody (Table 2) was incubated for 90 minutes at 37 °C, followed by 100 µL of a secondary antibody (anti-mouse-IgG-HRP from goat) diluted 1:5000 (incubated at 37 °C for 60 minutes). Finally, TMB was added to wells and the plates were incubated for 20 minutes in dark. The reaction was stopped with 50 µL of 1N sulphuric acid. The plates were read at 450 nm using a SpectraMax® M2e Multimode Microplate Reader.

Table 2: Primary antibodies for indirect ELISA.

Marker	Source	Dilution	Reference
TNF-α	Mouse	1:400	(29)
IL-10	Mouse	1:400	(58)

4.7. **Data analysis**

For data analysis (means, standard deviation and multiple t test) and graphical presentation of the results (physical parameters, cortisol, glucose, Gpx activity and immune markers), GraphPad Prism 8.0.2 was used. Differences were considered significant when p-value was < 0.05. The physical parameters, namely weight and length, were measured.

Correlation coefficients were calculated using corrplot package in R (available on CRAN: http://cran.r-project.org/package=corrplot) using the averages of each parameter by diet. The correlations were considered significant when p-value was < 0.01.

5. Results

5.1. Physical parameters of experimental fish

Per each dietary group, the measured length (Figure 8A) was 20.77 ± 0.86 cm (control diet), 20.64 ± 1.03 cm (L4) and 20.38 ± 1.21 cm (L6). On the other hand, the weight (Figure 8B) for the same groups were 105.44 ± 16.53 g (control diet), 109.33 ± 15.47 g (L4) and 104.41 ± 18.83 g (L6). No significant differences were detected between the different groups for each parameter.

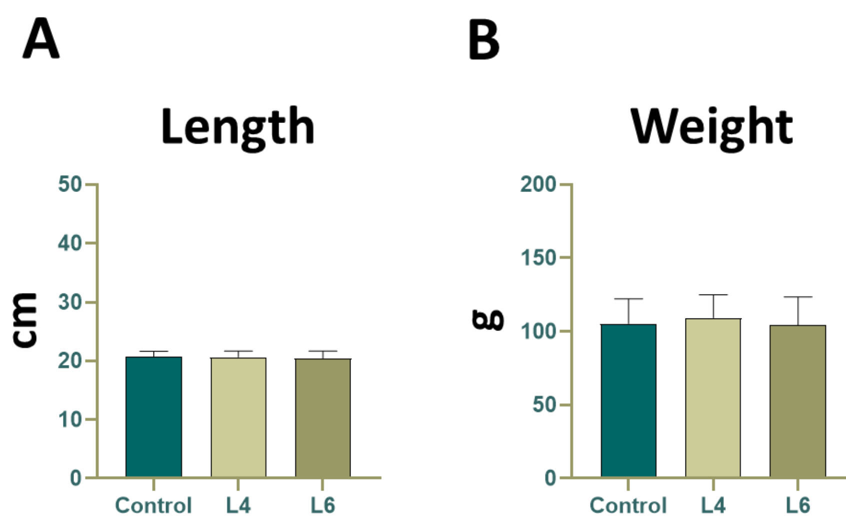

Figure 8. Physical parameters. Length (A) and weight (B) of the fish divided into different diet groups namely control (dark green), L4 (olive green) and L6 (pickle green).

5.2. Plasma parameters

5.2.1. Cortisol levels

Cortisol levels in the plasma (Figure 9) showed that the base cortisol levels (before stress) were 169.08 ± 77.38 ng mL^{-1}, 152.38 ± 97.67 ng mL^{-1} and 57.04 ± 21.58 ng mL^{-1} for control diet, L4 and L6 group respectively.

Control diet group showed a significant increase in the levels of cortisol in the 1h group (1749.68 ± 806.99 ng mL^{-1}) compared with the NS group ($p<0.05$). This was followed by a significant decrease at 12h (41.97 ± 18.68 ng mL^{-1}) and 24h post-stress (20.62 ± 16.48 ng mL^{-1}) compared with the NS group.

Regarding L4 group, there was no significant difference between different time-points compared to the NS group. However, in L6 group, there was a significant decrease of cortisol immediately after stress (0h: 22.24 ± 8.99 ng mL^{-1}) and a significant increase at 1h (1238.52 ± 419.41 ng mL^{-1}) and 3h post-stress (279.76 ± 134.77 ng mL^{-1}) compared with the NS group ($p < 0.05$).

By comparing the different diets in the same sampling time, the 1h post-stress showed a significant decrease of cortisol in the L4 group (34.83 ± 9.54 ng mL^{-1}) compared with both control diet (1749.68 ± 806.99 ng mL^{-1}) and L6 group (1238.52 ± 419.41 ng mL^{-1}) (p-value < 0.05).

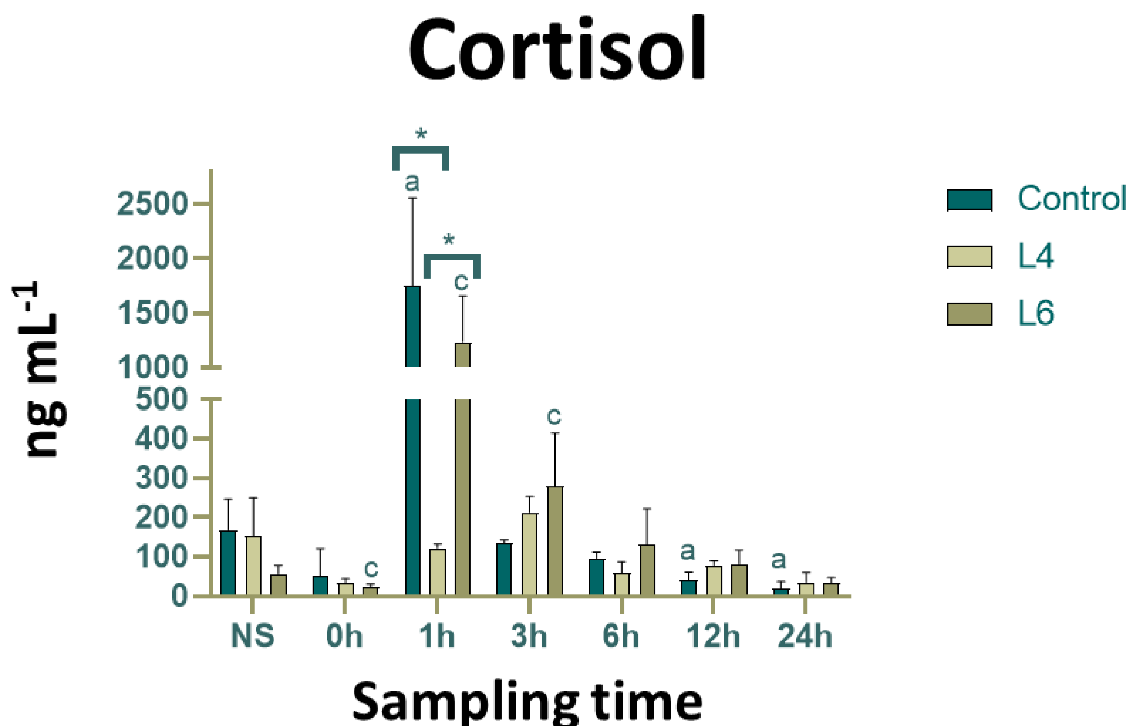

Figure 9. Cortisol levels in plasma samples of Atlantic salmon. Control diet (dark green), L4 (olive green) and L6 (pickle green). *: Significant differences ($p<0.05$) between dietary groups (at the same sampling time). Lowercase: a, b and c when compared with the initial control (NS) of the diets groups control, L4 and L6 respectively.

5.2.2. Glucose level

The results for glucose level (Figure 10) showed that at 24h post-stress, a significant decrease of glucose level was detected in L4 group compared with the control group ($p<0.05$). In addition, the control group showed a significant decrease 3h post-stress compared to NS. Similarly, L4 also showed a decrease in glucose levels at 3h post-stress compared to NS.

Moreover, this trend was repeated at 24h post-stress. The L6 group did not show significant differences between the values of glucose levels

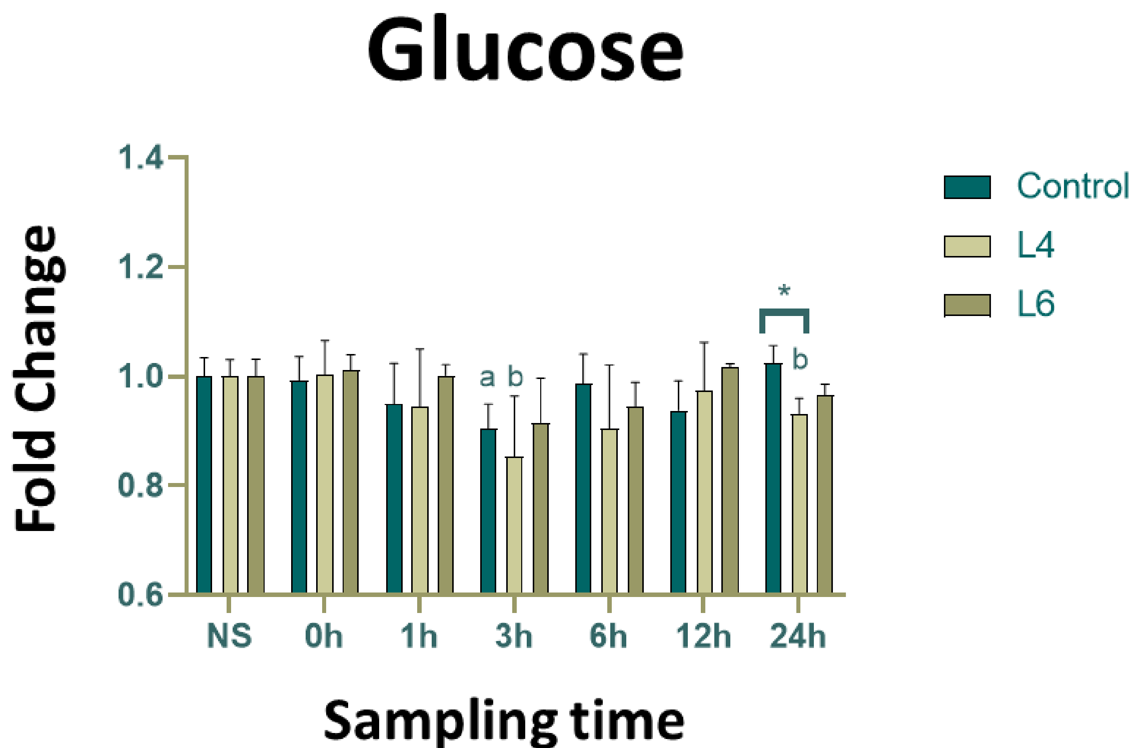

Figure 10. Glucose levels in fold change (compared to initial control value) at different time-points control diet (dark green), L4 (olive green) and L6 (pickle green. *: Significant differences ($p<0.05$) between dietary groups (at the same sampling time). Lowercase: a, b and c when compared with the initial control (NS) of the diet's groups control, L4 and L6 respectively.

5.2.3. Glutathione Peroxidase activity

Gpx activity results (Figure 11) showed that the base levels were 2.05 ± 0.72 nmol mL^{-1}, 1.43 ± 0.27 nmol mL^{-1} and 1.18 ± 0.18 nmol mL^{-1} for control, L4 and L6 groups respectively.

Among diets, at 12h post-stress, the L4 group showed a significantly lower Gpx activity levels ($p<0.05$) compared to the control diet. Moreover, the L4 group, at 12h post-stress (0.95 ± 0.2 nmol mL^{-1}) showed a significantly lower Gpx activity levels ($p<0.05$) compared to the NS group (1.43 ± 0.27 nmol mL^{-1}). On the other hand, the L6 group at 3h post-stress (1.77 ± 0.33 nmol mL^{-1}) had a significantly higher Gpx activity levels ($p<0.05$) compared with the NS group (1.18 ± 0.18 nmol mL^{-1}).

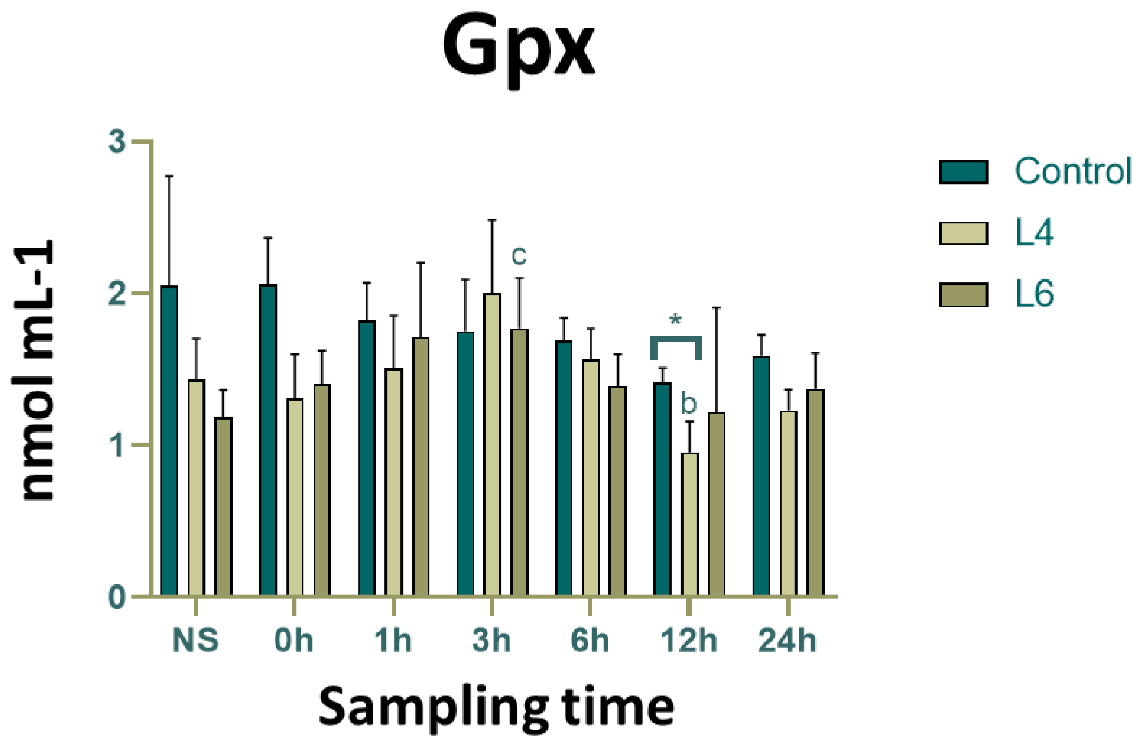

Figure 11. Gpx activity levels at different time-points control diet (dark green), L4 (olive green) and L6 (pickle green). *: Significant differences ($p<0.05$) between dietary groups (at the same sampling time). Lowercase: a, b and c when compared with the initial control (NS) of the diets groups control, L4 and L6 respectively.

5.2.4. Immune marker

The results of immunological markers at the protein level in plasma samples (compared between the different diets) showed that IL-10 fold change levels (Figure 12) were increased in the L6 group at 0, 1, 3, 6, 12 and 24 hours post-stress compared to the other diets (control diet and L4). In addition, when comparing the IL-10 values with the NS group, a significant decrease at 0-, 1-, 3- and 12-hours post-stress was detected in the control diet, while at 6h post-stress IL-10 was increased.

The same analysis in the L4 group showed that IL-10 levels were lower at 0, 1- and 12-hour post-stress. A different trend was observed in the L6 group, here at 6- and 24-hours post-stress an increase in IL-10 was detected compared to NS. The fold change in the L6 group was 0.80 ± 0.04, 1.05 ± 0.04, 1.31 ± 0.06, 1.68 ± 0.03, 0.84 ± 0.09 and 1.47 ± 0.06 for 0h, 1h, 3h, 6h, 12h and 24h respectively. These were significantly higher compared with the fold change of control and L4 diets at the respective time points.

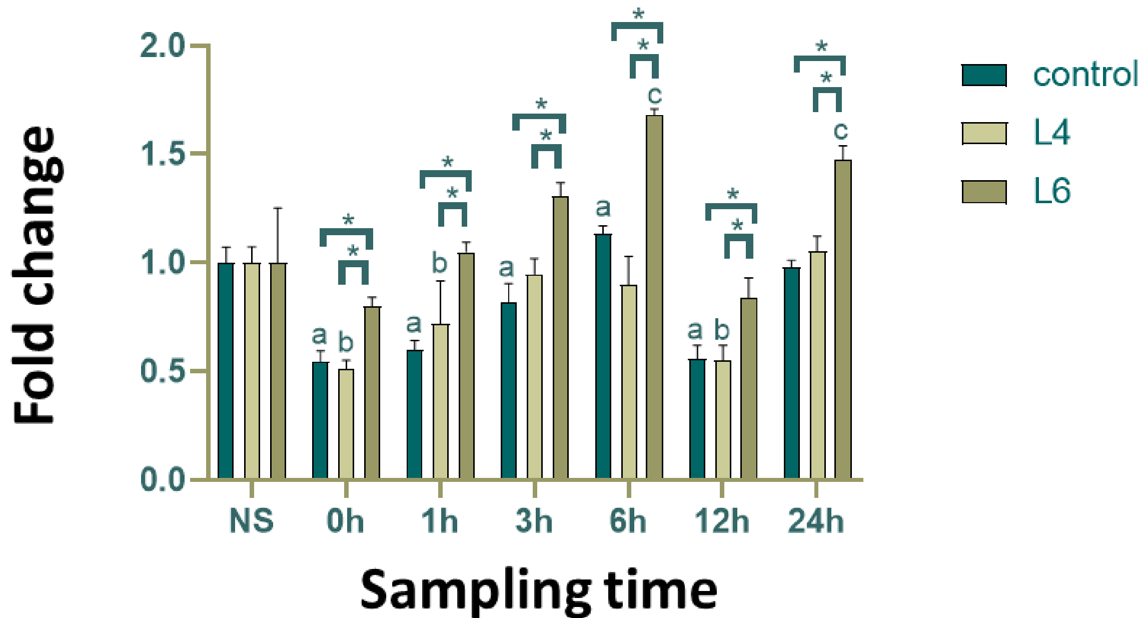

Figure 12. IL-10 levels at different time-points. Control diet (dark green), L4 (olive green) and L6 (pickle green). *: Significant differences ($p<0.05$) between dietary groups (at the same sampling time). Lowercase: a, b and c when compared with the initial control (NS) of the diets groups control, L4 and L6 respectively.

Regarding TNF-α (Figure 13), only significant differences were detected comparing NS with the other values for each diet. The three diets showed a similar pattern in the detection of this marker, which showed that at 3, 6 and 24 hours post-stress there was a significant increase in TNF-α levels namely 2.15 ± 0.38, 1.90 ± 0.26 and 1.78 ± 0.13 respectively for Control; 2.00 ± 0.09, 1.39 ± 0.16 and 1.57 ± 0.15 respectively for L4; and 1.89 ± 0.23, 1.75 ± 0.12 and 1.85 ± 0.23 respectively for L6.

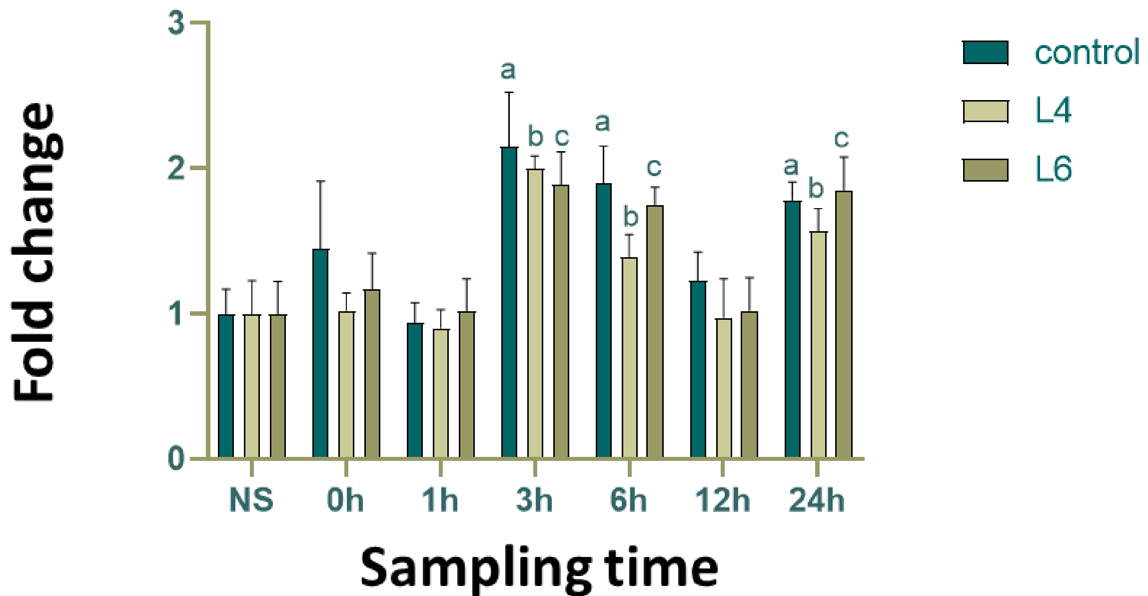

Figure 13. TNF-α levels at different time-points control (dark green), L4 (olive green) and L6 (pickle green). Significant differences ($p<0.05$) are denoted by * when compared with the TNF-α values of the different diets at the same time point and denoted by a, b and c when compared with the initial control of the diets groups control, L4 and L6.

5.3. Correlations

The correlation among the different parameters for each diet (Figure 14) showed that cortisol levels in control diet and L6 were significantly positively correlated. In addition, glucose levels in L4 and L6 were also positively correlated. Regarding immunological markers, TNF-α levels showed a significantly positively correlation among all different diets.

In L6, the plasma glucose levels were significantly negatively correlated with the TNF-α levels. Gpx and IL-10 did not show significant correlations among the different diets.

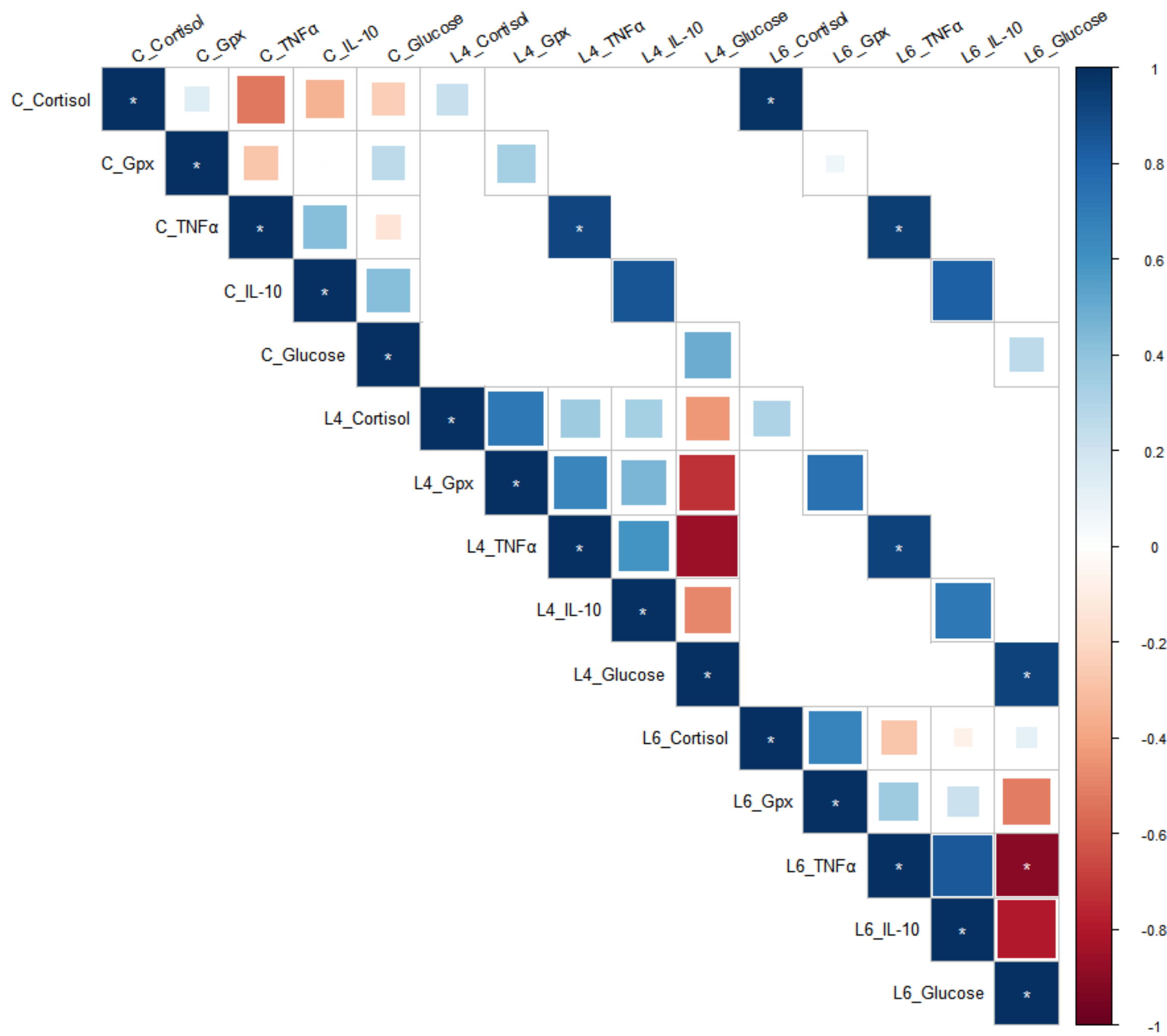

Figure 14. Correlation analysis among the different parameters. The prefix 'C_', 'L4_' and 'L6' refers to the diet groups Control, L4 and L6 respectively. All the parameters that are significantly correlated (p-value < 0.01) are denoted by '*'. Degrees of freedom = 5.

6. Discussion

The rapid growth in the aquacultural industry can lead to increased problems and challenges mainly related with multiple-stressors conditions during different stages of fish farming, which impact health and welfare (infectious diseases, suboptimal nutrition, environmental and handling stress) (6). Stressful conditions are in many cases unavoidable; however, they are one of the main impediments to the sustainable growth of this industry. Therefore, it is critical that these challenges are addressed effectively.

In intensive aquaculture, optimal nutrition is important for the coordination of the response of fish to stressful conditions, because the energy and nutrients provided by the feeds are essential to maintain an optimal immune function (22). Moreover, different studies have already investigated the role functional ingredients as immunostimulants (89–92). In fish, MI such as yeast cell wall components have shown to modulate stress responses. However, stress and immune modulatory functions of yeast cell wall components vary depending on the species and strain of yeast and the method used for downstream processing (29,78). To find an ideal candidate for commercial use, *in vivo* feeding experiments combining challenges with stressful conditions are necessary.

β-glucans and MOS, which are yeast cell wall components have shown to have an effect on the stress response in several fish species (87,93,94). They are non-soluble polysaccharides that act as prebiotics which promote the growth of gut microbiota. Prebiotics have been found to alleviate chronic gastrointestinal inflammations (93), which could be associated with reduced intestinal permeability. Another plausible mechanisms for the regulation of gut conditions are the reduction in inflammation due to changes in gut microbiota (95,96) and increased production of mucus. Nevertheless, reports describing the systemic effect of the use of immunostimulants during stressful conditions are still scarce.

Stress induces an increase in cortisol in fish (58). Cortisol is a classical marker for measuring stress response in vertebrates and its typical pattern is that plasma levels of this hormone peak 1 to 3 hours after exposure and return to normal within 6 to 8 hours (58,97). In the current study, we evaluated the effects of two yeast cell wall candidates, namely L4 and L6, on immune and stress response in Atlantic Salmon when exposed to acute hypoxic stress. The results of this study showed that a short-term hypoxia induced an increase in plasma cortisol levels 1 hour post-stress in fish fed the control and the L6 diet, which is similar to what was recently described in Atlantic salmon (58) using the same stress model. Interestingly, the inclusion of L4 in diets appears to prevent the cortisol peak in the fish (compared to the other groups). This phenomenon has been observed before where β-glucans or other yeast cell wall components have reduced the cortisol levels in response to stress (21,94,98). However, to our knowledge, this is the first time this phenomenon is observed in Atlantic salmon. It has also been reported that stress and the subsequent increase in cortisol has been found negatively correlated with the number of taxa of the gut microbiome (99). A proposal to explain the absence of cortisol peak in response to stress could be that the addition of prebiotics, which are known to enhance the growth of specific microbiota (100), prevent the increase of cortisol levels in the blood plasma (93), as observed in the present study (with L4 diet). This might suggest that use of a diet with the inclusion of L4 would improve the

resistance of the fish to acute stress, enhancing the welfare of the animal. However, further research needs to be done to elucidate the effect of L4 on the gut and microbiome.

One of the effects of cortisol in vertebrates is that it increases the glucose circulation in the blood. This phenomenon usually occurs at the same time that the cortisol peak is observed, but is not always the case (21). In this study, glucose levels had a flat behaviour without marked variations (except at 3 and 24 hours post-stress in the group of fish fed L4), which does not allow proposing the evaluation of glucose levels as a standard parameter for characterizing the effect of acute hypoxia stress in Atlantic salmon.

Additionally, stressor conditions also causes redox imbalances which can be studied by the activity of redox enzymes such as Gpx (60). Cortisol has two main pathways for exerting its effects. One is the genomic pathway where cortisol increases the transcription and translation in the cell and the subsequent increase in protein synthesis (49). The other is the non-genomic pathway in which cortisol is known to increase the reactive oxygen species (ROS) and reactive nitrogen species (RNS) (40,101). This increase of ROS and RNS are known as oxidative stress and are countered by the antioxidants of the defence mechanism of the animal. One such pathway is the glutathione pathway which increases the activity of Gpx in response to oxidative stress. It is described that acute hypoxia stress increases Gpx activity 12 hours post-stress (58). Nevertheless, no such peak was observed in this trial. One possible explanation for the absence of the peak in the present study compared with (58) could be the differences in genetic makeup of the fish. Even the results showed that Gpx activity was significantly lower compared to the control group at 12h after stress (in L4 group), which may have been due to the reduced levels of cortisol or the absence of a peak in the fish fed the L4 diet.

Stress has shown to modulate cytokine levels such as that of IL-1β (42) and IL-10 (58). Hence it would be interesting to look at the downstream effects of stress on immune markers and how these markers respond differently when given yeast cell wall components as functional feed. TNF-α and IL-10 are classical pro and anti-inflammatory molecules, respectively, and they are ideal candidates to study the interaction between stress, nutrition, and immune response. A rise in TNF-α and IL-10 levels in response to β-glucans have been observed before (102). In this study, there was no difference in TNF-α levels among the diets. However, an increase of TNF-α was detected at 3, 6 and 24 hours post-stress in all diets (compared to the initial control without stress). This could suggest an activation of pro-inflammatory pathways in fish after short-term stress, which should be further investigated in future research, as this could be related to an attempt by the fish to control immunosuppressive profiles. In addition, IL-10 showed significant increase in the L6 group compared with both control and L4 group. This could either be beneficial or detrimental as immunity is a double-sided sword. Inflammation helps effectively counter pathogenic invasion. But, if the inflammation is not controlled, it could be detrimental and damage the tissues (69). Hence, future studies with pathogenic trial will help us understand L6's effect on disease resistance. This understanding might help reduce mortality in aquaculture, thereby, reducing production costs and improving fish welfare.

It is again interesting that the L4 group did not show any significant change in IL-10 levels compared with the control and that L4 did not show a surge in cortisol levels compared to the control. This phenomenon suggests the activation through different effector pathway(s)

(other than through cortisol) in response to hypoxia stress. To further support this conclusion, in the present study, the peak cortisol levels varied among the diet, whereas the TNF-α levels did not. An *in-vitro* study on head kidney cells of gilthead seabream suggest that ACTH, acting independently of cortisol, can increase the TNF-α mRNA levels (103). While L6 appears to have a similar effect on cortisol as the control diet, L4 shows a relatively flat response over time. L4 can be useful as a functional feed ingredient, capable of preventing or reducing the response of salmon to hypoxic stress.

From the data of this work, it can be hypothesized that different strains or downstream processing can have different proportions of MAMPs or different bioavailability of functional molecules. These differences could lead to variations in host's stress and immune response (29,78). In any case, understanding the interplay between the biomarkers and functional ingredients will help us get a better picture of the immune reaction in response to stress.

Given these results, there are two possible approaches for further studies regarding nutrition and stress. The first option is that, we can take a fundamental approach, characterize the different components in the functional feeds and identify which component(s) is/are responsible for the stress resistance and cytokine level changes. This approach will help us understand the exact underlying mechanisms of the stress response. Alternatively, we can take an application-oriented approach and identify the window in which the functional feed is most potent and duration for which its effects last. If the effects can be induced within a short span of time and the effects on stress and immune response are transient, the functional diets could be used before situations that require the handling of salmon such as vaccination, delicing and transfer where the fish are taken out of the water. Also, it would be interesting to look at whether the same functional ingredients can have a stress resistance effect on other species of fish.

In the stress model used in this study, the trend of cortisol levels in the control diet and L6 follows a similar pattern observed by a previous hypoxic stress test conducted by Djordjevic et al (58). This similar behaviour proves the robustness and repeatability of the hypoxic stress test. Some of the contemporary methods for stress test include crowding and transport (87,94,97,98,104). Nevertheless, these methods are inflexible in the sense that they are either time-consuming or require a lot of fish or both. The hypoxic stress test, on the other hand, is quite simple and easily controlled. This opens an array of further studies such as identifying non-invasive markers for detecting stress. This simple hypoxic stress test can also be coupled with other stressors to create a multi-stressor effect, which would help us better simulate fish farm conditions (105). Also, the repeatability of this test would allow us to validate and standardise non-invasive stress measurements eg. cortisol from skin mucus.

Future studies should include a stress-free control for each time point. This setup would require an additional 21 tanks with 6 fish in each tank. Also, this setup would enable us to make stronger interpretations about different time points for the same diet. Also, a wider variety of antibodies for salmon would have given us a better selection of immune markers to analyse.

7. Conclusion

Atlantic salmon fed two different functional ingredients based on yeast cell wall from non-saccharomyces (L4 and L6) showed some distinctive differences in their stress and immune responses after hypoxia stress. The use of a L4 diet enabled the Atlantic salmon, to control the surge in cortisol levels in the plasma after exposure to acute stress. Therefore, the null hypothesis can be rejected. On the other hand, the L6 diet, did not prevent the surge in cortisol levels in response to stress of the fish. Nevertheless, these fish showed a higher availability of IL-10 in plasma samples (compared to the control and L4 diets), so the null hypothesis can be rejected for immune response and accepted for stress response.

This study is the first step towards a more resilient salmon capable of coping with the challenges presented by a more intensive aquaculture. Further studies should be conducted before these functional feeds can be applied in the industry.

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