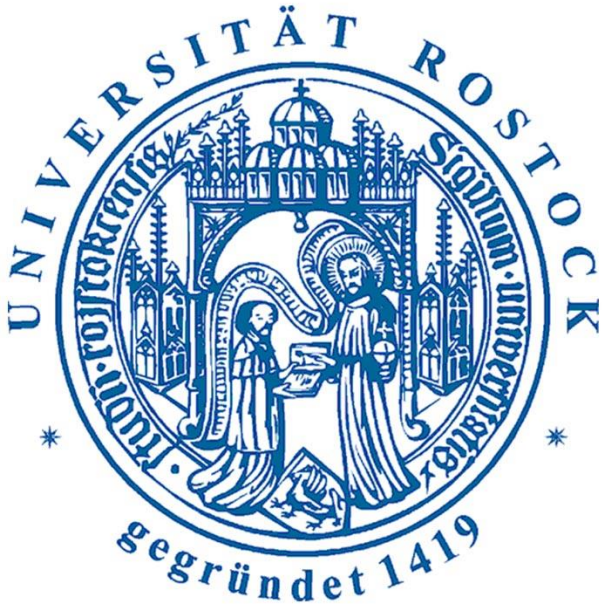
Bioindicators to evaluate welfare status of Atlantic salmon *Salmo salar* under changing farming conditions and the potential of microalgae-enriched diets



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Doret Rosie van Muilekom, geb. am 03.04.1995 in Deventer

aus den Niederlanden

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Supervisors:

Prof. Dr. Tom Goldammer

University Professor for Molecular Biology and Fish Genetics, Faculty of Agricultural and Environmental Sciences, University of Rostock, Germany
Head of Fish Genetics Unit, Research Institute for Farm Animal Biology (FBN), Dummerstorf, Germany

Prof. Dr. Inna Sokolova

Professor and Chair of Marine Biology, Institute for Biological Sciences, University of Rostock, Rostock, Germany

Reviewers:

Prof. Dr. Tom Goldammer

Prof. Dr. Inna Sokolova

Prof. Dr. Matthias Stöck

Summary

The welfare of farmed fish is a major concern in sustainable aquaculture. New farming strategies associated with changing husbandry conditions may disturb the fish's homeostasis and, thus, their health and welfare. Only informative bioindicators reflect the fish's health and welfare status. The present thesis aimed to identify bioindicators affected by changing farming conditions and dietary components. Indicative parameters were measured at various levels, from morphometric indices and feed intake to blood parameters and expression profiles of stress- and immune-related genes, to determine the status of Atlantic salmon *Salmo salar*. The fish were exposed to potentially challenging farming conditions by transfer from brackish water to full-salinity seawater and treatment of the recirculating aquaculture system (RAS) with peracetic acid (PAA)-based disinfection. Alongside, the beneficial effects of diets enriched with microalgae on the health status of Atlantic salmon and the potentially mitigating role of microalgae on the effects of changing farming conditions were evaluated. In addition, this thesis characterized the family of NF-κB inhibitors (IκB), which have emerged as informative bioindicators as key regulators of stress and immune responses in salmonid fish.

Expression patterns were identified in Atlantic salmon under changing farming conditions in combination with feeding diets enriched with microalgae. The most significantly affected indicators were involved in stress and immune responses such as antiviral defense, acute phase, T helper 1 (Th1) immunity, and the transition from innate to acquired immunity. The potential as bioindicators of NF-κB inhibitors was confirmed by the involvement of salmonid IκBα and IκBε proteins in immune regulatory processes. Plasma cholesterol levels and myeloperoxidase concentrations in the liver were specifically affected by microalgae-enriched diets, and the fatty acid and carotenoid content of fish muscle reflected the fatty acid and carotenoid composition of the microalgae species fed to the fish. Since no harmful levels of indicative parameters were measured, we concluded that the fish were in good health. The measured mild stress and immune responses were most likely necessary to restore homeostasis after transfer from brackish water to seawater and after PAA-based disinfection. Supplementation of diets with selected microalgae had no significant beneficial effect on simultaneous growth performance, product quality, and immune and stress status of fish. However, microalgae species-specific effects were found on certain parameters, such as growth performance, body composition, diversity of gut microbiota, and liver health. At the same time, our research also indicated that not only microalgae species but also preparation, inclusion levels, and feeding frequency influence the potency of dietary microalgae. No major influence of microalgae on fish health status was observed after changes in farming conditions. Therefore, salmon feed may be supplemented with microalgae after carefully considering the eventual product quality and either excluding or targeting modulation of certain stress or immune pathways.

In conclusion, this thesis advances the assessment of the welfare status of Atlantic salmon, evaluates new farming strategies, and outlines the potential of microalgae-enriched diets for salmon health. In future research, the panel of indicative parameters can be further expanded through transcriptomic, proteomic, and behavioral analyses to provide a more thorough examination of innovative farming techniques.

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Abbreviations

arg2	Arginase 2
A. platensis	*Arthrospira platensis*
b2m	Beta-2 microglobulin
BSC	Brain-sympathetic-chromaffin
C. vulgaris	*Chlorella vulgaris*
c1ql2	Complement C1q like 2
c4b	Complement C4b
camp	Cathelicidin
cat	Catalase
ccl4	CC chemokine 4
CD	Control diet
cd28	CD28 (cluster of differentiation) T-cell-specific surface molecule
cd4	CD (cluster of differentiation) 4 T-surface molecule
cebpb	CCAAT/enhancer-binding protein beta-encoding gene
CHSE-214	Chinook salmon embryonic cell line
CNS	Central nervous system
clra	C type lectin receptor A
cu/znsod	Copper- and zinc-containing superoxide dismutase
cxcl8a	Interleukin-8-like a
drtp1	Differentially regulated trout protein 1-encoding gene
fcgr1a	High affinity immunoglobulin gamma Fc receptor I precursor
FCR	Feed conversion ratio
gpx	Glutathione peroxidase
gr	Glutathione reductase
hamp	Hepcidin
HPI	Hypothalamic-pituitary-interrenal
HSP	Heat shock protein
hsp1a1	Heat shock 70 kDa protein 1-encoding gene
hspa5	Heat shock 70 kDa protein 5(glucose-regulated protein 78 kDa)-encoding gene
ier2-2	Immediate early response 2-2
ifit5	Interferon-induced protein with tetratricopeptide repeats 5-2-encoding gene
IKK	IκB kinase
ikba	NF-kappa-B inhibitor alpha
il10	Interleukin 10
il10rb	Interleukin-10 receptor beta chain precursor
il18	Interleukin-18
il15rα	Interleukin 15 receptor subunit alpha
il1b	Interleukin-1 beta
isg15	Interferon-stimulated gene 15
il1r2	Interleukin-1 receptor type II
IκB	Inhibitor of NF-κB
lao1	L-amino-acid oxidase
lyzc2	Lysozyme C II precursor
marco	Macrophage receptor with collagenous structure
mnsod	Manganese superoxide dismutase

mpo	Myeloperoxidase
muc2	Mucin-2
muc5ac	Mucin-5AC
muc5b	Mucin-5B
muc7	Mucin-7
NF-κB	Nuclear factor kappa-light chain-enhancer of activated B cells
nfkbia	NF-kappa-B inhibitor alpha
nkaα1a	Na(+)/K(+)-ATPase subunit alpha 1a
nkaα1b	Na(+)/K(+)-ATPase subunit alpha 1b
PAA	Peracetic acid
PRE	Protein retention efficiency
psu	Practical salinity unit
RAS	Recirculating aquaculture system
ROS	Reactive oxygen species
RNA-Seq	RNA-sequencing
rsad2	Radical S-adenosyl methionine domain-containing protein 2-encoding gene
saa5	Serum amyloid A-5 protein-encoding gene
sod1/Sod1	Superoxide dismutase 1
SWT	Seawater transfer
S. limacinum	*Schizochytrium limacinum*
TAC	Total antioxidant capacity
TCA	Tricarboxylic acid cycle/ citric acid cycle
T. chuii	*Tetraselmis chuii*
Th	T-helper
tnfrsf14	TNFR superfamily member 14
UV	Ultraviolet
znfx1	NFX1-type zinc finger containing protein-encoding gene

1. Introduction

1.1. Aquaculture and salmonid farming

Aquaculture production continues to grow worldwide and is therefore playing an increasingly important role in food security and nutrition for the growing global population. In 2020, the global aquaculture production was 122.6 million tons, of which 87.5 million tons consisted of aquatic animals (FAO, 2022). The growth in marine aquaculture production is mainly attributable to the resilient salmon. Due to its suitability for large-scale aquaculture, this fish holds a strong position in the world market. This high-value species is a great source of protein, omega-3 fatty acids, and key nutrients such as minerals and vitamins, which support a healthy diet for humans (FAO, 2022; Mettu et al., 2017; USDA - Food Composition Databases, 2019).

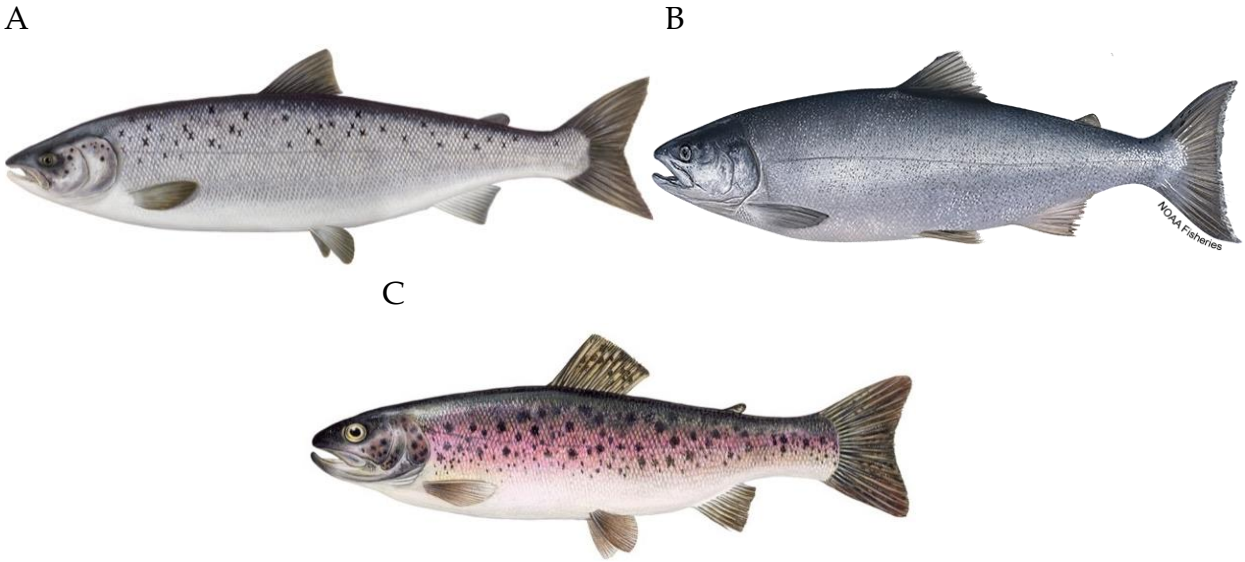

Figure 1. Adult individual of A) *Salmo salar*, B) *Oncorhynchus kisutch* and C) *Oncorhynchus mykiss* (Scandinavian Fishing Year Book and NOAA Fisheries).

The salmonid family comprises many members, of which Atlantic salmon *Salmo salar* (Linnaeus, 1758), coho salmon *Oncorhynchus kisutch* (Walbaum, 1792) and the closely related rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) are the most commonly produced salmonids for aquaculture (FAO, 2022) (Figure 1). As their name suggests, Atlantic salmon are native to the North Atlantic, from the North American coast to Greenland, Portugal, Norway, and the Baltic Sea. Although there are some non-anadromous populations (Berg, 1985), the majority of Atlantic salmon follow anadromous migration and can reach rivers, coastal waters, and the open ocean in these areas (Ó Maoiléidigh et al., 2018). Due to its favorable geographic location, most salmon are farmed in these areas (European coast and Canada), as well as on the Chilean coast, where Atlantic salmon was introduced (Asche et al., 2013; FAO, 2022).

1.1.1. The life cycle of wild salmon

In the wild, anadromous salmon return from the ocean to freshwater to spawn in autumn, after which eggs laid by females in gravel nests are fertilized. The following spring, the eggs hatch as alevins that absorb nutrition from the yolk sac and then develop into fry. From this stage, the fry start feeding and further develop into parr. These freshwater phases last a varying number of years in total (Mobley et al., 2021). Subsequently, when fish reach an appropriate size, the parr can go through so-called smoltification, a process that prepares the

fish for transfer into seawater (Björnsson et al., 2000; Hoar, 1988). During this parr-smolt transformation, fish go through different physiological, behavioral, and morphological changes. Smoltification is initiated by environmental cues such as photoperiod and temperature, which activate the endocrine system and then drive the different processes of smoltification (Björnsson et al., 2000; Ebbesson et al., 2003; Hoar, 1988). These changes involve, for example, a change in the quantity and distribution of body lipids and an enhanced metabolism, giving fish a leaner, longer shape. In addition, a smolt can be recognized by a silver glow and a behavioral shift to schooling with other smolts, both to reduce predation risk in the pelagic environment (Björnsson et al., 2011; Hoar, 1988). Furthermore, to cope with the higher salinity in seawater, physiological changes concerning ion regulation take place in the gill epithelium (Morera et al., 2021). The seawater transfer is an important stage in the life cycle of salmon, as they have a rich food supply of marine organisms in the oceans, allowing them to feed and grow well.

1.1.2. The production cycle of salmon

In salmon farming, the biological life cycle is maintained in the production process. Commonly, the process in salmon aquaculture can be divided into three different phases (Figure 2) (Asche & Bjorndal, 2011). During the first step, adult salmon are selected as broodstock and transferred to freshwater tanks. Here, eggs are fertilized and hatched, and the fish grow from alevin to fry, to eventually feeding parr. Often, at this second step of the production process, fish are transferred to tanks with either a recirculating aquaculture system (RAS) or flow-through system. As in nature, farmed salmon also prepare for life in seawater by smoltification. Once the fish have reached the correct size, parr-smolt transformation will commence either naturally or by artificial photoperiod (Morera et al., 2021; Myklatun et al., 2023). Subsequently, as the fry have developed into full smolts, the salmon are transferred in the third step of the production process to large cages in the sea. This phase lasts the longest, but the fish also grow substantially to about 3- 5 kilograms in 14- 24 months (Morera et al., 2021). After this, the fish are harvested and further processed for market.

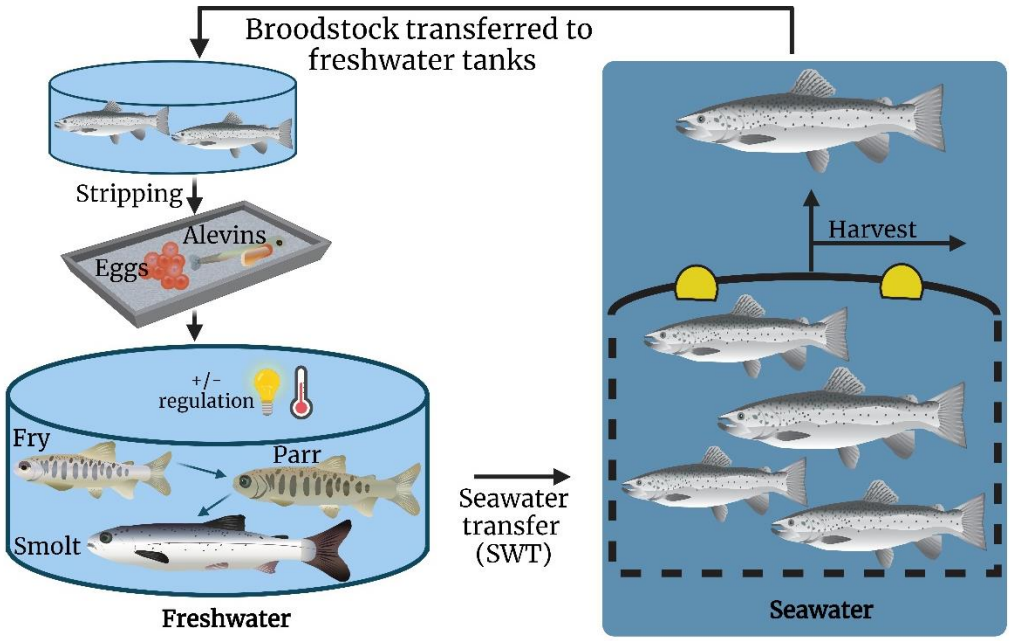

Figure 2. Production cycle of salmon, adapted from: (Fisheries and Aquaculture - Cultured Aquatic Species - Salmo salar (fao.org))

1.1.3. Developments in salmon farming

Despite the potentially complicated anadromous life cycle of salmonids, fish are very advantageous as farm animals due to their low energy consumption because of their cold-blooded homeostasis physiology and their gravitational resistance to underwater buoyancy (Macaulay et al., 2020; Philis et al., 2019; Torrissen et al., 2011). Over time, many investments have been made in salmon farming that have resulted in innovations in different areas within the industry.

Originally, salmon were farmed on a small scale by fishermen. In the 1970s, the introduction of sea cages enabled large-scale salmon aquaculture to be produced. Since then, the salmon industry has grown tremendously, mainly due to innovations that have been applied (Afewerki et al., 2023; Asche, 2008). These innovations affect the entire salmon production process, starting from egg growth to smolt. Initially, the young fish were kept in the tanks for only a few days, and then they continued to grow into smolts in freshwater. During the 1970s, this whole process was moved to land-based farms, where fish could be monitored and controlled much more closely. Here, the fish resided in the so-called flow-through system where fresh water from a river passes through the facility (Sandvold & Tveterås, 2014). Later, in the mid-2000s, an innovative technology was implemented, the RAS, in which water is recycled, nitrogenous metabolites and carbon dioxide are filtered out, and oxygen is added (Espinal & Matulić, 2019). Besides a huge reduction in water use, this closed system also allows more control of the environment, such as temperature regulation and water quality (Bergheim et al., 2009; Espinal & Matulić, 2019; Kristensen et al., 2009). Currently, many farms produce smolts entirely in RAS. Here, for example, artificial light can also be used to control smoltification, leading to smolt production throughout the year (Sandvold & Tveterås, 2014).

During the grow-out phase, the salmon are transferred to seawater, which allows them to grow and get ready for harvesting (4-6 kg). At the beginning of salmon farming in 1970, salmon stayed during the grow-out phase in simple salmon cages made of wood with a width of 5-10 m and 4 m depth that were easily accessible from land. With the growth of salmon farms, many innovations were applied, such as the enlargement and improvement of salmon cages (currently averaging 50 m in diameter and 40 m in depth), the relocation of salmon cages to the open sea and fjords, and the use of boats with advanced instruments for feeding, activity, and health tracking (Afewerki et al., 2023; Asche et al., 2013).

Already during the early days of salmon farming, a breeding program was implemented, primarily to improve fish growth (Næve et al., 2022). Nowadays, other features such as product quality and the robustness of the fish are also very valuable. In terms of end product quality, the fish's body shape, fat percentage, and fillet color are traits of interest (Powell et al., 2008). High robustness of fish is important as it reflects good health, expressed by disease resistance and stress tolerance (Gjedrem & Rye, 2018). Throughout the production process, the health of fish is also monitored. During the huge growth of salmon farming in the 1980s, the production of the fish in high densities started, which was eventually accompanied by high death rates due to bacterial and viral diseases. Where antibiotics were first widely used, vaccines were soon developed, which is currently still an important development within salmon farming for the prevention of disease outbreaks and further dramatic consequences (Sommerset et al., 2020; Tveterås, 2002).

Feed is one of the most important factors in salmon farming, mainly because of its major impact on the output of production but also because feed represents around 50% of the costs

for farmers (FAO, 2022; Fauske, 2022; Torrissen et al., 2011). For those reasons, a lot of financial investment has gone into constantly improving feed quality. Shortly after salmon farming began, farmers had already switched from feeding raw fish and semi-moist pellets to innovative high-energy extruded dry pellets (Tacon et al., 2011). Over the years, these pellets have been further developed to determine the best ratio of ingredients for the salmon's health and production efficiency, as well as the pellet's environmental impact (Afewerki et al., 2023). With the continued growth of salmon aquaculture, challenges keep arising within feed development. These vary from uneaten feed ending up in the open sea, fishmeal, and fish oil, which need to be limited, to alternative ingredients with an imbalance or too low values of certain nutrients (Afewerki et al., 2023; Bandara, 2018). The main concern is the reduction and replacement of fishmeal and fish oil in feed because of the adverse environmental impact of the large input of pelagic fish stocks and the high financial cost (Olsen & Hasan, 2012). However, research continues to improve feeds in order to achieve optimal fish performance at the lowest possible cost (Karalazos et al., 2011; Opstvedt et al., 2003; Weihe et al., 2018; Ytrestøyl et al., 2015).

1.2. Challenges in salmon farming

In the short age of salmon aquaculture, many innovations have already taken place through modern technologies, which have made the industry grow enormously and become more efficient. However, there are still many challenges in salmon farming that need attention. One of the major challenges remains salmon farming's intensification, which causes environmental problems in open seas such as microbe and salmon lice outbreaks, escapes of farmed fish, and water pollution by uneaten feed and feces (Naylor et al., 1998; Torrissen et al., 2013). Besides being a burden on the surrounding nature, it also affects both wild and farmed salmon, as they share the same habitat or ecosystem during the grow-out phase. Wild salmon populations are primarily the source of pathogens that infect farmed salmon. However, the high density of farmed salmon provides ideal conditions for further spread of the diseases, not only within farmed salmon but also again to wild salmon populations (Johansen et al., 2011; Liu et al., 2011). This susceptible environment creates uncertainties and ongoing challenges in salmon farming.

Nowadays, some farms are using a new strategy of retaining smolts in RAS for a longer time, even during the initial stages in saltwater, allowing the fish to start the grow-out phase as larger, more robust post-smolts (Afewerki et al., 2023). Despite the fact that RAS is a closed system where the environment can be well controlled, farming salmon in RAS may pose challenges to salmon health and welfare due to high stocking densities (Calabrese et al., 2017) and maintenance of RAS with, for example, routine disinfection (Pedersen & Lazado, 2020; Soleng et al., 2019). The optimal conditions for growing post-smolts in RAS are still under investigation, addressing these challenges by evaluating the best water treatment processes (Xiao et al., 2019) and water quality parameters, including nitrite levels (Mortensen et al., 2022), but also factors such as salinity and water velocity (Ytrestøyl et al., 2020).

To preserve and maintain a healthy and clean living environment in RAS, the circulating water can be cleaned by many different treatments, such as physical filtration, biological filtration and disinfection and sterilization with UV irradiation, ozonation, or the use of chemicals as disinfectants (Carletto et al., 2022; Gonçalves & Gagnon, 2011; Mota et al., 2022; Xiao et al., 2019). One example of an agent used to clean the habitat within RAS is peracetic acid (PAA), a disinfectant with strong oxidizing power and biocidal activity (Finnegan et al., 2010). Besides

its effectiveness against pathogens, PAA also seems like a welfare-friendly disinfectant for fish. In previous research, an adaptive stress response was measured in the plasma and mucosal organs of fish to therapeutic doses of PAA, which could be handled well and did not further flow into chronic stress (Lazado et al., 2020, 2021; Osório et al., 2022; Soleng et al., 2019). However, there is still little knowledge about the consequences in, for example, the head kidney that plays an important role in both systemic stress and immune response. This work extends the understanding of peracetic acid's effects on salmon in RAS by examining its effects on the immune system and stress response on both local and systemic levels.

After smoltification in closed systems, the fish are transferred for the grow-out phase to sea cages in open water. This seawater transfer is important for fish growth and maturation but also very susceptible to fish due to a suppressed immune system of the fish combined with increased pathogen pressure in the marine environment (Bang Jensen et al., 2020; Jansen et al., 2010; Johansson et al., 2016). This leads to high mortality rates during the first three months after seawater transfer (Bang Jensen et al., 2020; Oliveira et al., 2021). Apart from large economic losses, these death rates must be reduced for more sustainable salmon production. Besides focusing on salmon growth and low production costs within salmon farming, good fish health and welfare are also necessary for high-quality farmed fish products.

Considering this, new production strategies should be explored to improve the robustness and resilience of salmon. Therefore, we studied the effect of salinity change from brackish water to full-salinity seawater on the immune and health status of Atlantic salmon. Brackish water has a higher salinity than freshwater, making the transition to seawater more gradual and closer to the natural life cycle of salmon, where the fish swim from low salinity in river water to salty seawater. In this way, when smolts are transferred to seawater after brackish water acclimation, they may consume less energy in adapting to higher salinity and can use the saved energy for further adaptation to the open sea, such as building resistance to the many pathogens. In previous research, Atlantic salmon reared in brackish water had the best growth rate (Bœuf & Payan, 2001; Ytrestøyl et al., 2022), suggesting that energy savings in osmoregulation by the isosmotic environment are invested in growth, which could consequently lead to more robust fish after brackish water acclimation (Potts, 1954). However, salinity also has an effect on food intake, which in turn can play a role in the growth rate of fish (Bœuf & Payan, 2001). In another study, moderate exercise training in combination with an isotonic salinity (12 psu) showed, in addition to improved post-smolt growth and feed efficiency, positive effects on the welfare and survival of salmon (Calabrese, 2017; Ytrestøyl et al., 2020).

The intensification of salmon farming not only calls for innovative farming methods but also novel feed ingredients that are less dependent on finite marine resources. In recent years, microalgae have emerged as valuable feed ingredients. These are single-cell photosynthetic microorganisms having a rich nutritional profile including lipids, proteins, carbohydrates, pigments, minerals, and vitamins (Vaz et al., 2016) and are known to have immune-stimulating and stress-modulating properties (Carballo et al., 2018; Cerezuela, Guardiola, González, et al., 2012; Riccio & Lauritano, 2020; Teimouri et al., 2019). Because of this advantageous composition, much research has already been done to replace conventional fishmeal and fish oil with microalgae, which can be produced in a more sustainable manner (Ahmad et al., 2022; Ansari et al., 2021). The different types of microalgae vary in chemical composition. Green microalgae such as *Chlorella vulgaris* contain high concentrations of

carbohydrates, whereas *Spirulina platensis* contains a lot of protein (Rocca et al., 2015), and *Tetraselmis chuii* is high in polyunsaturated fatty acids (Mohammadi et al., 2015). Due to the different nutritional profiles, microalgae also have a wide array of immune and stress-modifying features affecting fish, ranging from increased white blood count (Yeganeh et al., 2015) to enhanced phagocytosis and complement activity (Cerezuela, Guardiola, González, et al., 2012) and higher antioxidant levels (Mahmoud et al., 2020). Beyond the role of microalgae as an alternative ingredient, little research has been done on the added value of microalgae in mitigating the effects of changing farming conditions. Therefore, this thesis investigated the effects of diets enriched with microalgae and their mitigating potential on the effects of peracetic acid-based disinfection and salinity change.

1.3. Health and welfare of salmon and their immune and stress responses

As mentioned in Section 1.2, salmon farming today is not only about production output but also about the health and welfare of the fish. To measure health and welfare status, it is important to define what is considered a "good level" of health and welfare. One way to classify animal welfare is through the three different categories: feelings, nature, and function (Segner et al., 2012; Turnbull & Kadri, 2007). Since animals are sentient (Chandroo et al., 2004), the first category of 'feelings' is concerned with animals not having negative but positive emotions. The 'nature' category includes the importance of being able to exhibit natural behavior for good animal welfare. Within the latter category, the importance of functions is also taken into account, meaning the ability of animals to adapt to environmental influences that do not involve crossing physical boundaries (Segner et al., 2012). In other words, in this category, good welfare is defined as preserving homeostasis and biological functions, which is also reflected in good health and a lack of illness. This thesis mainly focuses on the functional aspect of fish welfare, thereby addressing the health status of fish, which is a fundamental component of good welfare (Duncan, 2005). Specifically, we look at the effects of changes in farming conditions through peracetic-based disinfection and salinity change, and the potential of diets enriched with microalgae on the health and immune status of fish reared in RAS.

1.3.1. Stress response

Changes in the environment can create stressors that disrupt the internal homeostasis of animals. This triggers a stress response, a biological process that is conserved in vertebrates and is an interplay between the nervous, immune, and endocrine systems. The stress response can be subdivided into the primary, secondary, and tertiary responses (Wendelaar Bonga, 1997). The primary response is established in the hypothalamus, which then activates the brain-sympathetic-chromaffin (BSC) axis and hypothalamic-pituitary-interrenal (HPI) axis, resulting in the production of stress hormones such as adrenaline and cortisol. These stress hormones trigger a secondary stress response, which leads to a reaction at tissue levels. Here, physiological changes take place in metabolism, oxygen uptake, hydromineral balance, acid-base status, and immune and cellular responses, which are reflected, for example, by an increase in lactic acid, glucose, and heat shock proteins (HSPs) (Barton, 2002; Wendelaar Bonga, 1997). As a result of these adaptations, energy is released and defense systems are activated, allowing the fish to cope with the stressor. In the situation where the fish eliminates the stressor during this process, this type of stress is defined as acute stress, as it lasts at most minutes to hours.

The wide range of types of stressors challenging fish demands complex physiological mechanisms and behavioral changes to counteract. Based on the type, duration, and intensity of the stressor, an appropriate stress response is initiated, which aims to restore homeostasis (Balasch & Tort, 2019). This is an energy-consuming process that increases the allostatic load, also known as the energy required to adapt to the new circumstances and restore homeostasis (McEwen & Wingfield, 2003). The organism can adapt to an increased allostatic load up to certain limits. However, when the energy demand for the stress response becomes too high, allostatic overload occurs. Hereby, the tertiary stress response at the whole-animal level is induced, which requires energy budgeting from growth, reproduction, and immunity (Schreck, 2010; Wendelaar Bonga, 1997). In the long term, this allostatic overload, or chronic stress, leads to an impairment of the condition and health status of fish, resulting in a higher susceptibility to pathologies and poor welfare (Chiappelli et al., 1993; Korte et al., 2007; Pickering & Pottinger, 1989; Segner et al., 2012; Wendelaar Bonga, 1997).

1.3.2. Immune response to stress

There is a close cooperation between the immune and neuroendocrine systems since teleost stress hormones from both BSC and HPI axes are produced in the head kidney, an organ that also performs immune functions (Verburg-Van Kemenade et al., 2009). The fish's response to a stressor also involves an immune reaction, which starts as early as the secondary stress response. After the activation of glucocorticoid receptors by the binding of stress hormones, two types of processes are induced: a non-genomic and a genomic signaling cascade. Non-genomic signaling occurs rapidly through, for example, second messengers that activate effector proteins. The genomic signaling takes hours to days, promoting the expression of transcription factors that induce the expression of immune- and stress-response genes (Buchmann & Secombes, 2022; Das et al., 2018).

After an acute stressor, the innate immune system is activated to induce immediate responses to danger signals. In teleost, this includes, for example, an enhanced synthesis of acute-phase proteins and a quick release of different stress proteins, pro-inflammatory cytokines, and hormones (Buchmann & Secombes, 2022; Tort, 2011; Wendelaar Bonga, 1997). In addition, neutrophil and granulocyte levels are increased in the blood, while lymphocyte levels reduce, probably due to migration to impacted organs (Seibel et al., 2021; Tort, 2011; Wojtaszek et al., 2002). However, previous studies also show contrasting activity levels of non-specific immune markers such as lysozyme and complement system and conflicting results on phagocytic activity upon cortisol exposure (Guo & Dixon, 2021; Seibel et al., 2021; Tort, 2011; Verburg-Van Kemenade et al., 2009). This is mainly due to the high plasticity of the stress response, which varies, for example, between fish species and types of stressors. Furthermore, as stated in Section 1.3.1, during the stress response, energy is also redistributed from the immune system to mechanisms that are more important for survival. In particular, the acquired immune system can be suppressed during acute stress, such as suppression of antibody development and leukocyte synthesis (Buchmann & Secombes, 2022; Cain & Cidlowski, 2017; Maule et al., 1989). However, in the case of an acute stressor, the innate immune system often manages to cope and handle the stressor carefully.

In situations of chronic stressors or a combination of different stressors that are leading to allostatic overload, generally adverse and suppressive immune effects are detected on fish health (Dhabhar, 2008; Tort, 2011). This immune suppression results from a prolonged and/or very strong elevation of stress hormones, especially cortisol, which puts a lot of energy into

the stress response and thereby neglects parts of the immune system (Balasch & Tort, 2019; Tort, 2011; Wendelaar Bonga, 1997). In the short term, this suppression prevents an overstimulated and/or prolonged immune response (Buchmann & Secombes, 2022). However, in the long term, this suppressed immune status increases the fish's susceptibility to diseases and weakens their health.

Besides stress factors such as temperature change, handling, or crowding, fish are also exposed to pathogens and chemicals. The immune system recognizes these hazards and communicates them to the central nervous system (CNS), which triggers a neuroendocrine response. Here again, it becomes clear that there is close cooperation between nervous, immune, and endocrine systems during stress. The different pathogen agents induce a specific immune response in addition to a stress reaction, where viruses, for example, induce an antiviral response and the immune system promotes the synthesis of antioxidants to an oxidative stressor. During these immune responses, glucocorticoids act as important regulators and controllers to maintain the balance between pro-inflammatory and anti-inflammatory actions, preventing overstimulation of the immune system. For example, in the case of overstimulation, glucocorticoids inhibit the transcription of immune genes through the induction of anti-inflammatory genes (such as *nfkbia*) and counteracting pro-inflammatory transcription factors (such as NF-κB) (Buchmann & Secombes, 2022; De Bosscher et al., 2000). Moreover, in mammals, during the advanced stage of the tertiary stress response, a modulation is thrust towards a T helper 2 (Th2)-targeted immune response, protecting the organism from excess Th1 pro-inflammatory cytokines (Elenkov & Chrousos, 1999). Next to glucocorticoids, many other factors influence the regulation of the immune response, for example, also previous experience, biological rhythm, and catecholamines. The effects of catecholamines such as adrenaline have not yet been extensively studied in fish, but it seems that they are highly variable and time-dependent (Buchmann & Secombes, 2022).

1.3.3. Bioindicators for welfare and health status

As described in Section 1.2, many challenges remain to improve the fish's welfare and health status in salmon farming. New production strategies being implemented must also be carefully analyzed. To evaluate the welfare status from a functional point of view (so the ability to cope with stressors, to maintain homeostasis, and thereby a good health status), bioindicators are needed that give a good reflection of the health and immune status of the fish. Since stress responses are highly adaptive, based not only on the type, intensity and duration of the stressor but also on the fish species, it is not easy to define bioindicators that can consistently be applied in aquaculture. This thesis investigated potentially useful bioindicators for determining the health and immune status of Atlantic salmon, as well as bioindicators that give indications about distress levels due to changing farming conditions.

1.3.4. NF-κB inhibitors

One family of proteins strongly related to stress and immunity is the well conserved NF-κB (nuclear factor kappa-light chain-enhancer of activated B cells)/ Rel transcription factors. Upon a broad range of stimuli, ranging from pathogens and stress hormones to cytokines and physical stress, NF-κB is activated and induces the expression of immune- and stress-related genes (Figure 3). Hereby, NF-κB strongly drives and controls immune and stress responses (Hayden & Ghosh, 2011). Inhibitors of NF-κB (IκB) and IκB kinases (IKKs) control the pro-inflammatory transcription factor NF-κB. Through an interplay between these two factors, a sufficient immune and stress response is induced but also remains under control without

excessive overstimulation (Baeuerle & Baltimore, 1988; DiDonato et al., 1997; Rebl & Goldammer, 2018). By binding of IκB protein to NF-κB factors, the transcription factor NF-κB is kept inactive in resting cells. Upon activation, the IκB protein is phosphorylated by IKK, which results in the migration of NF-κB into the nucleus, where it induces gene expression (Ghosh et al., 1998).

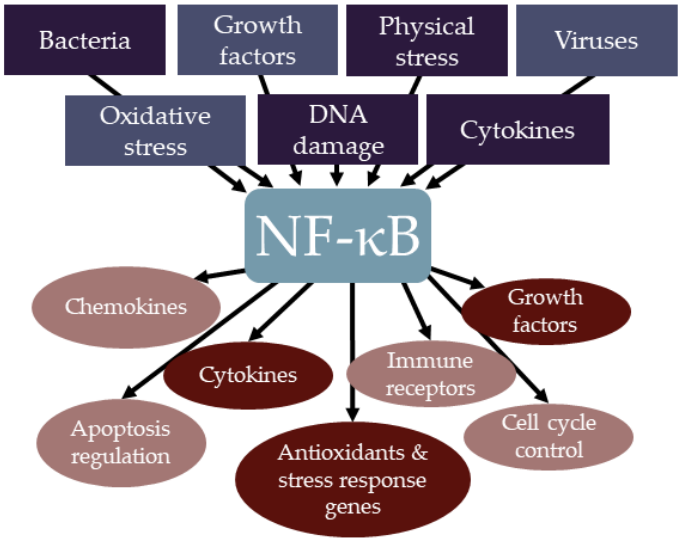

Figure 3. The nuclear factor-κB (NF-κB)/REL family is activated by numerous stressors and coordinates immune and stress responses. Adapted from: (Tilstra et al., 2011).

This controller of NF-κB, IκB, consists of different family members, each with their own characteristics and varying affinities for the NF-κB dimers. As shown in figure 4, the different IκB proteins are involved in the regulation of many different immune and stress factors. In this thesis, I research the IκB proteins in salmonid fish. This could provide insight into the regulation of NF-κB as a key regulator of immune and stress responses, and the potential of IκB proteins as bioindicators of immune and stress status.

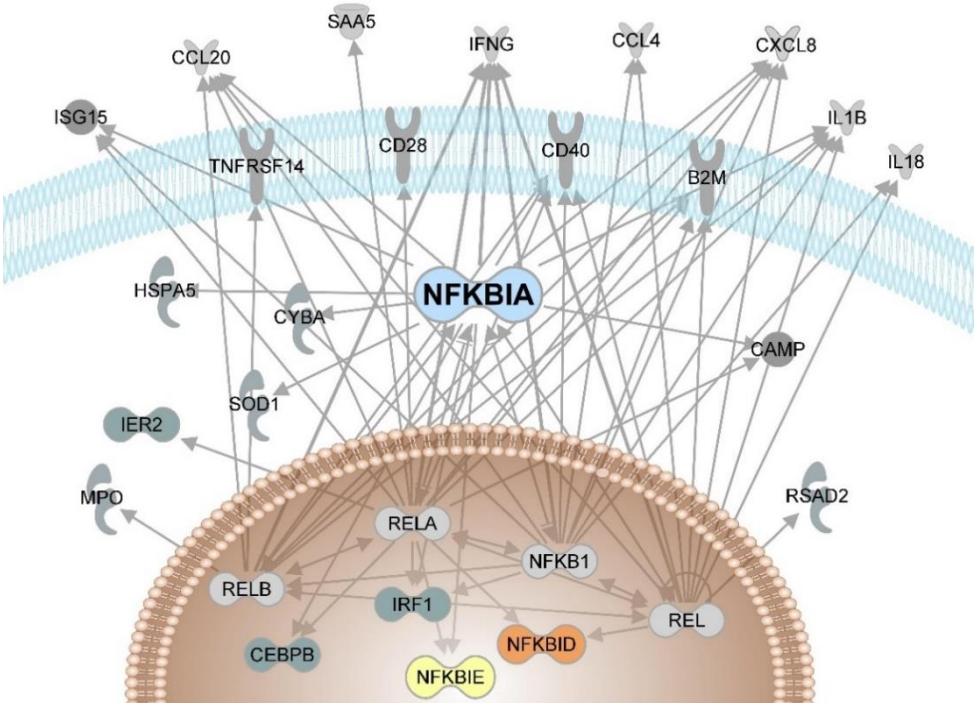

Figure 4. Ingenuity Pathway Analysis (IPA) of NF-κB encoding genes (*RELA, REL, RELB, NFKB1*), *NFKBI* genes and immune- and stress-related genes measured in this thesis. Note that the illustration is based on a QIAGEN software application that displays biological pathways and regulatory networks.

1.4. Hypotheses and aims

The present thesis arose from the joint research project BioFiA funded by Federal Ministry of Education and Research, Germany (BMBF; project number 031B09151E1), which aimed to develop and validate bioindicators to evaluate fish farming, health, and product quality.

The overarching hypotheses were as follows:

Bioindicators predict the health and welfare status of Atlantic salmon under changing farming conditions in RAS. Microalgae-enriched diets mitigate these effects and improve the health and welfare status of Atlantic salmon.

The main aims of the present work were:

- To identify the effects of diets enriched with microalgae on Atlantic salmon and to derive bioindicators that reflect the fish health and welfare status.
- To determine the mitigation potential of microalgae-enriched diets on the effects of potentially challenging farming conditions (PAA-based disinfection of RAS and salinity change) in Atlantic salmon farming and to derive suitable bioindicators reflecting fish status.
- To characterize a family of NF-κB inhibitors, key regulators in the stress and immune response of salmonid fish.

The present thesis includes four scientific papers that contribute to the main aims:

- **Paper I** investigated the beneficial effects of diets enriched with different microalgae species on the performance and health status of Atlantic salmon.
- Based on the microalgae species-specific effects detected in paper I, we researched in **paper II** how different inclusion rates and applications of *C. vulgaris* affect the performance and health of Atlantic salmon. In addition, the mitigation potential of *C. vulgaris* on the effects of PAA-based disinfection of RAS was examined.
- **In paper III**, the effects of salinity change to seawater after brackish water acclimation on the hypo-osmoregulatory capacity and health of Atlantic salmon were investigated. Alongside, the potentially mitigating role of microalgae on the effects of salinity change on the health status of Atlantic salmon was evaluated.
- In the above studies, many indicative parameters were measured at different levels and under different conditions to understand the status of the fish and to determine which indicators provide a sensitive and adequate representation of the health status of the fish. In this thesis, I additionally examined in **paper IV** more specifically, a family of important regulators of immune and stress responses, the NF-κB inhibitors (IκB). These proteins are potentially important bioindicators of immune and stress status, with the salmonid IκB family each having specific structural and functional properties important for coping with a variety of environmental conditions.

2. Discussion

The welfare of farmed fish is one of the main concerns in sustainable aquaculture production. Indicators for determining welfare status are evolving, with different approaches to assessing them (Raposo de Magalhães et al., 2020). However, these methods are not always reliable, so new bioindicators are needed that are easy to use, reliable, and cover various behavioral and physiological aspects. The current concept of animal welfare also takes into account the adaptive and dynamic nature of organisms (Raposo de Magalhães et al., 2020). This includes the ability to achieve homeostasis through endogenous change in the presence of altering environmental conditions. Because of the potentially subtle changes through new farming strategies, the broad panel of parameters measured in this thesis is important to provide a complete picture of the impact of these new strategies and the potentially beneficial role of dietary stimulants.

Indicative parameters measured in this thesis reflected the health and welfare status of farmed salmon at different levels, from growth performance and feed intake to organ indices, blood parameters, and expression of stress- and immune-related genes. The genes measured in our studies are selected from an established set of genes to evaluate immunocompetence in farmed Atlantic salmon (Krasnov et al., 2020) and cover distinct functional groups and pathways from the whole immune system. Moreover, they were selected not only for differential expression by immune challenges but also for important immune roles that do not necessarily have strong transcriptional regulation, such as genes involved in acquired immunity (Krasnov et al., 2020). This was why this gene set was well applicable to our studies, which investigated realistic conditions in aquaculture and did not necessarily expect extremely harmful effects and a compromised immune system with greatly changing immune and stress parameters. Gene expression analysis complements traditional indicators such as plasma levels of cortisol and glucose, which were shown to be unreliable as sole parameters and prone to bias due to stress during sampling itself. In addition, by measuring the expression of a wide range of immune- and stress-related genes in different tissues, we gain insight into the dynamics and affected pathways. Especially under changing conditions, as in our studies, gene expression profiling gives an understanding of the immune and stress status, and thus the immune competence of the fish.

Figures 5-9 list all bioindicators significantly affected by distinct conditions and diets researched in this thesis. Overall, it can be concluded that the fish were in good health, as no harmful levels of the indicative parameters were found in our studies. In addition to providing an indication of general health, bioindicators can reflect interactions between particular biological systems and a change or agent in the environment (World Health Organization, 1993).

Bioindicator	Treatment	Direction	Effect specifications
Whole body indicators			
DFI	Salinity	⇩	In all groups compared to before SWT
CF	CVB	⇩	Compared to CD
FCR	CVB	⇩	Compared to CD
	CV14	⇧	Compared to CD
SGR	CV14	⇩	Compared to CD
PER	CV14	⇩	Compared to CV2 + CV14w
PRE	CV2	⇧	Compared to CD + CV14
	CV14w	⇧	Compared to CD + CV14
Ash %	CV14	⇧	Compared to CD

Figure 5. Whole body indicators significantly affected in this thesis.
CD (control diet); CF (Fulton's condition factor); CV14 (*C. vulgaris* 14% daily); CV14w (*C. vulgaris* 14% once weekly); CV2 (*C. vulgaris* 2% daily); CVB (*C. vulgaris* broken 8% daily); DFI (daily feed intake); FCR (feed conversion ratio); PER (protein efficiency ratio); PRE (protein retention efficiency); SGR (specific growth rate); SWT (seawater transfer)

Bioindicator	Tissue	Treatment	Direction	Effect specifications
Muscle composition indicators				
Muscle fat content	Muscle	AP	⇩	Compared to CD
		SL	⇩	Compared to CD
Fatty acid composition	Muscle lipids	All diets		Reflection of microalgae in feed
Carotenoid: Lutein	Muscle + plasma	CVI	⇧	At 8 wks in BW, compared to CD
	Muscle + plasma	CVB	⇧	At 8 wks in BW, compared to CD
	Muscle + plasma	TC	⇧	At 8 wks in BW, compared to CD
Carotenoid: Violaxanthin	Muscle	TC		Detectable
Carotenoid: Zeaxanthin	Muscle	AP		Detectable
		TC		Detectable

Figure 6. Muscle composition indicators significantly affected in this thesis.
AP (*A. platensis* 8% daily); BW (brackish water); CD (control diet); CVB (*C. vulgaris* broken 8% daily); CVI (*C. vulgaris* intact 8% daily); SL (*S. limacinum* 8% daily); TC (*T. chuii* 8% daily); wks (weeks)

Bioindicators specifically affected by dietary stimulants (microalgae in our studies) were plasma cholesterol levels and myeloperoxidase concentrations in the liver (Figures 7, 8). In addition, the fatty acid and carotenoid content of the fish muscle reflected the fatty acid and carotenoid composition of the microalgae species fed to the fish (Figure 6). These parameters can therefore be used as bioindicators, indicating how, for example, meat is affected by microalgae-enriched feed. The bioindicators that were most responsive and significantly affected by changing rearing conditions in combination with dietary stimulants used in our studies were plasma glucose levels, abundance of Sod1 protein in organs, and expression of gene-based indicators *c1ql2*, *drtp1*, *ifit5*, *il18*, and *saa5* (Figure 7 – 9). Furthermore, there is a clear effect of the changing rearing conditions on cortisol, confirming the role of stress indicator.

Bioindicator	Tissue	Treatment	Direction	Effect specifications
Physiological indicators				
AST	Plasma	CVI	⇩	At 8 wks in BW, compared to CD
ALT	Plasma	CVI	⇩	At 8 wks in BW, compared to CD
Cholesterol	Plasma	CVI	⇧	Between 2 wks and 8 wks in BW, compared to CD
		CVB	⇧	Between 2 wks and 8 wks in BW, compared to CD
		TC	⇧	Between 2 wks and 8 wks in BW, compared to CD
Glucose	Plasma	SL	⇧	Between 2 wks and 8 wks in BW, compared to CD
		AP	⇧	Between 2 wks and 8 wks in BW, compared to CD
		PAA	⇧	At 1 h after PAA exposure (for all diet groups)
Sodium	Plasma	Salinity	⇧	At 20 h after SWT
Chloride	Plasma	Salinity	⇧	At 20 h after SWT
Cortisol	Plasma	Salinity	⇧	At 20 h in SW compared to fish in BW
		Salinity	⇩	Between 20 h to 2 wks after SWT
		PAA	⇧	At 1 h after PAA (for all diet groups)

Figure 7. Physiological indicators significantly affected in this thesis.
ALT (alanine aminotransferase); AP (*A. platensis* 8% daily); AST (aspartate aminotransferase); BW (brackish water); CD (control diet); CVB (*C. vulgaris* broken 8% daily); CVI (*C. vulgaris* intact 8% daily); h (hours); PAA (peracetic acid); SL (*S. limacinum* 8% daily); SWT (seawater transfer); TC (*T. chuii* 8% daily); wks (weeks)

These different indicators can generally be categorized into stress indicators (glucose, cortisol, *drtp1*) and indicators of immune response such as antiviral defense (*ifit5*), acute phase (*saa5*), Th1 immunity (*il18*) and a connecting bridge between innate and acquired immunity (*c1ql2*). Our studies show that these bioindicators are sensitive to change, although it is important to emphasize that this is specific to changing rearing conditions and dietary stimulants used in our studies. Nevertheless, it can be concluded that these bioindicators are useful to include in future research investigating the impact of other conditions on fish welfare.

In the case of a compromised immune system, either a reduction in gene expression (immune suppression), induction in gene expression (infection or overstimulation), or imbalance (strong suppression and overstimulation of several genes) is expected (Krasnov et al., 2020). In our studies, we could not speak of a compromised immune system but rather mild immune and stress reactions that were most likely necessary to restore homeostasis after changes in environmental conditions. Due to the slight responses, the interpretation of differential expressed genes was not always straightforward. In addition to an adaptive response to changing conditions, such slight reactions can also result from diurnal rhythms or the growth of the fish (Kajita et al., 1992; Montero et al., 2020; Sakai et al., 1996; Wang et al., 2022).

Bioindicator	Tissue	Treatment		Direction	Effect specifications
Organ protein indicators					
Mpo	Liver	CD		↑	Between 20 h to 2 wks after SWT, compared to fish fed CD
		CVB		↑	Between 20 h to 2 wks after SWT, compared to fish fed CVB
		AP		↑	Between 20 h to 2 wks after SWT, compared to fish fed AP
		AP		↓	At 2 wks in BW, compared to CD
Sod1	Liver	CVI		↓	Between 20 h to 2 wks after SWT, compared to fish fed CVI
		TC		↓	Between 20 h to 2 wks after SWT, compared to fish fed TC
		CV2	PAA	↓	Compared to before PAA
		CV14	PAA	↓	Compared to before PAA
	Gill	CV2	PAA	↓	Compared to before PAA
		CV14	PAA	↓	Compared to before PAA
Cat	Liver	CD	PAA	↑	Compared to before PAA
		CV14w	PAA	↑	Compared to before PAA
	Gill	CV2	PAA	↑	Compared to before PAA

Figure 8. Organ protein indicators significantly affected in this thesis.
AP (*A. platensis* 8% daily); cat (catalase); CD (control diet); CV14 (*C. vulgaris* 14% daily); CV14w (*C. vulgaris* 14% once weekly); CV2 (*C. vulgaris* 2% daily); CVB (*C. vulgaris* broken 8% daily); CVI (*C. vulgaris* intact 8% daily); h (hours); Mpo (myeloperoxidase); PAA (peracetic acid); Sod1 (superoxide dismutase 1); SWT (seawater transfer); TC (*T. chuii* 8% daily); wks (weeks)

Furthermore, it is also difficult to determine whether differential gene expression is the cause or consequence of a condition. For example, since a stimulatory effect on immune response and/or a modulating effect on stress response by microalgae was expected, interpretation was difficult in the case of a reduction in gene expression after supplementing diets with microalgae. Reduction in gene expression in this case seems to be caused either by bioactive molecules from the microalgae or that the bioactive molecules inhibit the function of the gene, which possibly impairs fish health. Only assumptions can be made about this, as well as about which specific bioactive molecules of the microalgae are responsible for specific effects. More research is needed to determine, for example, the norms in differential gene expression that also take into account the transcriptional regulation of each gene.

Furthermore, important regulators of immune and stress responses are the NF-κB transcription factors. In response to a wide range of stimuli, NF-κB is activated and induces the expression of many different genes. This thesis investigated and characterized IκB in salmonids, one of the major regulators of NF-κB, which may serve as a potentially useful bioindicator of immune and stress responses. In my study, a wide range of salmonid IκB proteins have been identified with different characteristics, functions, and tissue-specific expression patterns (van Muilekom et al., 2023). In previous studies, *nfkbia* expression has been reported as an indicative parameter of immune challenge, toxicity exposure, or specific diets (Eslamloo et al., 2016; Kaleo et al., 2019; Sood et al., 2021; C. Zhang et al., 2021). Our findings are in line with this, with an increase in *nfkbia* expression after fungal stimulation in vitro and PAA exposure in vivo (Mueller et al., n.d.; van Muilekom et al., 2023). Further research on the

exact role and function of the other members of the IκB family in salmonids is needed for possible use as bioindicators.

Bioindicator	Tissue	Treatment		Direction	Effect specifications
Gene-based indicators					
arg2	Gill	CD	PAA	↑	Compared to before PAA
	Head kidney	CV14	PAA	↓	Compared to before PAA
b2m	Gill	CV14	PAA	↑	Compared to before PAA
	Gill	CD	PAA	↑	Compared to before PAA
c1ql2	Liver	CVI		↑	At 8 wks in BW, compared to CD
		SL		↑	At 8 wks in BW, compared to CD
	Gill	Salinity		↓	At 20 h + 2 wks after SWT, compared to 8 wks in BW
		AP	Salinity	↓	At 20 h after SWT compared to CD
		CD	PAA	↑	Compared to before PAA
c4b	Gill	CD	PAA	↑	Compared to before PAA
camp	Head kidney	Salinity		↑	At 20 h +2 wks after SWT, compared to 8 wks in BW
	Gill	AP	Salinity	↓	At 20 h after SWT compared to CD
cat	Gill	CV14	PAA	↑	Compared to before PAA
		CD	PAA	↑	Compared to before PAA
ccl4	Head kidney	CD	PAA	↓	Compared to before PAA
cd28	Head kidney	CD	PAA	↓	Compared to before PAA
cd4	Gill	CD	PAA	↑	Compared to before PAA
cebpb	Gill	CV14	PAA	↑	Compared to before PAA
		CD	PAA	↑	Compared to before PAA
clra	Gill	AP	Salinity	↓	At 20 h after SWT compared to CD
		CV14	PAA	↑	Compared to before PAA
		CD	PAA	↑	Compared to before PAA
cxcl8a	CHSE-214 cells	Zymosan		↑	
drtp1	Anterior intestine	CVB		↑	At 8 wks in BW, compared to CD
	Head kidney	Salinity		↑	At 20 h after SWT, compared to 8 wks in BW
	Gill	CV14	PAA	↑	Compared to before PAA
		CD	PAA	↑	Compared to before PAA
fcgr1a	Gill	Salinity		↓	At 20 h after SWT, compared to 8 wks in BW
	Head kidney	CV14	PAA	↓	Compared to before PAA

Figure 9. Gene-based indicators significantly affected in this thesis.

AP (*A. platensis* 8% daily); CD (control diet); CV14 (*C. vulgaris* 14% daily); CV14w (*C. vulgaris* 14% once weekly); CV2 (*C. vulgaris* 2% daily); CVB (*C. vulgaris* broken 8% daily); CVI (*C. vulgaris* intact 8% daily); h (hours; PAA (peracetic acid); SWT (seawater transfer); TC (*T. chuii* 8% daily); wks (weeks)

Bioindicator	Tissue	Treatment		Direction	Effect specifications
Gene-based indicators					
hamp	Gill	Salinity		⇩	At 20 h + 2 wks after SWT, compared to 8 wks in BW
hsp1a1	Gill	CV14	PAA	⇩	Compared to before PAA
hspa5	Head kidney	CD	PAA	⇩	Compared to before PAA
		CV14	PAA	⇩	Compared to before PAA
ier2-2	Head kidney	CV14w		⇧	At both time points, compared to CD
		CD	PAA	⇩	Compared to before PAA
	Gill	CD	PAA	⇧	Compared to before PAA
ifit5	Head kidney	Salinity		⇩	At 2 wks after SWT, compared to 8 wks in BW
		CV14	PAA	⇩	Compared to before PAA
	Gill	CD	PAA	⇧	Compared to before PAA
		Salinity		⇩	At 2 wks after SWT, compared to 8 wks in BW
ifn2a	Head kidney	CD	PAA	⇩	Compared to before PAA
ikba or *nfkbia*	Gill	CV14	PAA	⇧	Compared to before PAA
		CD	PAA	⇧	Compared to before PAA
	CHSE-214 cells	Zymosan		⇧	
il10rb	Anterior intestine	CVI		⇩	At 8 wks in BW, compared to CD
		TC		⇩	At 8 wks in BW, compared to CD
il18	Head kidney	CD	PAA	⇩	Compared to before PAA
		CV14w	PAA	⇩	Compared to before PAA
		CV14	PAA	⇩	Compared to before PAA
	Gill	CD	PAA	⇧	Compared to before PAA
il1b	Gill	Salinity		⇩	At 20 h + 2 wks after SWT, compared to 8 wks in BW
	CHSE-214 cells	Zymosan		⇧	
il1r2	Anterior intestine	CVI		⇩	At 8 wks in BW, compared to CD
isg15	Gill	Salinity		⇩	At 2 wks after SWT, compared to 8 wks in BW
	Head kidney	Salinity		⇩	At 2 wks after SWT, compared to 8 wks in BW
lao1	Gill	CV14	PAA	⇧	Compared to before PAA
	Head kidney	Salinity		⇩	At 2 wks after SWT, compared to 8 wks in BW
lyzc2	Liver	TC		⇧	At 8 wks in BW, compared to CD
marco	Gill	CV14	PAA	⇧	Compared to before PAA

Figure 9. (*Continued*)

Bioindicator	Tissue	Treatment		Direction	Effect specifications
Gene-based indicators					
mpo	Head kidney	CV14	PAA	⇩	Compared to before PAA
nkaα1b/nkaα 1a	Gill	Salinity		⇧	After SWT
rsad2	Gill	Salinity		⇩	At 2 wks after SWT, compared to 8 wks in BW
		CV14w	PAA	⇧	Compared to before PAA
	Head kidney	Salinity		⇩	At 2 wks after SWT, compared to 8 wks in BW
saa5	Liver	CVB		⇩	At 8 wks in BW, compared to CD
		SL		⇩	At 8 wks in BW, compared to CD
	Spleen	CVI		⇧	At 8 wks in BW, compared to CD
	Head kidney	Salinity		⇧	At 20 h after SWT, compared to 8 wks in BW
sod1	Head kidney	Salinity		⇩	At 20 h + 2 wks after SWT, compared to 8 wks in BW
		CV14w	PAA	⇩	Compared to before PAA
tnfrsf14	Head kidney	CV14	PAA	⇩	Compared to before PAA
	Gill	CD	PAA	⇧	Compared to before PAA
znfx1	Head kidney	Salinity		⇩	At 2 wks after SWT, compared to 8 wks in BW

Figure 9. (*Continued*)

The approach of measuring a broad panel of indicative parameters at different levels to determine effects on health and welfare status can be applied to evaluate many types of potential challenges, stimulants, and new farming strategies. In our studies, we focused specifically on the identification of bioindicators in Atlantic salmon fed microalgae-enriched diets and, according to new farming strategies, kept in RAS with changing conditions such as PAA-based disinfection and salinity change. Below, I outline the indicative parameters that are significantly affected per changing rearing condition and/or by microalgae-enriched diets.

2.1.New farming strategies

Routine disinfection is performed in RAS in order to maintain a low pathogen pressure. During this process, the fish are exposed to the disinfectant, with more exposures when fish are kept longer in RAS. In this thesis, the effects on the immune and stress responses of salmon exposed in RAS to the oxidative disinfectant PAA were investigated.

By analyzing the expression of immune- and stress-related genes before and after PAA treatment, a local response in the gill of fish fed with control diet (CD) was observed (Mueller et al., n.d.). A local immune response was reflected by an immigration of T cells and phagocytes to the mucosal tissue of the gill (*il18, cd4, clra, tnfrsf14)* and activation of transcription factors affecting immune and stress responses (*cebpb, ikba*). Moreover, the chemical oxidant PAA induced a slight stress response in fish fed CD, demonstrated by

increased cortisol and glucose levels in plasma 1 h after PAA treatment compared to the control and an induction of genes involved in stress-related pathways (*drtp1, ier2-2, hspa5*) in the gill. Since the gill is in direct contact with the water containing PAA, a response was expected in order to cope with the altered environment. The return of plasma cortisol 18 h later to levels equal to before PAA treatment, indicates an adaptive response of the salmon to PAA without further chronic consequences. In addition, the increased catalase concentration in the liver confirmed a counter-reaction against the oxidative disinfectant. The poor modulation of selected genes in the head kidney of fish fed CD affirmed minimal effects on the systemic immune and stress response by PAA.

Consistent with our findings, previous studies found a systemic and mucosal adaptive response to PAA in Atlantic salmon, reflected by increased total antioxidant capacity (TAC), altered plasma cortisol, glucose, and lactate levels, differential expression in the gill of genes coding for antioxidant defense molecules (*gr, cu/znsod, gpx, mnsod*), cytokines (*il1b, il10*) and mucins (*muc5ac, muc5b, muc2*) (Osório et al., 2022; Soleng et al., 2019). Another study identified key pathways that contribute to the physiological adaptation of Atlantic salmon to PAA exposure, including internal ROS (reactive oxygen species) homeostasis, remodeling of gill mucosal cells, gill mucosal immunity with particular mucosal defense (*il15rα, muc7*) and expression of ribosomal genes involved in adaptive cellular responses (Lazado et al., 2021). Moreover, Lazado et al. (2020) found no effects on external welfare status and plasma metabolomes but a protective immune response in the gill of Atlantic salmon smolts. Most notably, the expression of genes involved in immune regulation, cellular signaling, and metabolism was induced in the gill. Although in the different studies varying exposure periods and concentrations of PAA were used, the overarching conclusion is that Atlantic salmon is able to cope with the oxidative disinfectant by activating an adaptive response, antioxidant activity, and immune response in the gill. By addressing, for example, the imbalance of ROS and ROS-induced DNA damage with physiological adaptations, internal homeostasis in the fish is restored without further long-term harming consequences. From this, it can be concluded that the use of PAA has no adverse effects on the health and welfare of Atlantic salmon reared in RAS. The effectiveness of PAA against pathogens in water also outweighs its short and poor effects on salmon health, indicating its suitability for use in RAS water treatment strategies.

To implement new farming strategies that keep smolts in RAS longer, conditions such as salinity must be considered too. Therefore, this thesis researched the effects of a new rearing strategy with a change in salinity from brackish water to full-salinity seawater on the osmoregulatory ability and immune and stress responses of Atlantic salmon.

After smoltification in fresh water, the fish were reared for several months in brackish water before they were transferred to full-salinity seawater. In brackish water, the fish already had hypo-osmoregulatory capacity, which was reflected in a positive *nkaα1b*/ *nkaα1a* ratio measured in the gill. After seawater transfer (SWT), this ratio increased further, indicating a good regulation of their ion balance within physiological limits (van Muilekom et al., 2024). These results also confirm that salmon retain osmoregulatory capacity in brackish water and

that desmoltification is not initiated, a process in which the smolt loses its hypo-osmoregulatory capacity when it is not moved to seawater in the 'smolt window' (Hoar, 1988).

The change in salinity from brackish water to full-salinity seawater reduced the transcript levels of four immune genes (*fcgr1a, c1ql2, il1b, hamp*) in the gill 20 h after SWT. This reduction is likely related to an acute response to the salinity change, with a sustained decreased effect on *c1ql2, il1b* and *hamp* gene products. Reduced expression of *fcgr1a* probably indicates a reduced number of phagocytes in the gill shortly after SWT, suggesting impaired antigen recognition. In the head kidney, salinity change caused an impaired antioxidant defense (*sod1*) at both 20 h and two weeks after SWT and diminished antibacterial and antiparasitic capacity (*lao1*) two weeks after SWT. In both the gill and head kidney, a clear systemic effect of salinity change on the antiviral defense was demonstrated, with a reduction of several antiviral genes (*isg15, rsad2, ifit5, znfx1)* two weeks after SWT.

Previous research reported impaired immunocompetence during smoltification, which was further exacerbated after SWT (Johansson et al., 2016). The high-energy consumption required for smoltification and adaptation to the marine environment is therefore likely to come at the expense of energy for defense against pathogens. Johansson et al. (2016) demonstrated systemic immune suppression in several organs, with in particular antiviral immunity and immune signaling, which is in line with our results. In our study, however, salmon had already completed smoltification some time before SWT, indicating a suppressive effect of salinity change *per se* on the immune response in Atlantic salmon. Moreover, change in salinity also induced the expression of genes involved in acute phase and stress response (*drtp1*, *saa5*) shortly after SWT and enhanced antimicrobial capacity (*camp*) 20 hours and 2 weeks after SWT in the head kidney. This shows that, despite mainly a reduced expression of immune genes, salinity change also induces a minor stress response in the head kidney. Since the fish were kept in the protected RAS environment, pathogen pressure was relatively low. The induced transcript levels of *camp* are therefore striking, particularly since this is the only immune gene of all selected genes that was upregulated after SWT. Salmon cathelicidins (encoded by *camp*) have been reported to be primarily expressed in response to pathogens (Brunner et al., 2020), but our study suggests that cathelicidins also play a role in response to environmental changes such as salinity change.

Overall, only a modest change in transcript levels was measured in both the head kidney and gill in our study. One reason for this may be that the transfer from brackish water to seawater allows for a more gradual change to full salinity water, resulting in a smaller change in environmental conditions. Consequently, the energy required for the fish to achieve renewed homeostasis is also smaller, possibly resulting in a lower stress response with only a slight suppression of host immunity. Previous studies also found positive effects on growth performance after an acclimation period in brackish water, possibly caused by lower energy expenditure on osmoregulation (Ytrestøyl et al., 2020, 2022). More research is needed to gain insights into the effects of brackish water acclimation, with in comparison also acclimation in freshwater for a longer period after smoltification before making further conclusions about the benefits of brackish water acclimation before SWT. Another reason for the minor change in transcript levels in our study is that the fish were moved from brackish water in RAS, to full

salinity water also in RAS. This gave us the opportunity to investigate the specific effect of salinity change on the immune and stress responses of the fish. However, this is not a realistic reflection of the production cycle, including the transfer to sea cages in the marine environment, where there are uncontrolled living conditions with higher pathogen pressure. To gain further insights into the potential benefits of using brackish water acclimation in salmon farms, more research is needed in which salmon are transferred to the marine environment after brackish water acclimation.

2.2. Potential of microalgae as functional feed ingredient

Microalgae have been used as a replacement for fishmeal, fish oil, and vegetable protein in previous studies (Gong et al., 2020; Marchi et al., 2023; Sarker et al., 2020). Besides serving as bulk ingredients in fish feed, the bioactive compounds of microalgae can also improve host defenses and the color and quality of fillets due to their rich source of nutrients and their stress-modulating and immune-stimulating effects (Carballo et al., 2018; Riccio & Lauritano, 2020; Teimouri et al., 2019). However, current studies on the functional properties of different microalgae species and their effects on Atlantic salmon under challenging environmental conditions are limited (de Mattos et al., 2019; Plaza et al., 2018; Sayed & Fawzy, 2014). Therefore, this thesis investigated the mitigation potential of different microalgae on the effects of changing rearing conditions on Atlantic salmon.

During our studies, basal feed components were not replaced by microalgae in the diet formulation, but were kept at constant levels. By maintaining this balance in nutrients, the direct effects of microalgae as a functional feed ingredient could be examined. Microalgae species were chosen based on their volume of production (with the possibility of industrial scale-up) and their source and diversity of functional compounds. Since each species of microalgae has its own unique composition, each species may have different effects on fish performance and health. However, in previous studies, often only a few indicative parameters were measured to evaluate the effects of microalgae, for example, growth and blood parameters, phagocytic activity, respiratory burst activity, concentration of immune proteins in blood, and expression of only a small panel of immune genes (Abdel-Tawwab & Ahmad, 2009; Cerezuela, Guardiola, Meseguer, et al., 2012; Q. Zhang et al., 2014). To provide a more comprehensive overview, our study measured many indicative parameters with different methods to present the effects of microalgae-enriched diets as fully as possible at different levels.

Based on all results, it can be concluded that dietary enrichment with selected microalgae has no significantly beneficial effects on simultaneous growth performance, product quality, and immune and stress status of fish. However, certain indicative parameters were positively influenced by the supplementation of specific microalgae in the diet. Consequently, the type of microalgae to be added to the diet should be determined by the specific needs of the fish or the wishes of the farmer.

When microalgae were used as a replacement in feed composition in previous studies, different effects on growth performance were measured, ranging from negative to neutral and positive effects (Gong et al., 2020; Ma et al., 2022; Messina et al., 2019; Sarker et al., 2020; Teimouri et al., 2013). In our studies, feeding salmon with microalgae-enriched diets had no

significant effect on growth performance as well as on feed intake (Mueller et al., n.d., 2023). During the critical period after SWT, feed intake decreased for all groups, with the enrichment of microalgae in the feed having no beneficial effect (Mueller et al., 2023). However, inclusion of 8% broken *C. vulgaris* in the diet had a positive effect by decreasing FCR (Mueller et al., 2023), while inclusion of 14% *C. vulgaris* in the diet caused an increase in FCR (Mueller et al., n.d.). Furthermore, salmon fed diets containing only 2% *C. vulgaris* daily and 14% *C. vulgaris* once weekly improved their protein retention efficiency (PRE) compared to fish fed 14% *C. vulgaris* daily (Mueller et al., n.d.). First, from this can be concluded that there may be a limit in microalgae inclusion proportion, which, if exceeded, could have a negative effect on feed conversion and therefore growth performance ultimately. Second, treatment of *C. vulgaris* by breaking the cell wall has a positive effect on FCR. Possibly the better fermentable *C. vulgaris* can be used more efficiently, stimulating the growth of beneficial gut bacteria, as has been found in previous research (Huang et al., 2023). Ultimately, this has a positive effect on food digestion and nutrient absorption from the overall feed. Finally, the inclusion of low percentages (2% daily and 14% once weekly) of *C. vulgaris* positively affected feed utilization, although unfortunately no significantly improved growth was observed in these groups. Overall, no convincingly favorable results were found that fish fed diets enriched with selected microalgae had improved performance. One possible reason for this is that it is more challenging for the carnivorous salmon to digest and utilize plant-based ingredients, such as carbohydrates (Colombo, 2020). This includes breaking down the cell wall of microalgae cells, which prevents the microalgae's bioactive molecules and carbohydrates from being used efficiently as a source of energy. The positive effect on FCR by *C. vulgaris* with a broken cell wall confirms the importance of diligent preparation of functional feed ingredients for optimal use.

The composition and quality of fish flesh are determined by several factors, such as environment, genetic features, and fish feed (Horn et al., 2018; Johnsen et al., 2011; Ørnholt-Johansson et al., 2017). Besides the fact that a well-balanced body composition of fish is important to ensure a high nutritional product quality for human consumption (Ahmed et al., 2022), it also reflects the physiological condition and health of the fish. Although plant-based microalgae are difficult to digest, they can positively affect the metabolism of carnivorous fish, with possible effects on body composition (Perera et al., 2020; Sheikhzadeh et al., 2012). In our study, supplementation of *A. platensis* and *S. limacinum* reduced muscle fat content after eight weeks of feeding period in brackish water compared to the control diet (Mueller et al., 2023), indicating an effect of these microalgae on lipid metabolism in salmon. Moreover, two weeks after SWT, muscle fat content was reduced in all diet groups (Mueller et al., 2023). The change in salinity induces a response that demands energy. Possibly, in our study, the energy was supplied mainly by the degradation of monounsaturated fatty acids (Henderson, 1996) in the muscle, where supplementation of microalgae may have no further influence. However, supplemented microalgae species were clearly reflected by a corresponding fatty acid and carotenoid profile in the fish muscle observed after 8 weeks of feeding time (Mueller et al., 2023).

The bioactive molecules of microalgae also have a modulating effect on stress response and a stimulatory effect on the immunity of fish, according to previous research (Abdel-Tawwab &

Ahmad, 2009; Cerezuela, Guardiola, Meseguer, et al., 2012; de Mattos et al., 2019; Grammes et al., 2013; Kousoulaki et al., 2020). In our studies, microalgae species-specific effects on the immune and stress status of Atlantic salmon have been observed after feeding microalgae-enriched diets. At the local level in the anterior gut, only minimal effects with an increased stress response (*drtp1*) in fish fed with broken *C. vulgaris* and reduced expression of genes encoding cytokine receptors (*il1r2, il10rb*) in fish fed with intact *C. vulgaris* and *T. chuii* were detected (Mueller et al., 2023). Daily supplementation of 14% *C. vulgaris* significantly affected microbial beta diversity in both the digesta and mucosa in the intestine (Mueller et al., n.d.). In detail, several taxa were enriched in the mucosa and digesta of fish fed daily with 14% *C. vulgaris*, of which the enrichment of *Trichococcus* seems to be directly related to *C. vulgaris* because of their ability to break down complex carbohydrates (Parshina et al., 2019). On the other hand, administration of *C. vulgaris* reduced the abundance of several taxa in both mucosa and digesta. The reduction in digesta of *Lactobacillus*, a bacterium that plays a protective role against bacterial infections (Giri et al., 2013; He et al., 2017), may have adverse effects on fish. Moreover, supplementation with *C. vulgaris* was predicted to enrich microbial pathways in digesta, such as aerobic respiration, the TCA cycle, and the degradation of glucose, cylose, and inositol (Mueller et al., n.d.).

Also on the systemic level, microalgae species-specific effects were demonstrated. Supplementation of intact *C. vulgaris* improved liver health (lower levels of aspartate- and alanine aminotransferase activity and increased activity of the antioxidative enzyme superoxide dismutase 1), induced signaling in the liver between innate and adaptive immunity (*c1ql2*) and enhanced acute phase response (*saa5*) in the spleen (Mueller et al., n.d., 2023). In the liver of salmon fed diets enriched with broken *C. vulgaris* and *S. limacinum*, a reduced acute-phase response (*saa5*) was observed, probably due to their bioactive molecules with anti-inflammatory activity. Supplementation of *T. chuii* did not significantly modulate the expression of stress- and immune-relevant genes, but increased antimicrobial capacity (*lyzc2*) in the liver (Mueller et al., 2023). No further substantial effects of microalgae-enriched feed were found on organ indices, selected plasma levels, protein abundance of myeloperoxidase and superoxide dismutase 1 in the liver or stress- and immune-related gene expression in the liver, spleen, and intestine.

The stress-modulating and immune-stimulating properties of microalgae can be of benefit, especially when fish are exposed to challenging conditions and are able to cope with them more efficiently. In this thesis, fish fed diets enriched with microalgae were exposed to a change in salinity or treatment with PAA, after which their stress and immune responses were measured. The primary stress response (plasma cortisol and glucose levels) and plasma electrolyte profile (sodium, potassium, chloride levels) were not affected by microalgae-enriched diets (Mueller et al., n.d.; van Muilekom et al., 2024). Moreover, several microalgae altered the activity of enzymes in the liver of fish in seawater in a species-specific manner. During their stay in seawater, myeloperoxidase activity increased in fish fed broken *C. vulgaris* and *A. platensis*, as well as control diet, indicating an increased antimicrobial activity in their liver (van Muilekom et al., 2024). In addition, supplementation of intact *C. vulgaris* and *T. chuii* appears to improve the oxidative status in the liver of salmon during their time in seawater, as demonstrated by a reduced Sod1 activity (van Muilekom et al., 2024). In contrast, when

exposed to PAA, diets enriched with *C. vulgaris* appear to have hardly any effect on antioxidant activity by Sod1 (Mueller et al., n.d.). However, after PAA treatment, increased antioxidant activity was observed, demonstrated by catalase abundance in the liver of fish fed 14% *C. vulgaris* once weekly and 2% *C. vulgaris* daily, and in the gill of fish fed 2% *C. vulgaris* daily (Mueller et al., n.d.).

In general, no major influence of microalgae on gene expression was detected after changes in conditions through salinity change and PAA treatment. Supplementation of *A. platensis* reduced antimicrobial capacity (*camp*) and pathogen recognition (*clra*, *c1ql2*) in the gill after salinity change (van Muilekom et al., 2024). Possibly, the bioactive molecules of *A. platensis* support the salmon's antimicrobial defense, reducing the host immune response of the fish. Moreover, supplementation of 14% *C. vulgaris* once weekly resulted in an induced immediate early response (*ier2-2*) in the head kidney compared to fish fed control diet (Mueller et al., n.d.). This possibly implies an activated, more potent immune response to PAA treatment in the head kidney. However, this is questionable because of the lack of other activated stress or immune pathways that could provide indications of this. In both studies, no further significant effects were found in fish fed microalgae-enriched diets relative to fish fed control diets. The reduced feed intake after salinity change (and thus microalgae intake) of fish fed microalgae-enriched diets may have contributed to their minimal effects on immune and stress responses. PAA exposure decreased the expression of several immune genes (*arg2*, *fcgr1a*, *ifit5*, *mpo*, *tnrsf14)* in the head kidney of fish fed diets supplemented with daily 14% *C. vulgaris* compared to levels before PAA treatment. These effects were not observed in fish fed control diet, indicating that supplementing 14% *C. vulgaris* daily either results in diminished immune protection in the head kidney or supports these immune functions by the bioactive compounds of *C. vulgaris*. When 14% *C. vulgaris* was supplemented only once per week, *sod1* expression in the head kidney reduced after PAA exposure, indicating reduced antioxidant capacity, but no further suppressed host immune responses were observed. In the gill, supplementation of 14% *C. vulgaris* daily led to enhanced antibacterial capacity (*marco*, *lao1*) and supplementation of 14% *C. vulgaris* once weekly enhanced antiviral capacity (*rsad2*) in the gill compared to before PAA exposure.

Taken together, very similar effects on immune and stress status were observed after salinity change and PAA treatment between fish fed with control diet or with microalgae-enriched diets. Based on our studies, it can be recommended to supplement microalgae in salmon feed, but critical consideration should be given to the method of supplementation and what should be targeted in terms of product quality or stimulation or modulation of certain stress or immune pathways. Based on that, specific microalgae species can be chosen that have positive effects on these special needs of the fish. In future studies, several more aspects can be investigated, such as different inclusion rates of different microalgae species, modes of application (for example pelleted or extruded feed), and special preparations (for example, broken or intact cell wall) of microalgae so that the fish can make optimal use of the microalgae content. Possibly, this will increase the effectiveness of microalgae as a functional feed ingredient. Moreover, salinity change and exposure to PAA in our studies did not appear to elicit strong stress and immune responses in salmon. As a result, possibly the mitigating potential of microalgae on stress effects was not captured.

3. Conclusions and future perspectives

The aim of the present thesis was to identify suitable bioindicators for the health and welfare of Atlantic salmon. The analysis of many indicative parameters allowed for selecting bioindicators that were significantly affected by changing farming conditions through salinity change and PAA-based disinfection of RAS and/or microalgae-enriched diets. Furthermore, this thesis describes the important role of NF-κB inhibitors in controlling NF-κB activity in salmonid fish. In particular, the involvement of salmonid IκBα and IκBε proteins in immune regulatory processes, demonstrates their applicability as bioindicators of health and welfare in salmonids.

By assessing a broad panel of indicative parameters, this thesis provided a comprehensive picture of the status of salmon. However, when considering the large investments being made in the salmon industry, one could consider an even more comprehensive approach. For example, alternative to measuring the expression of a selection of immune- and stress-related genes, RNA-Seq can be used to analyze the entire transcriptome. Here, known and novel transcripts and transcript variants, as well as non-coding regulatory RNA species can be quantified, and insights can be gained into the enrichment of certain biological pathways. Moreover, in parallel to the restricted set of plasma proteins and expression of protein-coding genes already measured in this thesis, more plasma proteins can be included, or even more extensively, the entire proteome. This provides further insight into protein products, their post-translational modifications, and their interactions. In future research, behavioral analysis may also be valuable to assess fish health and welfare, as this method is non-invasive and non-lethal. In principle, our approach to determining salmon welfare status can be applied to other diadromous fish species that are domesticated. However, the primers for measuring gene expression in this thesis were developed for salmon specifically, and there is a difference in interpretation of results due to fish-specific responses to changing conditions.

Assessment of immunocompetence is important to evaluate the health and welfare status of the fish as well as to mitigate or avoid risks in farming. The new farming strategies examined in this thesis had a minimal impact on salmon welfare. Our approach can also be applied to evaluate other new farming strategies as well as immune challenges or toxicity studies in future research. On the other hand, all kinds of stimulants of health and welfare can be evaluated, such as in this thesis the addition of microalgae in diets. Based on this thesis, I conclude that there are microalgae-specific effects on fish health, and that this is influenced by inclusion rates and feeding frequencies. Only a limited impact of microalgae has been found on the effects of changing farming conditions. The mitigation potential of microalgae can be further explored in future research by using stronger challenges to salmon, which may allow the potential of microalgae to be better highlighted.

In conclusion, this thesis contributes to improving the evaluation of the welfare status of Atlantic salmon by developing useful bioindicators. In addition, it provides an assessment of new farming strategies and reports on the potential of microalgae-enriched diets for salmon health. This knowledge contributes to the constant improvement of production protocols for higher robustness, disease resistance, and the welfare of salmon.

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5. Publications

5.1. Publication I: Microalgae as functional feed for Atlantic salmon: effects on growth, health, immunity, muscle fatty acid and pigment deposition

Microalgae can serve as a valuable functional feed ingredient because of their rich source of nutrients. This study investigated the potential beneficial effects of diets enriched with 8% commercially relevant microalgae species on the performance and health status of Atlantic salmon reared in RAS. Based on performance indicators, fatty acid and pigment profile analysis, and putative biomarkers at transcriptional and protein levels in plasma, liver, intestine, and spleen, the health and immune status of salmon were evaluated.

Highlights

- Supplementation of broken *C. vulgaris* positively affected feed conversion ratio and protein retention but reduced condition factor.
- Feed intake decreased in all groups after seawater transfer.
- After eight weeks of supplementation with *A. platensis* and *S. limacinum*, fat content was reduced in the muscles of fish in brackish water.
- The added microalgae species were clearly reflected in a corresponding fatty acid and carotenoid profile in the fish muscles after eight weeks of feeding time.
- Adding intact *C. vulgaris* to the diet lowered aspartate aminotransferase and alanine aminotransferase in plasma, suggesting improved liver health.
- Supplementation of different microalgae species affected the expression of several immune- and stress-related genes in the anterior intestine, liver, and spleen.

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EDITED BY
Ji Hyung Kim,
Gachon University, Republic of Korea

REVIEWED BY
Adnan H. Gora,
Central Marine Fisheries Research Institute (ICAR), India
Carlos Alfonso Alvarez-González,
Universidad Juárez Autónoma de Tabasco, Mexico

*CORRESPONDENCE
Jonas Mueller
jmueller@tierzucht.uni-kiel.de

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Microalgae as functional feed for Atlantic salmon: effects on growth, health, immunity, muscle fatty acid and pigment deposition

Jonas Mueller[1,2]*, Matteo Pauly[2], Joachim Molkentin[3], Ute Ostermeyer[3], Doret R. van Muilekom[4], Alexander Rebl[4], Tom Goldammer[4,5], Jacqueline Lindemeyer[6], Thekla Schultheiß[6], Henrike Seibel[2] and Carsten Schulz[1,2]

[1]Department for Marine Aquaculture, Institute of Animal Breeding and Husbandry, Kiel University, Kiel, Germany, [2]Fraunhofer Research Institution for Individualized and Cell-Based Medical Engineering IMTE, Aquaculture and Aquatic Resources, Büsum, Germany, [3]Department of Safety and Quality of Milk and Fish Products, Max Rubner-Institut, Kiel, Germany, [4]Fish Genetics Unit, Institute of Genome Biology, Research Institute for Farm Animal Biology (FBN), Dummerstorf, Germany, [5]Faculty of Agriculture and Environmental Sciences, University of Rostock, Rostock, Germany, [6]Institute of Toxicology and Pharmacology for Natural Scientists, University Medical School Schleswig-Holstein, Kiel, Germany

Microalgae are increasingly being investigated as functional feed additives in a variety of fish species, but our knowledge on how microalgae supplementation affects Atlantic salmon remains limited. We hypothesized that microalgae inclusion of 8% in the feed would improve performance, fatty acid and pigment deposition as well as health and immunity of Atlantic salmon reared in recirculating aquaculture systems (RAS). We fed Atlantic salmon smolts with five different microalgae enriched diets containing *Tetraselmis chuii* (TC), *Arthrospira platensis* (AP), *Schizochytrium limacinum* (SL) or *Chlorella vulgaris*, either intact (CVI) or as broken cell wall derivative (CVB) or a control diet (CD). After eight weeks of feeding in brackish water (13 psu), all groups were transferred to seawater (32 psu) for additional two weeks. Our results indicate that CVB improved feed conversion and protein retention, but reduced condition factor ($p < 0.05$) compared to fish fed with a control diet. Voluntary feed intake decreased in seawater, but was similar among diet groups. The amount of docosahexaenoic acid was particularly high in SL-fed fish and alpha-linolenic acid was enriched in fish fed CVI, CVB and TC ($p < 0.05$). Following seawater transfer, fat content and monounsaturated fatty acids decreased in the muscle, while polyunsaturated fatty acids increased. Lutein was present in all muscle samples, but highest concentrations were found in CVB-, CVI- and TC-fed fish. In the anterior intestine, microalgae supplementation induced differentially regulated trout protein 1 (*drtp1*) expression in CVI- and CVB-fed fish, but reduced the expression of interleukin 1 and 10 receptor (*il1r2 & il10rb*) in CVI-fed fish. In the liver, feeding CVI and SL induced complement C1q like 2 (*c1ql2*) expression, while reducing serum amyloid A5 (*saa5*) expression. Superoxide-

dismutase protein concentration was induced in the liver of fish fed SL, while myeloperoxidase was reduced in most microalgae-fed groups. In conclusion, we show that commercially relevant microalgae can be used as functional feed additives for Atlantic salmon promoting different health aspects without negatively affecting their growth performance when cultivated in RAS.

KEYWORDS

microalgae, functional feed, bioactive compound, fatty acids, carotenoids, immunity, fish health, Atlantic salmon

1 Introduction

Feed in aquaculture is a finite resource, which provides nutrients but should also promote the growth and health of farmed fish. Consequently, interest is growing in developing functional feeds that guarantee good fish health, improve performance and mitigate farming related stressors. Although a variety of compounds have been investigated as functional ingredients in fish feed, microalgae have only recently been considered. These single cell algae contain different types of polysaccharides, sulfolipids, polyunsaturated fatty acids and pigments (Riccio and Lauritano, 2020). Green algae such as *Chlorella* sp. and *Tetraselmis* sp. are rich in pigments such as chlorophylls and carotenoids, while heterotrophic *Schizochytrium* (herein considered as microalgae) contain high amounts of docosahexaenoic acid (DHA; Nakahara et al., 1996; Ren et al., 2010).

The diverse chemical composition of microalgae holds potential for a variety of biological activities, including antioxidant (Carballo et al., 2018; Teimouri et al., 2019), antimicrobial (Guzmán et al., 2019) as well as anti-inflammatory activities (Fujii, 2000; Guzmán et al., 2003). Feeding microalgae to different fish species was found to affect their health and immunity. Including *Chlorella vulgaris* into the feed, for instance, counteracted soy-bean meal-induced intestinal inflammation in Atlantic salmon *Salmo salar* (Grammes et al., 2013) and zebrafish *Danio rerio* (Bravo-Tello et al., 2017). Dietary administration of *Chlorella sorokiniana* further stimulated humoral innate immunity in rainbow trout *Oncorhynchus mykiss* (Chen et al., 2021), while *Tetraselmis chuii* and *Phaedactylum tricornutum* increased phagocytotic and complement activity in gilthead seabream *Sparus aurata* (Cerezuela et al., 2012a).

Although health and immune promoting effects are described for different microalgae species, the majority of studies in fish has investigated microalgae as a source to replace fishmeal or fish oil in the feed (Carvalho et al., 2020; Kousoulaki et al., 2020; Sarker et al., 2020a). Studies evaluating the functional properties of different microalgae species under challenging environmental conditions are currently missing.

Such challenging environmental conditions can occur during the production of Atlantic salmon in recirculating aquaculture systems (RAS). Production of Atlantic salmon in RAS is globally expanding (Bergheim et al., 2009; Davidson et al., 2021). This is because these systems allow a controlled production environment with a high level of biosecurity and a significantly reduced discharge of waste products into the aquatic environment (Dalsgaard et al., 2013; Ahmed and Turchini, 2021). However, the RAS environment is considered particularly challenging for fish health due to higher stocking densities (Calabrese et al., 2017), accumulation of waste products (Ruyet et al., 2008), as well as water disinfection treatment (Soleng et al., 2019; Stiller et al., 2020). Dietary mitigation under these conditions may be a promising strategy to improve overall health and performance of salmon cultivated in RAS. Currently the use of a brackish water phase in RAS before seawater transfer is investigated as an alternative to shorten the production time in the sea (Ytrestøyl et al., 2020; Ytrestøyl et al., 2023). While it is well established that transfer to seawater of Atlantic salmon is associated with a drastic stress-related reduction in appetite (Usher et al., 1991), little is known about whether microalgae, which contain different amino and fatty acids, could potentially increase feed intake in this critical time period.

A thorough assessment of the fish's health and immune status in functional feeding studies requires investigating both transfer of functional components from feed to fish and their subsequent effects *in vivo*. The former is usually performed using analytical chemistry to trace fatty acids, pigments and other functional compounds. The latter requires investigating different aspects of the fish's health and immune status. Functional feeds are expected to provoke a local response in the intestine (Bae et al., 2020; López Nadal et al., 2020), but also systemic effects may occur. These are reflected in physiological alterations of the blood plasma, the spleen and the liver, being constantly exposed to antigens from the bloodstream (Bayne et al., 2001; Wu et al., 2016).

In this study, we aimed to elucidate whether functional diets enriched with different commercially relevant microalgae species at 8% inclusion in the feed can improve performance, health and immune status of Atlantic salmon reared in RAS. Since seawater transfer of salmon smolts is a particular critical time period following land-based rearing in RAS (Usher et al., 1991; Karlsen et al., 2018), we evaluated diet dependent effects during this time-period in addition. Alongside with performance indicators, fatty acid and pigment profiles, a set of putative biomarkers on gene and protein level in plasma, liver, intestine and spleen were used to evaluate the health and immune status of the salmon.

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2 Materials and methods

2.1 Feed formulation

Six isonitrogenous and isoenergetic (on dry matter basis; see Table 1) experimental diets were formulated based on the nutrient requirements of Atlantic salmon (National Research Council, 2011). The diets were designed to include one of the following microalgae: *Chlorella vulgaris*, *Tetraselmis chuii* (TC), *Arthrospira platensis* (AP) or *Schizochytrium limacinum* (SL) at an inclusion level of 8%. Two different *Chlorella vulgaris* were included in the experimental design, one had an intact (CVI) and the other a broken cell wall (CVB). The microalgae were obtained from a commercial supplier and were cultivated in both open and closed bioreactors under commercial settings (Supplementary Table 1). All microalgae were spray-dried after harvesting. Inclusion of the microalgae was done in exchange for wheat starch, wheat gluten and canola oil. In contrast to other studies basal feed components (e.g. fish meal and fish oil) were kept constant in the diet formulation and hence allowed to evaluate the direct effect of every microalgae ingredient. The experimental diets were pelletized (Type 14U175, Amandus Kahl, Hamburg, Germany) at temperatures below 60°C to pellets with 4 mm diameter and stored at 4°C in the dark before and during the trial.

2.2 Experimental setup

The experiment was conducted at the facilities of the Fraunhofer IMTE, Büsum, Germany. Atlantic salmon smolts were obtained from Jurassic Salmon, Poland and were acclimated for two months in a recirculating aquaculture system. During acclimation, the fish were fed a commercial salmon diet (Aller Aqua, Denmark). Water treatment of the RAS (7.6 m^3, turnover rate 4 times h^{-1}) consisted of a moving bed biofilter, a bead filter (PolyGeyser, Model DF-6, Aquaculture Systems Technologies, L.L.C., New Orleans, LA, USA), a protein skimmer and UV-light disinfection. Water quality parameters were measured on a daily basis (NH_4^+ and NO_2^- biweekly) and kept in a suitable range for Atlantic salmon (13.5 ± 0.4°C, 7.3 ± 0.1 pH, 10.3 ± 0.2 mg/L O_2, 0.2 ± 0.1 mg/L NH_4^+, 0.2 ± 0.04 mg/L NO_2^-, (Microquant test kit for NH_4^+ and NO_2^-, Merck, Darmstadt, Germany). Salinity was set to 13.0 ± 0.8 psu (HI 96822 Seawater Refractometer, Hanna Instruments Inc., Woonsocket-RI, USA) by mixing freshwater and seawater. Light was provided for 24 h throughout the experimental period. Prior to the start of the experiment Atlantic salmon smolts (mean body weight 82.32 ± 1.96 g) were randomly divided into six different groups in triplicate, each consisting of 28 fish and stocked into 18 tanks (300 L) of the RAS (Figure 1). Following rearing the fish for eight weeks in brackish water, all of the fish were transferred into a new RAS system, which was identical to the other, but operated with full strength seawater (salinity of 31.8 ± 0.5 psu). Water parameters for this system were as followed: 13.4 ± 0.3°C, 7.2 ± 0.1 pH, 10.4 ± 0.3 mg/L O_2, 0.2 ± 0.1 mg/L NH_4^+, 0.2 ± 0.07 mg/L NO_2^-. The fish were kept under these conditions for additional two weeks before the experiment was terminated. The fish were fed manually twice per day (8 a.m. and 2 p.m.) until apparent satiation during the entire experiment. Leftover pellets were collected, counted and used to calculate feed intake.

2.3 Fish sampling

Samples were collected before the onset of the experiment (T0), after two weeks (T1) and eight weeks of feeding the experimental

TABLE 1 Feed formulation of experimental diets in g/100g dry matter (DM).

Ingredients (g/100g DM)	CD	CVI	CVB	TC	AP	SL
Fish meal[1]	15	15	15	15	15	15
Microalgae	0	8	8	8	8	8
Blood meal[2]	6	6	6	6	6	6
Gelatine[3]	5	5	5	5	5	5
Pea protein isolate[4]	14	14	14	14	14	14
Soy protein concentrate[5]	11	11	11	11	11	11
Wheat gluten[6]	12	7.17	6.53	8.44	5.25	9.44
Wheat starch[6]	21.4	18.45	18.85	17.86	20.2	19.3
Canola oil[7]	5.5	5.5	5.5	6.1	5.5	2.5
Fish oil[1]	6	6	6	6	6	6
Methionine[8]	0.1	0.1	0.1	0.1	0.1	0.1
Vitamin & mineral premix[4]	0.5	0.5	0.5	0.5	0.5	0.5
$CaHPO_4$[9]	2	2	2	2	2	2
Bentonite[10]	1.5	1.28	1.52	0	1.45	1.16

[1]Bioceval GmBH & Co. KG, Cuxhaven; Germany; [2]Saria SE & Co. KG, Selm, Germany; [3]Gustav Ehlert GmbH & Co. KG, Verl, Germany; [4]Emsland-Aller Aqua GmbH, Golßen, Germany; [5]EURODUNA Rohstoffe GmbH, Barmstedt, Germany; [6]Kröner-Stärke GmbH, Ibbenbüren, Germany; [7]Cargill GmbH, Riesa, Germany; [8]Evonik Industries AG, Essen, Germany; [9]Lehmann & Voss & Co. KG, Hamburg, Germany; [10]Del Lago Bentonite, Castiglioni Pes y Cía., Buenos Aires, Argentina.

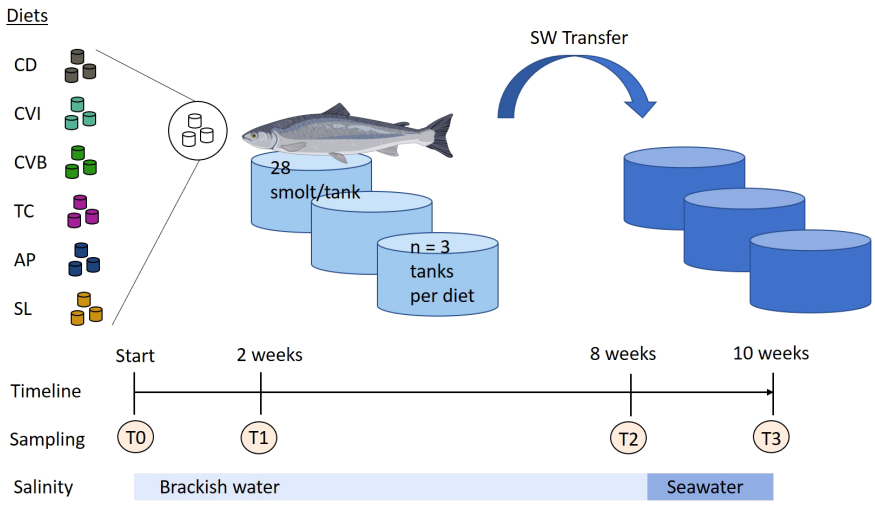

FIGURE 1
Experimental design of feeding trial with microalgae enriched diets. Atlantic salmon were first reared in brackish water and after eight weeks transferred to seawater (SW transfer) for additional two weeks. Throughout the trial the fish received six different experimental diets: control (CD), *Chlorella vulgaris* intact (CVI), *Chlorella vulgaris* broken (CVB), *Tetraselmis chuii* (TC), *Arthrospira platensis* (AP) and *Schizochytrium limacinum* (SL) at an inclusion level of 8%. Atlantic salmon image was created with BioRender.com.

diets in brackish water (T2) and two weeks following transfer into seawater (T3; Figure 1). At each sampling nine fish per treatment (three per tank) were randomly sampled. The fish were quickly netted from the experimental tanks and euthanized by an overdose of buffered MS-222 (0.3 mg/L). For each fish total length and total weight was recorded. 2 ml of blood was collected in heparinized syringes by caudal vein puncture. The blood was transferred into 2 ml Eppendorf tubes and centrifuged at 4000 g for 8 min. Aliquots of the plasma were flash-frozen on dry ice and stored at −80°C for the determination of plasma metabolites, total carotenoid content, and enzyme activities.

The liver and spleen were carefully removed and weighed for the calculation of organ specific indices. At the end of the brackish water phase (T2) a piece of the liver, anterior intestine and the spleen was placed in an RNase free tube and flash frozen in liquid nitrogen for gene expression analysis. In addition, after two (T1) and eight weeks (T2) of feeding the experimental diets a piece of the liver was flash-frozen on dry ice for later protein analysis using western blots.

At T0, T2 and T3 both fillets from every fish were taken, de-skinned, homogenized by means of a knife-mill (Grindomix GM200, Retsch GmbH, Haan, Germany) and stored at −40°C for later analysis of proximate composition, fatty acid profile, as well as carotenoid content. At T0 and T2 three additional fish per tank were sampled and pooled for the analysis of whole-body proximate composition.

2.4 Proximate composition of whole body and diets

Proximate composition was analyzed in microalgae (Supplementary Table 2), diets (Table 2) and whole-body homogenates in duplicates using the same methods. Whole body samples were freeze-dried (Alpha 1-2 LD plus and Alpha 1-4 LSC, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) until a stable weight was achieved and homogenized using a knife mill (GM 200, Retsch GmbH). Nutrients and gross energy were analyzed according to EU guideline (EC) 152/2009. Dry matter content was determined following drying of samples at 103°C in a drying oven for 4 h (ED 53, Binder GmbH, Tuttlingen, Germany). Ash content was determined after combustion in a combustion oven at 550°C (P300, Nabertherm, Lilienthal, Germany). Crude protein content was analyzed following the Kjeldahl method (InKjel 1225M, WD30, Behr, Düsseldorf, Germany). Crude lipid content was extracted with petroleum ether in a Soxhlet extraction system (Soxtherm, Hydrotherm, Gerhardt Königswinter, Germany) and quantified gravimetrically. Gross energy was determined using a bomb calorimeter (C 200, IKA, Staufen, Germany).

2.5 Diet and microalgae fatty acid composition

To physically break down the material, 10 g of each diet and microalgae (Supplementary Table 3) was ground with a mortar, mixed with 20 ml of distilled water and homogenised for 2 min with an Ultra-Turrax disperser (IKA). The slurry was lyophilised and mortared again. To determine the fatty acid composition, 20 mg sample (weighed to the nearest 0.1 mg) was then subjected to direct transesterification according to Griffiths et al. (2010). However, boron trifluoride-methanol was replaced by 3 M methanolic HCl (Sigma-Aldrich, Taufkirchen, Germany) and distilled water by 1 M aqueous NaCl (Merck, Germany). All other reagents were from Sigma-Aldrich. Each diet was transesterified in triplicate and the

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TABLE 2 Crude composition (percent dry matter) as well as fatty acid composition (mg/100g dry matter) and pigment composition (µg/100g dry matter) of the experimental diets given as mean of two and pigment concentrations as mean of four replicate analyses.

Chemical composition (% DM)	CD	CVI	CVB	TC	AP	SL
Dry matter (%)	90.03	91.52	91.59	92.90	92.08	93.02
Crude protein (%)	51.04	51.26	51.00	50.93	51.11	51.38
Fat (%)	16.18	16.38	16.60	16.87	16.19	15.11
Ash (%)	7.47	8.15	8.28	8.61	8.07	7.72
Crude energy (MJ/kg)	22.72	22.71	22.68	22.72	22.69	22.70
Fatty acids (mg/100 g DM)						
C14:0	321	317	320	320	312	336
C15:0	30	37	40	40	36	34
C16:0	1570	1600	1601	1645	1672	1943
C17:0	26	27	29	28	26	28
C18:0	335	335	339	347	333	317
C20:0	57	55	56	59	56	47
C22:0	30	30	29	30	26	25
C24:0	28	30	25	27	27	26
total SFA	**2397**	**2431**	**2438**	**2496**	**2488**	**2757**
C16:1n11	21	30	24	21	20	20
C16:1n9	21	32	37	27	27	21
C16:1n7	332	342	367	336	346	337
C16:1n5	18	18	17	17	18	18
C16:1n3	13	13	14	13	14	14
C17:1n8	21	23	24	22	25	19
C18:1n9	5128	5014	5011	5490	5000	3543
C18:1n7	395	402	443	448	390	331
C18:1n5	21	20	20	20	21	21
C20:1n9	550	538	537	560	531	513
C20:1n7	32	29	33	33	33	29
C22:1n11	687	679	678	689	668	688
C22:1n9	83	76	74	85	73	71
C24:1n9	58	56	57	58	56	55
total MUFA	**7380**	**7272**	**7337**	**7817**	**7222**	**5677**
C16:2n6	n.d.	37	51	13	11	11
C18:2n6	2409	2360	2371	2409	2307	1836
C18:3n6	11	11	11	23	93	13
C20:2n6	41	40	39	42	40	39
C20:3n6	13	13	12	14	14	18
C20:4n6	41	40	41	43	41	45
C22:5n6	22	22	21	22	21	401
total n6 PUFA	**2537**	**2524**	**2545**	**2567**	**2527**	**2362**

(Continued)

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TABLE 2 Continued

Fatty acids (mg/100 g DM)	CD	CVI	CVB	TC	AP	SL
C16:3n3	n.d.	71	96	n.d.	n.d.	n.d.
C16:4n3	n.d.	n.d.	12	89	n.d.	n.d.
C18:3n3 ALA	622	727	792	747	627	452
C18:4n3 SDA	143	144	146	178	142	154
C20:3n3	18	19	20	18	19	19
C20:4n3	48	47	50	49	48	70
C20:5n3 EPA	415	410	414	441	410	440
C22:5n3	86	84	86	88	85	89
C22:6n3 DHA	613	604	609	614	607	2518
total n3 PUFA	**1946**	**2107**	**2226**	**2223**	**1939**	**3742**
C16:2n4	24	24	28	24	26	26
C16:3n4	53	52	51	54	51	54
total PUFA	**4560**	**4706**	**4850**	**4868**	**4543**	**6184**
n3 HUFA	**1181**	**1165**	**1180**	**1209**	**1169**	**3136**
Carotenoids (µg/100 g DM)						
Lutein	29.8	3887	13251	3346	n.d.	36.9
Violaxanthin	n.d.	n.d.	n.d.	2186	n.d.	n.d.
Neoxanthin	n.d.	406	1548	4277	n.d.	n.d.
Zeaxanthin	n.d.	133	358	n.d.	3339	n.d.

n.d, not detected; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; HUFA, highly unsaturated fatty acids with 20 or more carbon atoms and 3 or more double bonds; ALA, α-Linolenic acid; SDA, stearidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

fatty acid methyl ester (FAME) extracts used for separate gas chromatography (GC) analysis. Fatty acid contents were calculated as mg FAME/100 g dry matter (Table 2). For GC conditions, see the section on muscle proximate and fatty acid composition.

2.6 Proximate and fatty acid composition of muscle

Moisture and ash content were determined by drying samples of around 5 g for 12 h at 105°C, followed by ashing at 550°C. The nitrogen content was measured by Dumas using a LECO TruSpecN (Leco Instruments GmbH, Mönchengladbach, Germany). Lipids were extracted according to Smedes (1999) using cyclohexane and 2-propanol (VWR, Darmstadt, Germany) with modifications by Karl et al. (2012). For GC analysis of fatty acids, lipids were obtained in a separate extraction without final drying at 105°C and transesterified into FAME using methanolic potassium hydroxide (ISO-IDF, 2002).

Fatty acid analysis was performed using a 7890A gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with a 7683B autosampler, a split injection port (injection volume 1 µL, split 1:100), flame ionisation detection and a 100 m x 0.25 mm i.d. x 0.20 µm - CP-Sil 88 column (Agilent Technologies). Hydrogen was used as the carrier gas with a constant flow of 1.6 mL min^{-1}. Two min after injection, the initial oven temperature of 175°C was increased by 1°C per minute to 190°C, held constant for 1 min, then increased by 5°C per minute to 225°C, held constant for 7 min, and finally increased by 1°C per minute to 237°C (1 min constant). Chromatograms were evaluated using EZChrom Elite 3.3.2 (Agilent Technologies). Identification of individual FAME was achieved by comparison to known standards (Supelco™ 37 Component FAME mix, PUFA No. 1, PUFA No. 3; all obtained from Sigma-Aldrich) in the range from C14:0 to C22:6n3. Fatty acid contents for fillet samples were calculated as weight percentage (g FA/100 g FA) and are given as means of duplicate analyses.

2.7 Carotenoid content

Carotenoids were extracted from the diets and microalgae with methanol using an Ultra-Turrax (IKA) at 24,000 rpm for two cycles of 45 s (Schüler et al., 2020). Samples were centrifuged and the supernatants of three repetitions were combined. During extraction the samples were kept on ice. The extraction of the fish fillet homogenate was done according to Ostermeyer and Schmidt (2004). After an initial evaluation of carotenoids in individual fish samples (n = 18), muscle samples were pooled on a tank level (n = 3).

For the quantitative determination of the carotenoids an aliquot of the extract was evaporated and the residue dissolved in mobile phase. HPLC was carried out using a C30 analytical column (5 µm, 250 x 4.6 mm i.d., YMC Europe, Dinslaken, Germany) preceding by a C30 guard column (5 µm, 10 x 4.0 mm i.d.) and a gradient of methyl tert-butyl ether, methanol with a small amount of an ammonium acetate buffer (pH 4.6), similar to (Rasmussen et al., 2012). A flow rate of 1.0 mL min^{-1} at 25°C with an injection volume of 100 µL was used. The detection was performed with a photodiode array detector (UV 6000 LP; Thermo Finnigan, San Jose, CA, USA) at 450 nm and 470 nm. Peaks were identified by comparison of the retention times and the absorption spectra (between 380 nm and 700 nm) with those of synthetic standards. The carotenoids were quantified using an external standard containing lutein, fucoxanthin, violaxanthin, neoxanthin, astaxanthin, zeaxanthin and canthaxanthin (ChromaDex, Irvine, CA, USA; Sigma-Aldrich; Dr. Ehrenstorfer, Augsburg, Germany).

2.8 Plasma metabolites and enzyme activities

Plasma glucose, triglycerides, total protein, alkaline phosphatase (ALP), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activity were measured on a Fuji Dry Chem NX500i (Fujifilm, Ratingen, Germany) using commercial kits and following the manufacturer´s instructions.

Total carotenoid content in plasma samples (Supplementary Figure 1) was measured after Donaldson (2012) with slight modifications. Briefly, 100 µL of plasma was mixed with 100 µL of 70% ethanol in a 1.5 mL reaction tube wrapped with aluminium foil and vortexed for one minute to precipitate the proteins. Then 300 µL of n-heptane (Roth, Karlsruhe, Germany) was added and the mixture was vortexed for four minutes at maximum speed. The mixture was centrifuged at 2000 g for two minutes. Following separation of both layers 290 µL of the heptane layer including the dissolved carotenoids were decanted and added to a 10 mm quartz cuvette (type 104-QS, Hellma, Müllheim, Germany) and further diluted with 410 µL of heptane. The absorbance was measured at 448 nm using a spectrophotometer (SPECORD210, Analytik Jena GmbH, Jena, Germany). The carotenoid content was then calculated according to Donaldson (2012). Spectral profiles of every sample (300 – 600 nm) confirmed the presence of a carotenoid peak at ~ 448 nm (Supplementary Figure 1B). Preparation of extracts and measurements were performed under reduced light conditions to minimize pigment degradation in the samples.

2.9 Western blots of liver proteins

Total protein from liver samples (n = 3 pool per tank) was extracted with Radioimmunoprecipitation (RIPA) lysis buffer according to the manufacturer's protocol (RIPA Lysis Buffer System, Santa Cruz Biotechnology, Dallas, Texas, USA). The proteins Cu, Zn superoxide dismutase (Sod1) and myeloperoxidase (Mpo) were analyzed in the salmon liver per SDS-PAGE and Western Blot. The protein ß-actin served as loading control. A no template control and one positive control per antibody were included, *Danio rerio* liver for Mpo, Bovine liver for Sod1 and HEK-293 cells for ß-actin. 20 µg total protein was processed in reducing conditions with SDS sample and reducing buffer (both TruPAGE, Sigma-Aldrich, Schnelldorf, Germany) at 70°C for 10 min. SDS-PAGE was performed in a Xcell SureLock Mini-Cell (Thermo Fisher Scientific, Waltham, Massachusetts, USA) using precast 4-12% gradient gels, TruPAGE running buffer and antioxidant (Sigma-Aldrich). Proteins were electro-transferred to a PVDF membrane. For parallel protein detection of Mpo and Sod1 the membrane was horizontally cut. Primary antibody incubation, with Mpo antibody (ab210563, Abcam, Cambridge., UK) in 1:5000 dilution in PBS-T containing 2.5% skim milk and Sod1 antibody (NBP2-24915, Novus Biologicals, Bio-Techne Ltd., Abingdon, UK) in 1:500 dilution in PBS-T containing 2.5% skim milk, was performed at 4°C overnight. Secondary antibody anti-rabbit IgG conjugated HRP (sc-2357, Santa Cruz Biotechnology) was incubated in a 1:5000 dilution for 90 min at room temperature. Detection was performed using ECL detection reagents (Amersham, Global Life Sciences Solutions USA LLC, Marlborough, MA, USA) and chemiluminescence film (Amersham, GE Healthcare Ltd, Little Chalfont, UK) with 40 sec exposure time for both Mpo and Sod1. For the subsequent detection of ß-actin the antibodies were stripped using 100 mM Glycin buffer (pH 2.5). The membrane was incubated in 1:5000 dilution of β-actin antibody (NB600-503, Novus Biologicals, Bio-Techne Ltd., Abingdon, UK) at 4°C overnight. Quantification of protein expression was done following densitometric analysis of the protein bands using GIMP and normalized to housekeeping (ß-actin) protein expression.

2.10 Gene expression in liver, spleen and intestine

Total RNA was extracted using TRIzol (ThermoFisher Scientific, Waltham, MA, USA) and further purified with the ISOLATE II RNA Micro Kit (Meridian Bioscience Inc., Cincinnati, OH, USA). The concentration and integrity of the extracted RNA was measured by NanoDrop One (Thermo Fisher Scientific). Subsequently, cDNA synthesis was performed using Reverse Transcription Master Mix (Fluidigm, San Francisco, CA, USA). The samples were preamplified by the PreAmp Master Mix (Fluidigm) and at last treated with exonuclease I (New England BioLabs, Frankfurt/Main, Germany). All steps have been carried out according to the manufacturer's instructions.

45 genes with tissue specific regulation were selected from an established gene set composed of key immune and stress regulated genes in Atlantic salmon (Krasnov et al., 2020; Lund et al., 2022; Supplementary Table 4). We extended this set by three immunogene-specific primers derived for *hamp*, *saa5* and *sod1* (Supplementary Table 4). The same 48 genes (45 target and 3 reference genes) were measured in the tissue anterior intestine, liver and spleen.

The 48.48 gene expression biochips were primed in the MX IFC Controller (Standard BioTools, San Francisco, CA, USA). The pre-amplified cDNA samples were pipetted to the sample inlets and the primers were loaded on the assay and finally, analyzed with the Biomark HD using the manufacturer's thermal protocol "GE Fast 48 × 48 PCR+Melt v2.pcl" (application type: gene expression; passive reference: ROX; assay: single probe).

The raw qPCR Ct values were obtained using the Fluidigm real-time PCR analysis software v. 3.0.2 (Munich, Germany). Relative expression was calculated based on ΔΔCt where three reference genes coding for b-actin, ribosomal protein L4 and ribosomal protein S20 (*actb*, *rpl4*, *rps20*) were used as internal normalisers. The mean Ct per gene for all samples was used as a calibrator during the calculation. Relative expression values were log2 transformed prior to statistical analysis. Four individuals with abnormal phenotypic signs and subsequently abnormally high gene expression profiles were removed from the dataset. The genes *cxcl8* and *il1b* in the liver and *cxcl8* in the spleen were removed from the dataset, as to many missing values hampered analysis of the data.

2.11 Statistical analysis

Statistical analysis and data visualization were conducted using the software R (R version 4.1.0) in the environment RStudio. For all test $\alpha = 0.05$ was used as the level of significance. Data is presented as mean ± standard error of mean (SEM). For the performance parameters, protein concentrations in the liver as well as carotenoid concentrations in the muscle an appropriate statistical model based on generalized least squares was defined (Carroll and Ruppert, 1988) which included the factor diet as well as timepoint for the latter two. The residuals were assumed to be normally distributed and to be heteroscedastic, which was based on a graphical residual analysis. Analysis of variance (ANOVA) was conducted, followed by multiple contrast tests for heteroscedastic data (Hasler and Hothorn, 2008) in order to compare the several diets with the control diet. For plasma parameters, proximate and fatty acid composition of the muscle, as well as gene expression data, mixed effect models (Laird and Ware, 1982; Carroll and Ruppert, 1988; Pinheiro and Bates, 2000) were used. The model included diet, timepoint and their interaction as fixed factors and tank as a random factor. The residuals were assumed to be normally distributed and to be heteroscedastic. Based on this model, a Pseudo R^2 was calculated (Nakagawa and Schielzeth, 2013) and an ANOVA was conducted, followed by multiple contrast tests in order to compare the several diets with the control diet, and the timepoints, respectively. If the factors diet and timepoint had no significant interaction, then corresponding multiple contrast tests were pooled over the levels of the remaining factor. Spearman correlation analysis was employed to relate muscle lutein and plasma carotenoid concentrations, since muscle lutein concentrations were not normally distributed. Pearson correlation analysis was applied to relate the increase in DHA with a decrease in fat content in the muscle.

3 Results

3.1 Fish performance and proximate body composition

Feeding the experimental diets for eight weeks to the salmon in brackish water revealed no difference in growth and feed intake among groups (Table 3). Feed conversion ratio (FCR) was highest in the control group (CD), and including broken *C. vulgaris* (CVB) in the feed significantly improved the feed conversion ratio ($p = 0.03$). Furthermore, feeding CVB slightly improved the protein efficiency ratio ($p = 0.11$) but reduced body condition ($p = 0.01$). Hepatosomatic index and spleen somatic index were not affected by the diet (Table 3). Mortality was low and not different among diet groups. Furthermore, the diet did not affect proximate whole-body composition of the salmon (Table 4) but feeding CVB slightly increased ash content ($p = 0.06$). After the fish were transferred to full strength seawater voluntary feed intake decreased to one third of the levels prior transfer but was not different among groups (Table 3).

3.2 Proximate and fatty acid composition of muscle

The diet did not affect protein, water and ash content of the muscle in brackish water (T2) and following transfer to seawater (T3; Tables 5; S5). Fat content was significantly reduced in brackish water in fish fed *A. platensis* (AP; 12% reduction) and *S. limacinum* (SL; 13.5% reduction) compared to CD (Table 5). Fat content decreased on average by 29% after transfer to seawater across all groups (Table 5). It decreased most in groups receiving intact and broken *C. vulgaris*, CVI (38%) and CVB (32.9%), but markedly less in fish fed AP (18.5%).

Generally, fatty acid composition in the muscle lipids reflected that of the diet (Table 5; Table 2). Both diet and timepoint significantly influenced the fatty acid composition, however, an interaction of both factors was absent in most cases (Tables 5; S5). *Alpha*-linolenic acid (LA) was significantly enriched in muscle lipids of CVB, CVI and *T. chuii* (TC) fed fish in brackish water and in CVB and CVI fed fish after transfer to seawater (Table 5). Steraidonic acid (SDA) content was higher in TC compared to CD at both timepoints. Eicosapentaenoic acid (EPA) levels were affected by an interaction of diet and timepoint and levels increased in CD by 11.3%, CVB by 16.2% and CVI 18.1% following transfer to seawater (Tables 5; S5). Docosahexaenoic acid (DHA) levels were significantly increased in SL compared to CD fed fish at both timepoints and relative levels increased following transfer to seawater in all groups by 23.8% (Figure 2A, Table 5). A significant negative correlation between the relative reduction in total fat content in the muscle and the relative increase in DHA based on diet group means was detected ($R = 0.89$, $p < 0.001$; Figure 2B).

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TABLE 3 Growth performance and organ specific indices of Atlantic salmon after eight weeks of feeding the experimental diets in brackish water and feed intake for the period of two weeks in seawater.

	CD	CVI	CVB	TC	AP	SL	ANOVA
IBW [g]	81.9 ± 0.5	80.6 ± 0.7	83.6 ± 0.6	80.6 ± 1.2	82.5 ± 0.5	81.6 ± 0.8	ns
FBW [g]	149.0 ± 2.1	149.4 ± 4.9	156.0 ± 2.3	147.7 ± 5.6	153.3 ± 1.8	147.9 ± 2.6	ns
WG [g]	67.1 ± 1.6	68.8 ± 4.6	72.5 ± 1.9	67.1 ± 6.1	70.7 ± 1.3	66.3 ± 1.8	ns
SGR	1.07 ± 0.01	1.1 ± 0.05	1.11 ± 0.02	1.08 ± 0.08	1.11 ± 0.01	1.06 ± 0.01	ns
DFI	1.18 ± 0.02	1.14 ± 0.08	1.14 ± 0.03	1.11 ± 0.05	1.19 ± 0.07	1.15 ± 0.03	ns
FCR	1.11 ± 0.01	1.03 ± 0.03	1.03 ± 0.01*	1.04 ± 0.03	1.08 ± 0.06	1.08 ± 0.04	0.01
PER	1.96 ± 0.02	2.07 ± 0.07	2.09 ± 0.02	2.05 ± 0.06	1.99 ± 0.11	1.94 ± 0.08	0.05
PRE	38.1 ± 0.1	40.3 ± 1.4	39.8 ± 0.6	39.0 ± 1.0	37.4 ± 1.7	37.0 ± 0.9	ns
CF	0.96 ± 0.01	0.90 ± 0.02	0.86 ± 0.01*	0.97 ± 0.02	0.95 ± 0.02	0.90 ± 0.02	0.01
HSI [%]	1.46 ± 0.10	1.42 ± 0.08	1.25 ± 0.04	1.35 ± 0.08	1.45 ± 0.09	1.28 ± 0.07	ns
SSI [%]	0.09 ± 0.00	0.09 ± 0.00	0.1 ± 0.01	0.1 ± 0.01	0.09 ± 0.01	0.09 ± 0.01	ns
Survival [%]	100 ± 0.0	97.6 ± 1.0	96.4 ± 1.7	95.2 ± 2.6	98.8 ± 1.0	95.2 ± 1.0	ns
DFI SW	0.44 ± 0.04	0.31 ± 0.04	0.39 ± 0.01	0.40 ± 0.05	0.53 ± 0.07	0.52 ± 0.04	ns

Data is presented as mean ± SEM, with n = 3 tanks per treatment for performance parameters and n = 9 individuals for organ specific indices. A significant difference ($p < 0.05$) compared to the control diet (CD) was assessed by Dunnett's multiple comparisons and indicated with a *; ns, not significant. Note that presented ANOVA results do not fully agree with results from multiple comparisons due to heteroscedasticity. IBW (initial body weight); FBW (final body weight); WG (weight gain); SGR (specific growth rate) = (ln (FBW) – ln (IBW))/experimental days * 100; DFI (daily feed intake) = daily feed intake in % body weight; FCR (feed conversion ratio) = total feed intake (g)/weight gain (g); PER (protein efficiency ratio) = weight gain (g)/crude protein intake (g); PRE (protein retention efficiency) = crude protein gained (g)/crude protein intake (g) * 100; CF (Fulton´s condition factor) = weight/fish length^3 *100; HSI (hepatosomatic index) = liver weight (g)/fish weight * 100; SSI (spleen somatic index) = spleen weight (g)/fish weight (g) * 100; SW seawater.

3.3 Muscle and plasma carotenoid content

Lutein was the main carotenoid present in all groups and significantly enriched in the muscle at both timepoints of fish fed CVI, CVB and TC (Table 5) reflecting the content of lutein in the respective algae (Supplementary Table 3). Lutein content in fish fed CVB was more than two times higher than in fish fed CVI and TC. Zeaxanthin was detected in muscle of fish fed AP and TC and violaxanthin was only detected in fish fed TC (Table 5).

The total carotenoid content in plasma of CVB fed fish was two times higher than in fish fed CVI ($p = 0.08$) and TC ($p = 0.05$; Figure 3A), which was also visible when comparing plasma samples of the respective groups directly (Supplementary Figure 1A). No carotenoid content was detectable in groups fed CD and SL (Supplementary Figures 1A, B), while values for AP were below the calculated standard curve and were subsequently excluded from further analysis. Muscle lutein concentrations significantly correlated with total carotenoid concentration in plasma samples (Spearman $R = 0.52$, $p = 0.03$; Figure 3B).

3.4 Plasma metabolites and enzyme activities

CVI inclusion significantly lowered aspartate aminotransferase and alanine aminotransferase activity in microalgae fed groups compared to CD ($p \leq 0.046$; Figures 4A, B). Furthermore, SL lowered aspartate aminotransferase activity ($p = 0.06$; Figure 4A). The highest variance in aspartate aminotransferase and alanine aminotransferase activity was found among fish fed CD, while it was lowest among fish fed CVI. Total plasma protein and alkaline phosphatase activity were not influenced by the diet but showed an

TABLE 4 Proximate body composition (percent OS) of Atlantic salmon after eight weeks of feeding the experimental diets.

[%] OS	CD	CVI	CVB	TC	AP	SL	ANOVA
Moisture	70.41 ± 0.44	70.23 ± 0.23	70.47 ± 0.04	70.38 ± 0.17	70.54 ± 0.13	70.60 ± 0.08	ns
Ash	2.33 ± 0.01	2.47 ± 0.05	2.54 ± 0.03(*)	2.22 ± 0.05	2.34 ± 0.07	2.39 ± 0.04	0.004
Crude protein	18.23 ± 0.10	18.3 ± 0.17	18.11 ± 0.07	18.09 ± 0.01	18 ± 0.06	18.11 ± 0.11	ns
Crude lipid	9.04 ± 0.37	9.00 ± 0.27	8.88 ± 0.06	9.31 ± 0.14	9.12 ± 0.13	8.90 ± 0.22	ns
Energy[MJ/kg]	7.88 ± 0.16	7.82 ± 0.09	7.71 ± 0.02	7.90 ± 0.04	7.82 ± 0.04	7.77 ± 0.06	ns

Data is presented as mean ± SEM as original substance (OS), with n = 3 tanks per treatment. A trend ($p < 0.1$) compared to the control diet (CD) was assessed by Dunnett's multiple comparisons and indicated with (*); ns, not significant. Note that presented ANOVA results do not fully agree with results from multiple comparisons due to heteroscedasticity.

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TABLE 5 Proximate, fatty acid and carotenoid composition of Atlantic salmon muscle fed microalgae enriched diets eight weeks in brackish water (T2) and two weeks following seawater transfer (T3).

Timepoint	T2						T3					
Diet	CD	AP	CVB	SL	CVI	TC	CD	AP	CVB	SL	CVI	TC
Proximate composition [% OS]												
protein [%]	19.93 ± 0.19	20.41 ± 0.10	19.86 ± 0.23	20.21 ± 0.17	19.73 ± 0.14	20.28 ± 0.09	20.90 ± 0.35	20.68 ± 0.17	20.60 ± 0.15	20.43 ± 0.21	20.57 ± 0.18 [b]	20.71 ± 0.13
water [%]	74.71 ± 0.22	74.99 ± 0.24	75.11 ± 0.27	75.31 ± 0.22	75.10 ± 0.21	74.43 ± 0.19	75.65 ± 0.35	75.97 ± 0.18	76.32 ± 0.16	76.48 ± 0.33	76.49 ± 0.27 [b]	75.89 ± 0.12
ash [%]	1.46 ± 0.03	1.46 ± 0.04	1.54 ± 0.05	1.43 ± 0.02	1.38 ± 0.02	1.39 ± 0.02	1.49 ± 0.02	1.44 ± 0.01	1.46 ± 0.01	1.46 ± 0.02	1.48 ± 0.01	1.48 ± 0.01
fat [%]	4.66 ± 0.12	**4.1 ± 0.06 [a]**	4.29 ± 0.19	**4.03 ± 0.14 [a]**	4.60 ± 0.26	4.68 ± 0.26	3.24 ± 0.15 [b]	3.34 ± 0.12 [b]	2.88 ± 0.17 [b]	2.97 ± 0.12 [b]	2.85 ± 0.16 [b]	3.48 ± 0.15 [b]
[% Fatty Acids]												
total SFA	17.87 ± 0.16	17.82 ± 0.27	**17.11 ± 0.07 [a]**	18.12 ± 0.21	17.76 ± 0.21	17.8 ± 0.24	17.67 ± 0.24	17.53 ± 0.32	17.23 ± 0.23	18.74 ± 0.23	17.06 ± 0.08	17.69 ± 0.22
total MUFA	49.25 ± 0.27	48.31 ± 0.12	48.51 ± 0.32	**42.16 ± 0.45 [a]**	48.71 ± 0.28	49.29 ± 0.21	47.71 ± 0.39 [b]	47.27 ± 0.24 [b]	46.3 ± 0.55 [b]	**39.89 ± 0.41 [a, b]**	46.33 ± 0.53 [b]	47.93 ± 0.43
LA	11.99 ± 0.07	11.9 ± 0.09	12.26 ± 0.04	**10.8 ± 0.12 [a]**	12.15 ± 0.12	11.9 ± 0.06	11.67 ± 0.11 [b]	11.65 ± 0.13	11.53 ± 0.03 [b]	**10.02 ± 0.11 [a, b]**	11.65 ± 0.09 [b]	11.49 ± 0.11 [b]
total n6 PUFA	14.86 ± 0.07	14.99 ± 0.06	**15.15 ± 0.05 [a]**	**14.41 ± 0.10 [a]**	14.98 ± 0.12	14.69 ± 0.07	14.77 ± 0.11	14.9 ± 0.12	14.56 ± 0.04 [b]	**13.99 ± 0.09 [a]**	14.75 ± 0.07	14.46 ± 0.08
ALA	2.66 ± 0.05	2.79 ± 0.04	**3.16 ± 0.06 [a]**	2.54 ± 0.06	**3.04 ± 0.04 [a]**	**2.97 ± 0.02 [a]**	2.57 ± 0.06	2.72 ± 0.06	**2.96 ± 0.03 [a]**	**2.26 ± 0.05 [a, b]**	2.81 ± 0.03 [b]	2.73 ± 0.04 [b]
EPA	1.59 ± 0.03	1.63 ± 0.03	1.67 ± 0.03	1.7 ± 0.04	1.6 ± 0.04	1.59 ± 0.03	1.77 ± 0.05 [b]	1.74 ± 0.04	1.94 ± 0.05 [b]	1.75 ± 0.03	1.89 ± 0.06 [b]	1.79 ± 0.07
DHA	8.65 ± 0.22	9.32 ± 0.18	9.16 ± 0.19	**16.08 ± 0.34 [a]**	8.69 ± 0.27	8.28 ± 0.29	10.58 ± 41 [b]	10.83 ± 0.36 [b]	11.98 ± 0.67 [b]	**18.76 ± 0.45 [a, b]**	12.14 ± 0.51 [b]	10.23 ± 0.42 [b]
total n3 PUFA	15.36 ± 0.28	16.26 ± 0.23	16.63 ± 0.27	**22.83 ± 0.35**	15.86 ± 0.35	15.43 ± 0.35	17.36 ± 0.52 [b]	17.8 ± 0.45	19.54 ± 0.72[b]	**25.16 ± 0.43 [a, b]**	19.43 ± 0.56 [b]	17.31 ± 0.51
total PUFA	30.96 ± 0.29	31.96 ± 0.26	32.49 ± 0.29	**37.91 ± 0.31 [a]**	31.53 ± 0.43	30.81 ± 0.39	32.83 ± 0.53	33.38 ± 0.54	34.74 ± 0.71	**39.8 ± 0.43 [a, b]**	34.84 ± 0.51 [b]	32.46 ± 0.51
n3 HUFA	11.97 ± 0.26	12.73 ± 0.20	12.73 ± 0.21	**19.64 ± 0.40 [a]**	12.1 ± 0.32	11.6 ± 0.35	14.16 ± 0.47 [b]	14.42 ± 0.44 [b]	15.92 ± 0.73 [b]	**22.34 ± 0.47 [a, b]**	15.96 ± 0.57 [b]	13.81 ± 0.51 [b]
[ng/g muscle]												
Lutein	11.1 ± 0.3	13.8 ± 1.6	670.6 ± 101.0	11.6 ± 0.4	**315.2 ± 3.7 [a]**	**229.2 ± 29.8 [a]**	15.4 ± 1.3	15.3 ± 0.7	**743.1 ± 64.9 [a]**	15.9 ± 1.7	**282.4 ± 17.9 [a]**	**203.1 ± 7.6 [a]**
Violaxanthin	n.d.	n.d.	n.d.	n.d.	n.d.	86.0 ± 14.9	n.d.	n.d.	n.d.	n.d.	n.d.	88.8 ± 5.0
Zeaxanthin	n.d.	68.9 ± 7.5	n.d.	n.d.	n.d.	14.8 ± 2.2	n.d.	98.2 ± 14.6	n.d.	n.d.	n.d.	14.9 ± 1.2

Data is presented as mean ± SEM, with n = 9 individuals per treatment for proximate and fatty acid composition and n = 3 (pooled on tank level) for carotenoids. Two-way ANOVA was used to assess the effect of diet and timepoint, as well as their interaction on the response variable. For ANOVA results see Supplementary Table 5. A significant difference ($p < 0.05$) compared to the control diet (CD) within one timepoint was assessed by Dunnett's multiple comparisons and indicated in bold with a, while differences between timepoints within one diet were assessed by Tukey´s multiple comparison test and indicated with b; ns, not significant, n.d. not detected. Note that presented ANOVA results do not fully agree with results from multiple comparisons due to heteroscedasticity.

overall increase between T1 and T2 ($p < 0.001$; Supplementary Figure 2). Total cholesterol increased significantly between the first two samplings for fish fed CVI, CVB and TC ($p < 0.05$; Supplementary Figure 2). Glucose furthermore increased over time only in fish fed SL ($p = 0.02$; Supplementary Figure 2).

3.5 Liver proteins

Abundance of myeloperoxidase (Mpo) in the liver of Atlantic salmon fed a microalgae-enriched diet was lower in most cases at both timepoints compared to CD (Figure 5A). This effect was

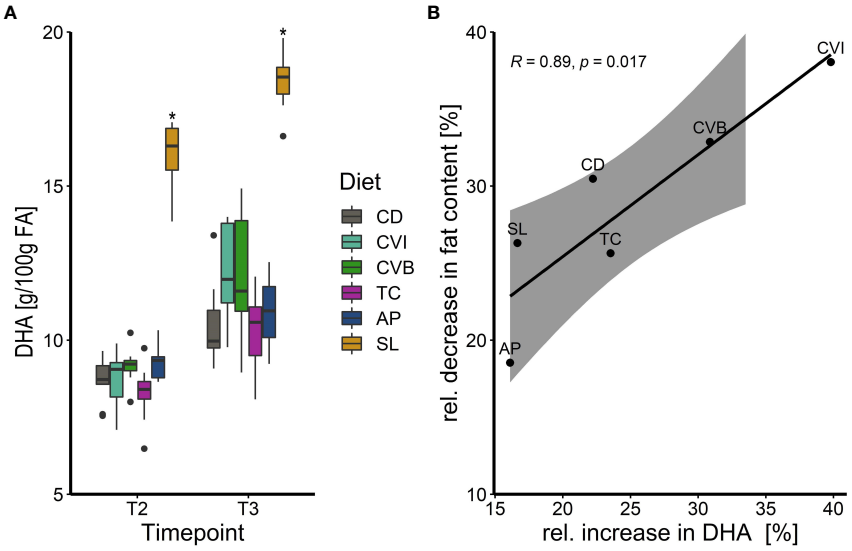

FIGURE 2
(A) Boxplot of docosahexaenoic acid (DHA) concentration in g/100g fatty acids (FA) of Atlantic salmon muscle fat following feeding the experimental diets for eight weeks in brackish water (T2) and transferred to seawater for two weeks (T3), n = 9. The fish received six different experimental diets: control (CD), *Chlorella vulgaris* intact (CVI), *Chlorella vulgaris* broken (CVB), *Tetraselmis chuii* (TC), *Arthrospira platensis* (AP) and *Schizochytrium limacinum* (SL) at an inclusion level of 8%. A significant difference ($p < 0.05$) compared to the control diet (CD) was assessed by Dunnett's multiple comparisons and indicated with a * **(B)** Linear relationship between the relative increase in muscle lipid DHA content in % in relation to the relative decrease (%) of muscle fat content following transfer to seawater (T3). Note that for Pearson´s correlation analysis group means per diet were used (n = 6) and diet groups are indicated next to each datapoint.

however only significant for fish fed AP at T1 ($p = 0.049$) with a 35% reduction compared to CD due to a large within group variation (Figure 5A). Though, AP at both timepoints showed the lowest variation. Sod1 protein level was induced by 3-fold in SL at T1 compared to CD ($p = 0.10$; Figure 5B).

3.6 Gene expression

Only few genes were significantly differentially expressed between microalgae fed groups compared to CD fish, due to a large overall variability in expression (Figures 6–8) although some

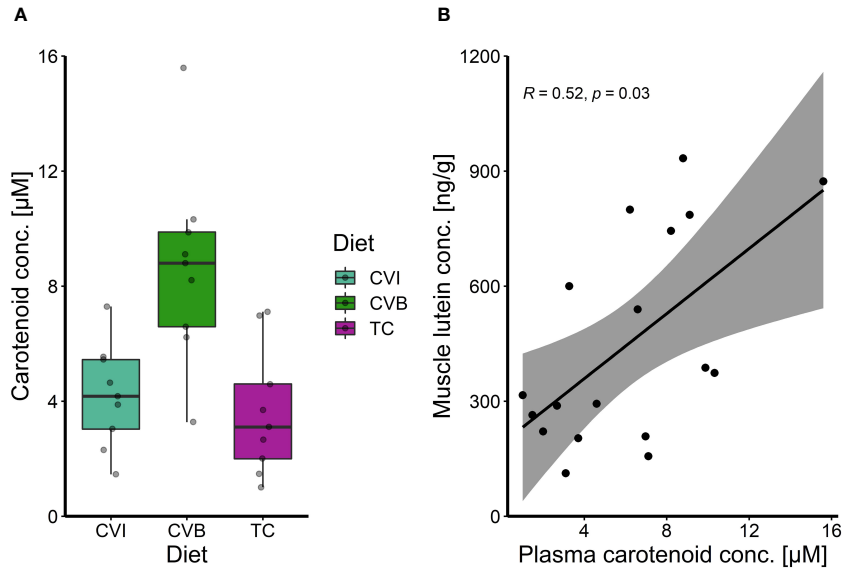

FIGURE 3
(A) Boxplot of carotenoid concentration in plasma of Atlantic salmon after eight weeks of feeding experimental diets enriched with *C. vulgaris* intact (CVI), broken (CVB) or *T. chuii* (TC) and **(B)** spearman correlation among plasma total carotenoid concentration and muscle lutein concentration after eight weeks of feeding experimental diets for fish where both parameters were measured on an individual level (n = 18).

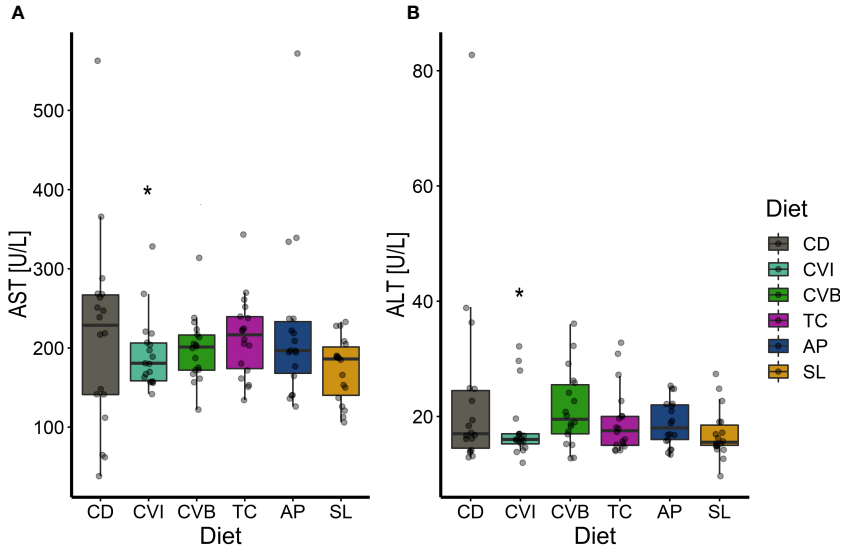

FIGURE 4
Boxplot of aspartate aminotransferase (AST) activity **(A)** and alanine aminotransferase (ALT) activity **(B)** in plasma of Atlantic salmon fed microalgae enriched diets for eight weeks (T2, n = 18). The fish received six different experimental diets: control (CD), *Chlorella vulgaris* intact (CVI), *Chlorella vulgaris* broken (CVB), *Tetraselmis chuii* (TC), *Arthrospira platensis* (AP) and *Schizochytrium limacinum* (SL) at an inclusion level of 8%. A significant difference ($p < 0.05$) compared to the control diet (CD) was assessed by Dunnett's multiple comparisons and indicated with a *.

large fold-changes were evident (Supplementary Figure 3). In the anterior intestine, increased *drtp1*-transcript levels were found in CVI- (2.8-fold, p = 0.065; Figure 6A) and CVB-fed salmon (7-fold; p = 0.023; Figure 6A). *isg15* transcript levels were induced in all microalgae fed groups except for CVI (Figure 6B), although not statistically significant. *il1r2* transcript levels were reduced by 2.7-fold in fish fed CVI (p = 0.039; Figure 6C) and *il10rb* levels were reduced in fish fed CVI (p = 0.028; Figure 6D) and TC (p = 0.039).

In the liver, the transcript levels of the acute-phase gene *saa5* were significantly reduced in fish fed CVB (0.37-fold; p = 0.016; Figure 7A) and SL (0.29-fold; p = 0.012, Figure 7A). *c1ql2* transcripts were significantly induced in fish fed CVI (p = 0.032)

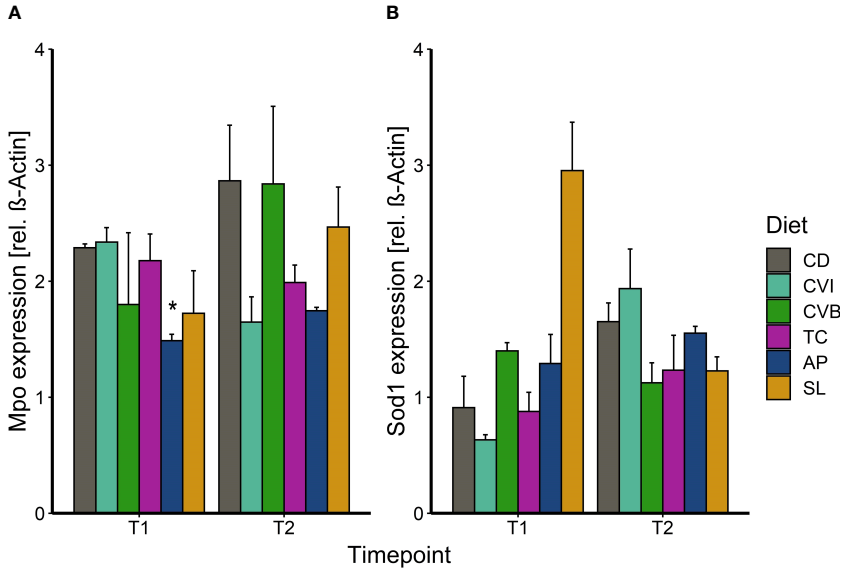

FIGURE 5
Protein expression of myeloperoxidase **(A)** and superoxide dismutase 1 **(B)** in liver tissue of Atlantic salmon following feeding the microalgae enriched diets for two weeks (T1) and eight weeks (T2) in brackish water. The fish received six different experimental diets: control (CD), *Chlorella vulgaris* intact (CVI), *Chlorella vulgaris* broken (CVB), *Tetraselmis chuii* (TC), *Arthrospira platensis* (AP) and *Schizochytrium limacinum* (SL) at an inclusion level of 8%. Data is presented as mean + SEM, n = 3 (pooled on tank level). A significant difference ($p < 0.05$) compared to the control diet (CD) was assessed by Dunnett's multiple comparisons and indicated with a *.

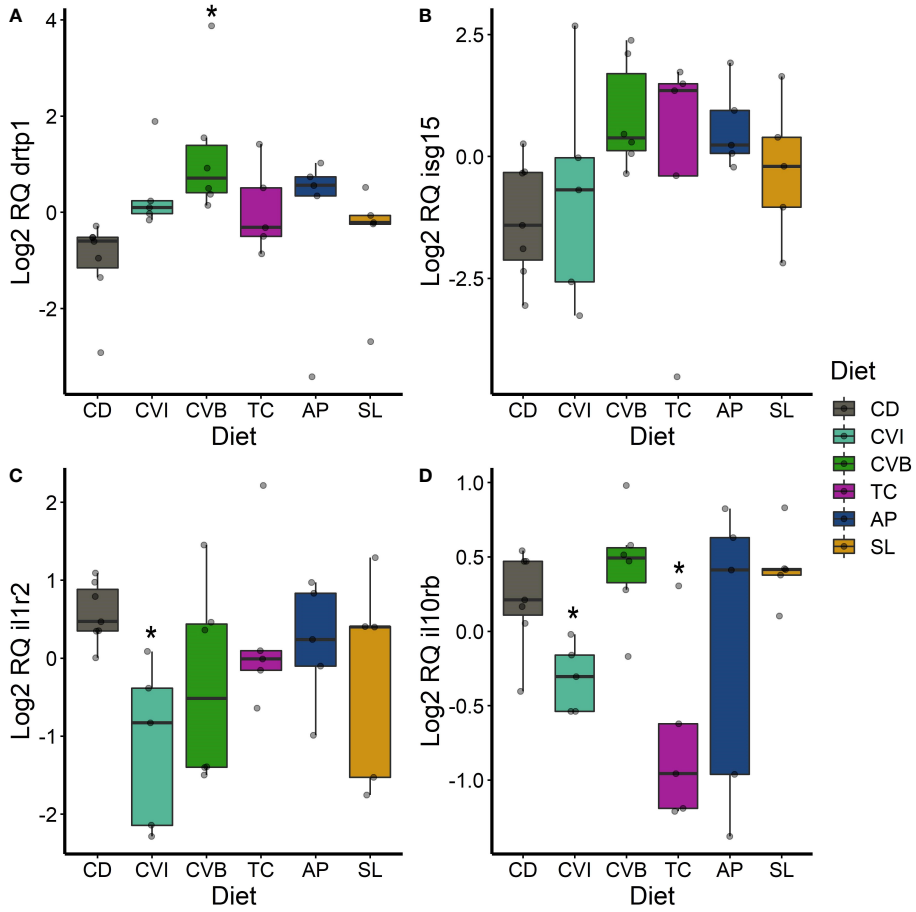

FIGURE 6
Boxplot of the gene expression in the anterior intestine of *drtp1* **(A)**, *isg15* **(B)**, *il1r2* **(C)** and *il10rb* **(D)** of fish fed microalgae enriched diets for eight weeks. The fish received six different experimental diets: control (CD), *Chlorella vulgaris* intact (CVI), *Chlorella vulgaris* broken (CVB), *Tetraselmis chuii* (TC), *Arthrospira platensis* (AP) and *Schizochytrium limacinum* (SL) at an inclusion level of 8%. Expression values were normalized relative to the mean expression of all samples and log2 transformed (n = 5 – 7). A significant difference ($p < 0.05$) compared to the control diet (CD) was assessed by Dunnett's multiple comparisons and indicated with a *.

and SL (p = 0.03; Figure 7B). *hamp* transcripts were 2.2-fold higher concentrated in the liver of fish fed CVB (p = 0.066; Figure 7C) and *lyzc2* transcripts were even 12-fold increased in fish fed TC (p = 0.04; Figure 7D) compared to the control group.

In the spleen, *c1ql2* transcripts were slightly 1.5-fold induced in fish fed CVB (p = 0.095) and TC (p = 0.097; Figure 8A). *ikba* transcripts were 0.59-fold reduced in the spleen of fish fed CVI (p = 0.06; Figure 8B). Furthermore, transcript abundance of *saa5* was 2.1-fold increased in fish fed CVI (p = 0.034; Figure 8C). The transcript level of *cd209d* was reduced across all microalgae-fed fish, although not statistically significant (Figure 8D).

Since the levels of plasma markers and selected transcripts varied largely, we conducted a correlation analysis to identify connections and validate the overall utility of the used health parameters. However only alkaline phosphatase (ALP) activity in plasma significantly correlated with *clra* (R = 0.46, p = 0.008; Supplementary Figure 4A) and *c4b* transcript levels in the liver (R = 0.45, p = 0.008; Supplementary Figure 4B).

4 Discussion

Microalgae are gaining attention as a sustainable ingredient to replace fishmeal or oil in aquaculture diets (Shah et al., 2018; Kousoulaki et al., 2020; Sarker et al., 2020a; Sarker et al., 2020b) and further as a functional supplement, prebiotic and immunostimulant for farmed fish (Reyes-Becerril et al., 2013; Rahimnejad et al., 2017; Messina et al., 2019; Sun et al., 2019; Teimouri et al., 2019). In this study microalgae inclusion did not negatively affect performance of Atlantic salmon reared in recirculating aquaculture systems. However, we found that health, immunity as well as fatty acid and pigment deposition were influenced in an algae specific manner.

Inclusion of microalgae in fish feed has been shown to affect the growth performance via increasing feed intake or improving feed conversion in a variety of fish species (Table 6). Including 5% *Chlorella sorokiniana* in the diet increased feed intake and thus growth in rainbow trout (Chen et al., 2021) and including *Chlorella*

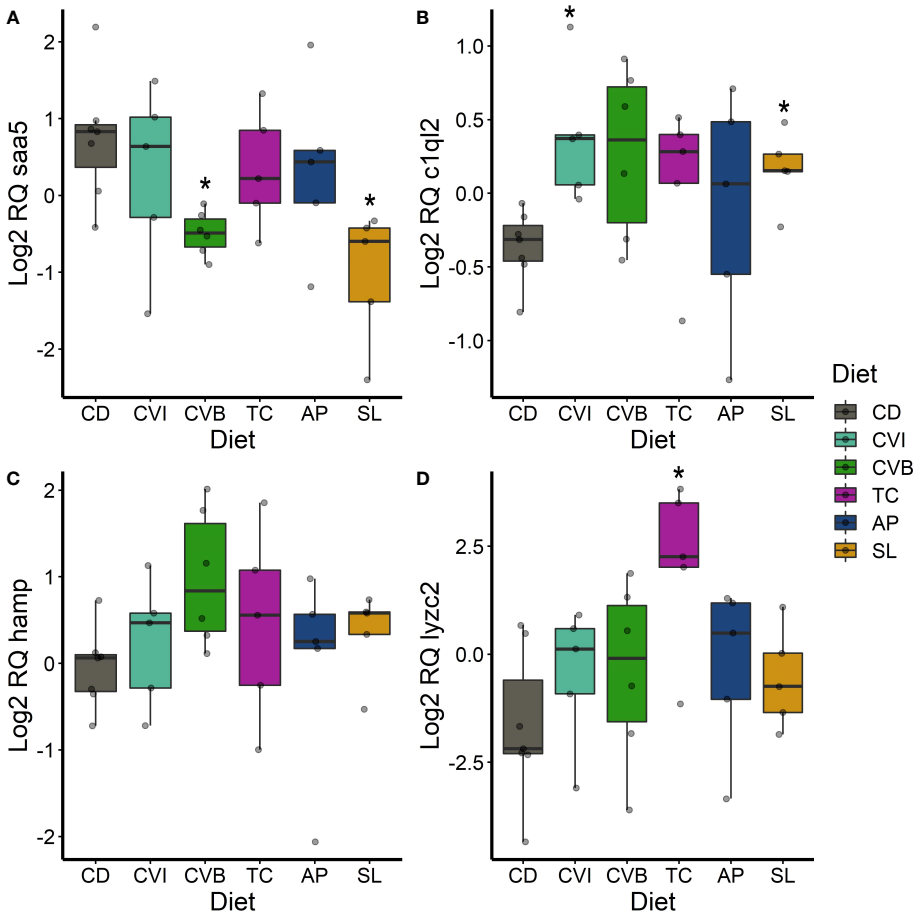

FIGURE 7
Boxplot of the gene expression in the liver of *saa5* **(A)**, *c1ql2* **(B)**, *hamp* **(C)** and *lyzc2* **(D)** of fish fed microalgae enriched diets for eight weeks. The fish received six different experimental diets: control (CD), *Chlorella vulgaris* intact (CVI), *Chlorella vulgaris* broken (CVB), *Tetraselmis chuii* (TC), *Arthrospira platensis* (AP) and *Schizochytrium limacinum* (SL) at an inclusion level of 8%. Expression values were normalized relative to the mean expression of all samples and log2 transformed (n = 5 – 7). A significant difference ($p < 0.05$) compared to the control diet (CD) was assessed by Dunnett's multiple comparisons and indicated with a *.

vulgaris at levels of 10 to 15% improved feed intake and conversion in olive flounder (Rahimnejad et al., 2017). In this line including broken *C. vulgaris* in our study improved feed conversion efficiency but not feed intake in Atlantic salmon. Nevertheless at 8% inclusion level the palatability of the feeds in our study was not negatively affected, likely because these microalgae do not contain high amounts of anti-nutritional factors, as observed for other plant-based ingredients (Nagel et al., 2012; von Danwitz and Schulz, 2020). Voluntary feed intake decreases in many fish species in response to stress (Kulczykowska & Sánchez Vázquez, 2010). Transferring the salmon into seawater reduced appetite as previously described (Usher et al., 1991). Although microalgae were shown to increase feed intake in many species (Table 6) and mitigate acute stress (de Mattos et al., 2019), we found no indications that microalgae could increase feed intake during the critical time period of the first weeks in seawater. In contrast, a diet enriched with the feeding stimulant squid extract was able to improve feed intake of Atlantic salmon in this time period (Toften et al., 2003) and other ingredients which act as feed attractants might be explored in the future. The overall lower growth performance compared to other studies (Kousoulaki et al., 2020; Ytrestøyl et al., 2020) is likely attributed to the use of pelletized feeds (Kiron et al., 2012), as compared to high performance extruded feeds.

Many microalgae species such as *Chlorella* and *Tetraselmis* contain a rigid cell wall, with a rigid cell wall fraction build of chitin- or chitosan-like polysaccharides (Domozych et al., 2012; Weber et al., 2022), which can reduce nutrient digestibility. Digestibility was reduced in Atlantic salmon fed with *Chlorella vulgaris* already at 6% inclusion (Tibbetts et al., 2017), but pre-extruded *Nanochloropsis* included at 10% improved dry matter digestibility and did not change protein digestibility in Atlantic salmon (Gong et al., 2020). The improved FCR found for fish fed broken *C. vulgaris* in our study, a species with a particularly rigid cell wall, is probably related to the destructed cell wall (Weber et al., 2022). The actual mechanism for improvements in feed conversion efficiency by microalgae is however not well understood, but seems to be related to promoting growth of beneficial intestinal bacteria (Ma et al., 2022), as well as improving intestinal health and nutrient uptake (Perera et al., 2020; Molina-Roque et al., 2022). Although

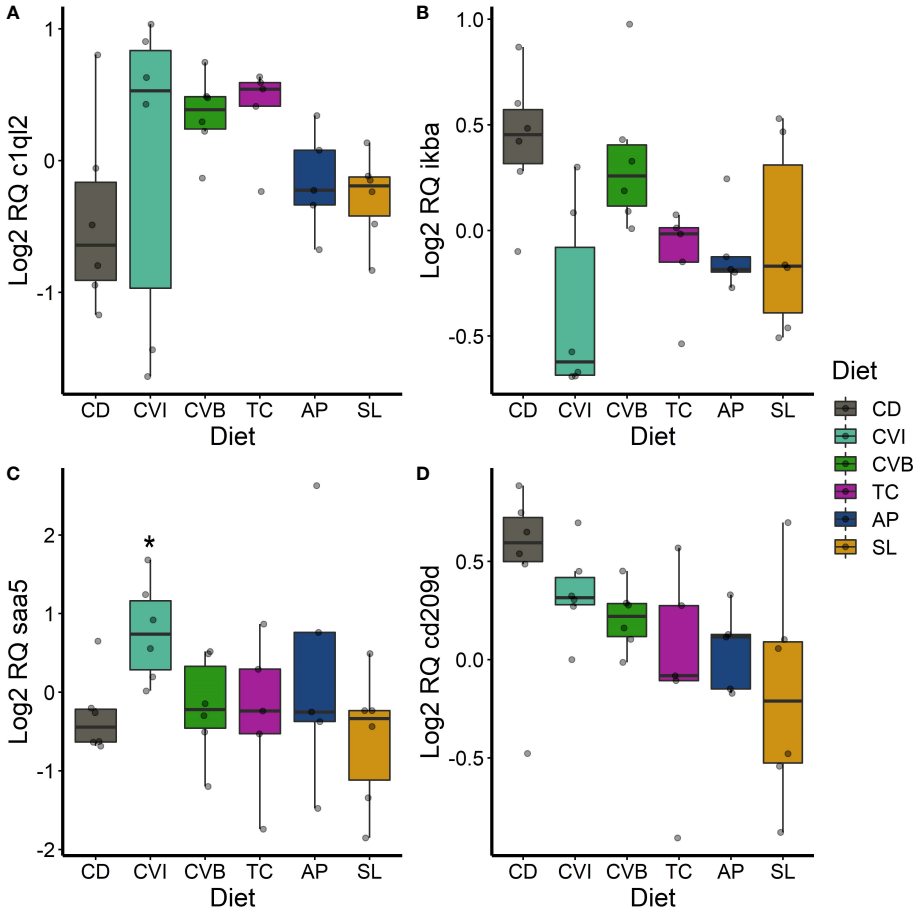

FIGURE 8
Boxplot of the gene expression in the spleen of *c1ql2* **(A)**, *ikba* **(B)**, *saa5* **(C)** and *lyzc2* **(D)** of fish fed microalgae enriched diets for eight weeks. The fish received six different experimental diets: control (CD), *Chlorella vulgaris* intact (CVI), *Chlorella vulgaris* broken (CVB), *Tetraselmis chuii* (TC), *Arthrospira platensis* (AP) and *Schizochytrium limacinum* (SL) at an inclusion level of 8%. Expression values were normalized relative to the mean expression of all samples and log2 transformed (n = 5 – 7). A significant difference ($p < 0.05$) compared to the control diet (CD) was assessed by Dunnett's multiple comparisons and indicated with a *.

inclusion levels of microalgae in diets vary greatly (Table 6) there seems to be a threshold at 10 – 15% inclusion for carnivorous fish, upon where growth performance is negatively affected likely because of reduced digestibility.

Besides its direct effects on growth, microalgae as a functional feed additive can influence body proximate composition and somatic indices by modulating energy metabolism. Low inclusion levels of microalgae derived nutraceuticals increased body proximate protein content, hepatosomatic index and glucose uptake capacity of the liver of gilthead sea bream, *Sparus aurata* (Perera et al., 2020). *C. vulgaris* in the diet was found to influence lipid metabolism in rats given a high fat diet, where it was able to lower triglycerides, total cholesterol and LDL cholesterol (Cherng and Shih, 2005). Similar results have been found in a study with humans (Ebrahimi-Mameghani et al., 2014). Reduced condition, hepatosomatic index and whole-body fat content in salmon fed diets containing broken *C. vulgaris* in our study also indicates interference with the lipid metabolism. It is however unclear whether increased metabolization or decreased deposition of lipid and glycogen took place as a response to the diet. However, in contrast to the above-mentioned studies, total cholesterol levels in plasma were unaffected.

Microalgae supplementation had a clear effect on the fatty acid profile of the fish muscle and levels found in the muscle of the salmon mirrored levels in the diets (Bell et al., 2001; Caballero et al., 2002). Increased levels of *alpha*-linolenic acid in the muscle of fish fed *C. vulgaris* (intact and broken) and *T. chuii*, as well as higher levels of DHA in fish fed *S. limacinum* resulted from higher contents of these specific fatty acids in the respective microalgae. This was evident although the experimental period of eight plus two weeks was rather short compared to other studies (Bell et al., 1993; Ruyter et al., 2000; Sissener et al., 2016). Atlantic salmon need to take up essential fatty acids via the diet and dietary requirements for EPA + DHA for Atlantic salmon post-smolts have been found to be ~ 0.5% of dry matter (Bou et al., 2017). All diets contained sufficient EPA and DHA, but high levels of DHA (2.5% of DM) in the diet containing *S. limacinum* were not reflected to the same degree in the muscle of the fish. The results must, however, be interpreted carefully, as preferential retention of specific fatty acids can influence the results in the muscle (Bell et al., 2003). Retention

TABLE 6 Growth and immune effects of microalgae species incorporated in the diet and fed to different fish species.

Microalgae species	Fish species	Days	Inclusion rate [%]	SGR	FCR	DFI	Antioxidant activity	Immune cells and markers	Antibacterial activity	Immune signaling	Source
A. platensis	*O. niloticus*	84	0.125 - 1	↑	↓	↑	↑	↑↓	↓		Abdel-Tawwab and Ahmad, 2009
A. platensis	*S. aurata*	30	42.6				↓	↑			de Mattos et al., 2019
A. platensis	*C. carpio*	40	1					↑			Khalil et al., 2017
A. platensis	*C. gariepinus*	84	12.5 -18.75	↑	↓		↑	↑			Raji et al., 2018
A. platensis	*O. mykiss*	70	2.5 -10.0	↓ ↑	↓		↑ ↓				Teimouri et al., 2019
A. platensis	*O. mykiss*	70	2.5 -10.0					↑			Yeganeh et al., 2015
C. vulgaris	*S. salar*	28	20						↑↓		Grammes et al., 2013
Chlorella sp.	*C. auratus*	56	1.0 - 4.0	↑	↓	↑		↑		↑	Luo et al., 2018
C. vulgaris	*O. niloticus*	60	5	↑	↓		↑	↑	↑		Mahmoud et al., 2020
C. vulgaris	*C. gariepinus*	84	12.5 - 18.75	↑	↓		↑	↑			Raji et al., 2018
Chlorella sp	*C. auratus*	60	0.4 - 2.0	↑				↑			Zhang et al., 2014
C. sorokiniana	*O. mykiss*	90	0 - 10			↑	↓↑	↑	↑↓		Chen et al., 2021
D. salina	*D. rerio*	30	15							↑	Fan et al., 2022
I. galbana	*D. rerio*	30	15				↑			↑	Fan et al., 2022
L. incisa	*D. rerio*	30	7.50 -15.0				↑		↑	↑	Nayak et al., 2020
N. gaditana	*S. aurata*	28	5.0 -10.0	↓					↑		Cerezuela et al., 2012b
N. gaditana	*S. salar*	84	10.0 - 20.0	↓	↑	↑	↑			↑	Sørensen et al., 2017
Navicula sp.	*L. peru*	28/ 56	10				↑	↑	↑		Reyes-Becerril et al., 2014
Navicula sp.	*S. aurata*	14/ 28	10							↑	Reyes-Becerril et al., 2013
P. tricornutum	*S. aurata*	28	10					↓			Cerezuela et al., 2013
P. tricornutum	*S. aurata*	14/ 28	10				↑	↑	↑	↑	Cerezuela et al., 2012b
P. tricornutum	*S. aurata*	28	5.0 - 10.0	↓ ↑			↑		↑	↑	Cerezuela et al., 2012b
P. tricornutum	*D. rerio*	30	15				↑			↑	Fan et al., 2022

(Continued)

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TABLE 6 Continued

Microalgae species	Fish species	Days	Inclusion rate [%]	SGR	FCR	DFI	Antioxidant activity	Immune cells and markers	Antibacterial activity	Immune signaling	Source
S. almeriensis	*O. niloticus*	30	25	–			↑		↑		García-Márquez et al., 2020
S. almeriensis	*O. niloticus*	60	5.0 - 22.0	↑	↑	–	↑	↑	↑		Abdel-Tawwab et al., 2022
Schizochytrium sp.	*M. salmoides*	84	5.7 - 14.1				↑↓	↓↑		↑	Habte-Tsion et al., 2020
Schizochytrium sp.	*O. niloticus*	105	1.2					↓			de Souza et al., 2020
S. limacinum	*S. salar*	330	2.62 - 6.25		↓	↓	↑	↓	↓	↑	Kousoulaki et al., 2020
S. limacinum	*C. altivelis*	30	1	↑			↑			↑	Sun et al., 2019
S. limacinum	*T. ovatus*	56	3	↑	↓	↑				↓	Xie et al., 2019
T. chuii	*S. aurata*	28	10					↓			Cerezuela et al., 2013
T. chuii	*S. aurata*	14/ 28	10				↑	↑	↑	↑	Cerezuela et al., 2012b
T. chuii	*S. aurata*	28	5.0 - 10.0	↓				↑	↑		Cerezuela et al., 2012b
T.suecica & T. lutea	*D. labrax*	105	6.0 - 18.0						↑		Messina et al., 2019
T. ultriculosum	*O. mykiss*	90	5.0 -10.0	↑	↑	↑		↓↑	↑		Chen et al., 2021

Note that only studies were included which reported effects on immunity and were not solely based on performance parameters. Responses marked with arrows are shown when at least one inclusion level caused a significant increase or decrease. In case different inclusion levels or diets caused divergent responses both arrows are shown. SGR, specific growth rate; DFI, daily feed intake; FCR, feed conversion ratio.

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efficiency of DHA was indicated to be dose dependent (Glencross et al., 2014; Emery et al., 2016) and high dietary DHA in the *S. limacinum* diet could have reduced the need for an efficient retention of DHA in the muscle. Furthermore diets rich in saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) improve long-chain polyunsaturated fatty acid (LC-PUFA) metabolism efficiency (Xu et al., 2014; Emery et al., 2016). The diet enriched with *S. limacinum* had a lower MUFA content due to reduced inclusion of canola oil. Together with a lower digestibility of palmitic acid, which is present in high concentrations in *Schizochytrium* (Kousoulaki et al., 2020; Hart et al., 2021), this could have led to an overall lower fat content in the muscle of fish fed with this microalgae. Although palmitic acid was enriched in the diet containing *S. limacinum*, it was only slightly increased in the muscle at the end of the trial and its metabolic fate requires more attention in future studies. Muscle fat content was also reduced in salmon fed *A. platensis* for eight weeks in our study and *Arthrospira platensis* has been found to reduce hyperlipidaemia in the rat model (Hua et al., 2018; Li et al., 2019). However, no effect was detected in our study on cholesterol levels in plasma, which has been described by Hua et al. (2018).

Seawater transfer results in increased energy demands and coupled with reduced feed intake caused a significant reduction in the muscle fat content, primarily in the relative abundance of specific fatty acids of salmon smolts (Woo et al., 1978; Sheridan, 1989; Usher et al., 1991). Since monounsaturated fatty acids are preferentially used as metabolic fuel (Henderson, 1996) polyunsaturated fatty acids such as DHA were protected from being metabolized and not oxidized during the early time window in seawater. Hence, relative abundance of DHA in the muscle of all groups increased significantly after transfer to seawater and was linearly related to the decrease in total fat content of the muscle.

Besides polyunsaturated fatty acids also pigments are important in maintaining health and immune function in fish (de Carvalho & Caramujo, 2017). Lutein, the dominating pigment present in all muscle samples, was found to improve growth and antioxidant status of whiteleg shrimp *Litopenaeus vannamei* (Fang et al., 2021) and improve survival of goldfish *Carassius auratus* (Besen et al., 2019). Differences in the carotenoid profile among the diet groups were directly related to its feed origin, with lutein found in highest concentrations in fish fed broken *C. vulgaris*, followed by intact *C. vulgaris* and *T. chuii*. Sørensen et al. (2023) also found high concentrations of lutein in the muscle of salmon fed *T. chuii* biomass, but in contrast to their study, we did not detect any astaxanthin in the muscle samples. This could be explained by the lower inclusion of fishmeal in our diet, which is a natural source of astaxanthin (Lim et al., 2018) and further by a difference in the pigment profile of the used microalgae products. Lutein concentrations in the muscle of the salmon in our study were linearly related to total carotenoid concentration found in plasma samples, which could be caused by a dynamic equilibrium of carotenoids between the bloodstream and muscle. However, it could also imply that fish that accumulated more carotenoids over the entire trial also ingested more during the days before sampling. Aside from lutein, the xanthophylls zeaxanthin and violaxanthin were present in the muscle of fish fed *A. platensis* and *T. chuii* respectively. All detected carotenoids have important functions in maintaining eye-health in mammals (Giordano and Quadro, 2018) and could be investigated for the prevention of eye health disorders such as cataract in fish, a common problem in salmonid aquaculture (Waagbø et al., 2003; Bjerkås and Sveier, 2004).

While several studies have investigated health and immune effects of microalgae in fish, detected responses seem to depend on both microalgae species as well as fish species investigated (Table 6). We detected microalgae specific influences on health and immunity in Atlantic salmon at both local (intestine) and systemic (plasma, liver, spleen) scales. Adding broken *C. vulgaris* to the diet in this study lowered aspartate aminotransferase and alanine aminotransferase activity levels in the salmon plasma which may indicate improved liver health. Activity of these two enzymes is primarily considered an indicator of liver damage, as higher levels result from destructed or damaged liver cells (Huang et al., 2006). A meta-analysis on the effect of *Chlorella* supplementation on liver health in humans found an overall reduction of aspartate aminotransferase levels, while no effect was detected on alanine aminotransferase serum levels (Yarmohammadi et al., 2021).

Oxidative stress resulting from the increased formation of reactive oxygen species was shown to be reduced by the dietary intake of antioxidants in various fish species (reviewed by Hoseinifar et al., 2021). Superoxide dismutase 1, which catalyses the breakdown of superoxide radicals (Fukai and Ushio-Fukai, 2011), was induced in salmon fed *S. limacinum* after two weeks of feeding the diets. High amounts of DHA from *S. limacinum* are prone to peroxidation resulting in the formation of 4-hydroxyhexenal (4-HHE) which in turn can activate the Nrf2 antioxidant pathway inducing expression of sod (Yang et al., 2019). Several studies with mammalian cell lines have indicated that DHA is able to increase GSH content (Arab et al., 2006) as well as intracellular Sod and Gpx concentrations (Clementi et al., 2019). Our results indicate an early effect of the diet; however, protein levels after eight weeks were similar to the control diet. This suggests that beneficial effects of a functional diet may change over administration time and the potential temporal "habituation effect" requires more attention in future studies. Myeloperoxidase is a characteristic enzyme of neutrophil granulocytes which is involved in the oxidative burst response, where it catalyses the oxidation of chloride ions (Klebanoff, 1999; Frijhoff et al., 2015). It has been further associated with inflammatory processes and various diseases in humans (Davies & Hawkins, 2020). Reduced protein concentrations in the liver of salmon fed *A. platensis* could be caused by a lower abundance of neutrophil granulocytes in the liver, indicating no acute inflammatory response of the liver. Granulocytes in the blood of Nile tilapia *Oreochromis niloticus* fed diets containing different levels of *A. platensis* decreased strongly after 12 weeks of feeding (Abdel-Tawwab and Ahmad, 2009), but no inferences about the concentration or activity of Mpo has been made in this study.

The liver furthermore produces acute phase proteins and levels of transcripts encoding the acute phase protein serum amyloid A (*saa5*) were downregulated in fish fed broken *C. vulgaris* and *S. limacinum*. This highlights the potential anti-inflammatory role of

these microalgae with described effects of their main chemical components in the literature; lutein in case of *C. vulgaris* (Chung et al., 2017; Demmig-Adams et al., 2020) and DHA in case of *S. limacinum* (Li et al., 2005; Mullen et al., 2010).

However, microalgae addition increased the expression of *c1ql2* in the liver and spleen. C1ql2 is closely related to the first subcomponent of the complement system and might be therefore involved in the response to a variety of environmental conditions in Atlantic salmon (Krasnov et al., 2020; Beemelmanns et al., 2021). The precise function of c1ql2 is still unknown (Lao et al., 2008; Köbis et al., 2017). In addition to *c1ql2*, the upregulated levels of transcripts coding for the anti-microbial peptide hepcidin (*hamp*) and lysozyme (*lyzc2*) in the liver indicate a potentially enhanced anti-microbial defense by microalgae-enriched diets (Messina et al., 2019; García-Márquez et al., 2020).

Functional feed additives are thought to modulate the local immune response in the intestine and several of the investigated genes in the anterior intestine were modulated by the diet. The increased expression of *drtp1* in all microalgae supplemented groups may be linked to a general response of the intestine towards novel antigens in the diet, but was also found to be induced after an acute phase response (Martin et al., 2006; Talbot et al., 2009). Interferon-stimulated gene 15 (*isg15*) was upregulated in the anterior intestine in response to the microalgae diets. This gene is induced by type 1 interferon and acts like a cytokine (Perng & Lenschow, 2018). Interestingly, other cytokine receptors, namely interleukin 1 receptor (*il1r2*) as well as interleukin 10 receptor (*il10rb*) were downregulated in most microalgae diets. This may indicate a reduced sensitivity of the intestine towards pro-inflammatory signals or a general reduction of pro-inflammatory signals present in the intestine. Grammes et al. (2013) showed that including *Chlorella vulgaris* in the diet had anti-inflammatory action and protected Atlantic salmon from developing a soybean meal-induced enteritis (SBMIE). In zebrafish *Danio rerio* feeding diets with PUFA-rich microalgae increased the expression of the anti-inflammatory cytokine *il10* (Nayak et al., 2020). Interference of the diet with pro-inflammatory signaling was also detected in the spleen where expression of NFKB inhibitor alpha (ikba; Wang et al., 2009) was reduced in fish fed intact *C. vulgaris*.

The future of using microalgae as a functional feed ingredient largely depends on its production cost and economic benefits when incorporated into diets for Atlantic salmon. Although we investigated microalgae which are already cultivated at commercial scale, the current price (~20 – 30€ per kg) permits its use at higher inclusion levels only in restricted time periods. These might be during the production of juveniles, where feed costs are generally lower or in the final stage of production. Enhancing the product quality before slaughtering by increasing the fillet DHA and carotenoid content, with known benefits for human health can represent an economically viable strategy which should be further explored.

Conclusion

Our study revealed that microalgae addition of 8% to the diet could have positive effects on the health of Atlantic salmon reared in RAS without affecting its growth performance. We confirmed the transfer of important functional components of microalgae (polyunsaturated fatty acids and pigments) into the fish muscle, but the role and function of many of the functional compounds present in microalgae remains elusive and needs further investigation. Our results further indicate that microalgae enriched diets induce a local anti-inflammatory response in the intestine, improve oxidative stress response and stimulate complement and antibacterial responses in liver and spleen. Based on our comprehensive data, we encourage future studies to provide a holistic view on the health status of fish when evaluating functional feeds in aquaculture and investigate the use of microalgae enriched diets in other economically important production phases.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The experiment was approved by the animal welfare officer of the "Fraunhofer IMTE Büsum" and the local authority of Schleswig-Holstein, according to the German animal welfare law (NTP – ID 00043858-1-0). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

JMu: Conceptualization, Data curation, Formal Analysis, Investigation, Visualization, Writing – original draft. MP: Data curation, Investigation, Writing – review & editing. JMo: Data curation, Methodology, Writing – review & editing. UO: Data curation, Methodology, Writing – review & editing. DRvM: Data curation, Writing – review & editing. AR: Data curation, Writing – review & editing. TG: Funding acquisition, Project administration, Writing – review & editing. JL: Writing – review & editing, Data curation, Methodology. TS: Data curation, Methodology, Writing – review & editing. HS: Conceptualization, Supervision, Writing – review & editing. CS: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2023.1273614/full#supplementary-material

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5.2. Publication II: Can dietary *Chlorella vulgaris* supplementation improve health of Atlantic salmon? Insights from combined analysis of microbiota, host immunity and the response to oxidative stress

The microalgae species *Chlorella vulgaris* is already widely used in aquaculture, but its health effects and mitigation potential on the effects of new farming strategies on Atlantic salmon are still unclear. This study examined the effects of supplementation of *C. vulgaris* with different inclusion rates and applications (daily or weekly supplementation) on the performance, proximal body composition, and gut microbiota of fish. Moreover, after PAA-based disinfection of RAS, the response of Atlantic salmon was examined by measuring plasma indicators, protein concentrations in the liver and gill, and the expression of stress- and immune-related genes in the head kidney and gill. In addition, the mitigation potential of *C. vulgaris*-enriched diets on these effects was analyzed.

Highlights

- Supplementation of 14% *C. vulgaris* daily increased feed conversion ratio and significantly affected microbial beta diversity in both the digesta and mucosa in the intestine.
- The relative abundance of *Paenarthrobacter* and *Trichococcus* in the digesta and mucosa increased when salmon were fed with 14% *C. vulgaris* daily, indicating an improved ability to metabolize complex carbohydrates.
- Supplementation of *C. vulgaris* increased superoxide dismutase abundance in the liver and gill in a dose-dependent manner.
- PAA-based disinfection induced a systemic stress response and a local response in the gill with induced expression of immune-related genes.
- Fish fed 14% *C. vulgaris* daily had reduced transcript levels of immune-related genes after PAA exposure in the head kidney, while in the gill the expression of two immune (*marco, lao1)* genes was induced.
- No induced immune or stress response at the transcriptional level after PAA exposure in fish fed 14% *C. vulgaris* once weekly.

Can dietary *Chlorella vulgaris* supplementation improve health of Atlantic salmon? Insights from combined analysis of microbiota, host immunity and the response to oxidative stress

Jonas Mueller[1,2*]†, Doret R. van Muilekom[3]†, Jannick Ehlers[2], Marvin Suhr[4], Stéphanie C. Hornburg[4], Corinna Bang[5], Marie Wilkes[6], Thekla Schultheiß[6], Edmund Maser[6], Alexander Rebl[3], Tom Goldammer[3,7], Henrike Seibel[2], Carsten Schulz[1,2]

[1]*Department for Marine Aquaculture, Institute of Animal Breeding and Husbandry, Kiel University, Kiel, Germany*

[2]*Fraunhofer Research Institution for Individualized and Cell-Based Medical Engineering IMTE, Aquaculture and Aquatic Resources, Büsum, Germany*

[3]*Working Group Fish Genetics, Research Institute for Farm Animal Biology (FBN), Dummerstorf, Germany*

[4]*Institute of Animal Nutrition and Physiology, Kiel University, Kiel, Germany*

[5]*Institute of Clinical Molecular Biology, Kiel University, Kiel, Germany*

[6]*Institute of Toxicology and Pharmacology for Natural Scientists, University Medical School Schleswig-Holstein, Kiel, Germany*

[7]*Faculty of Agriculture and Environmental Sciences, University of Rostock, Rostock, Germany*

† These authors contributed equally to this work and share first authorship

***Correspondence: Jonas Mueller: jmueller@tierzucht.uni-kiel.de**

Keywords: Atlantic salmon, Chlorella, microalgae, functional feed, microbiome, immunity, oxidative stress

Abstract

Microalgae are emerging as functional feed ingredients in aquaculture due to their immune-stimulating and stress-modulating properties. We investigated the potential of the microalgae *Chlorella vulgaris* as a feed supplement to improve the health of Atlantic salmon reared in a recirculating aquaculture system (RAS). Triplicate groups of Atlantic salmon received diets supplemented with 2% (CV2), 14% (CV14) *C. vulgaris* daily, 14% once weekly (CV14w), or a control diet (CD) for eight weeks. Subsequently, all groups were exposed to peracetic acid (PAA), a commonly used disinfectant in RAS. While CV14 reduced feed conversion and thus growth significantly, feeding the diets CV2 and CV14w improved protein retention efficiency. CV14 had the strongest effect on the microbiota in both the intestinal digesta and mucosa, resulting in a significantly modulated beta-diversity, but this effect was already visible in fish fed CV2. Feeding CV14 and, to a lesser degree, CV2 increased the relative abundances of *Paenarthrobacter* and *Trichococcus* in the digesta and mucosa, which are able to metabolize complex carbohydrates. However, the same diets reduced the abundance of the lactic acid bacteria *Lactobacillus* and *Weissella* in the digesta and *Floricoccus* in the mucosa. Peracetic acid (CH3CO3H; PAA) exposure induced systemic stress (increase in plasma glucose and cortisol) and a local immune response in the gill, with the most prominent upregulation of several immune- and stress-regulated genes (clra, cebpb, marco, tnfrsf14, ikba, c1ql2, drtp1) 18 h after exposure in fish fed the control diet. Fish receiving CV14 once a week showed a reduced transcriptional response to PAA exposure. Catalase protein abundance in the liver increased following exposure to PAA, while superoxide dismutase abundance in the gill and liver was increased in a dose-dependent manner in response to *C. vulgaris* inclusion before stress. Overall, the results highlight that the inclusion rate of *C. vulgaris* in feed for Atlantic salmon significantly influences growth and the intestinal microbiota. Despite the limited potential to mitigate the systemic stress effects of PAA treatment, feeding *C. vulgaris* once a week modulated local immune and stress responses in the gill.

1 Introduction

Aquaculture production continues to grow at a high rate, providing fish and seafood for a continuously increasing consumer demand globally [1]. A more sustainable intensification of fish farming is needed in order to ensure future growth of the industry. Implementing novel feed ingredients with less reliance on finite marine resources [2], as well as adopting new farming practices with minimized environmental impacts [3], are considered key to improve aquaculture sustainability. Furthermore, promoting fish health is a prerequisite for increasing production.

Atlantic salmon (*Salmo salar*) is one of the most valued fish species for human consumption, with a global production of 2.7 M tons in 2020 [4]. In recent years, Atlantic salmon farming has been suffering from compromised health and high mortality in the sea phase of production [5,6]. This has led to a strong increase in the use of land-based recirculating aquaculture systems (RAS), in which the production environment can be fully controlled [7,8]. However, the RAS environment can pose challenges related to fish health, e.g. caused by high stocking densities [9], reduced water quality [10] as well as routine disinfection [11,12]. While the latter is used to maintain a high level of biosecurity within the system, disinfectants and their derivatives can induce a stress and an immune response in treated fish. Peracetic acid (CH_3CO_3H; PAA) is an oxidative biocide with broad anti-bacterial activity, which is commonly used due to its low effective dose, rapid degradation, and minimal environmental impact [12]. However, acute exposure to PAA was shown to induce the expression of antioxidant genes (*gpx, sod*) in the gill and increase plasma cortisol and antioxidant capacity of Atlantic salmon [13]. Furthermore, chronic PAA exposure induced the expression of immune genes in the olfactory rosettes in Atlantic salmon [14] and increased serum antioxidant capacity and levels of free radicals in the gill and serum of rainbow trout [15].

Functional feeds are reported to improve the overall health status of fish. Their beneficial stress-mitigating and immune-modulating properties have the potential to counteract negative side effects when fish are treated with PAA [16]. Microalgae have emerged as valuable functional feed ingredients because of their high content of functional compounds and nutritional value [17]. A variety of vitamins, pigments, polyphenols, and complex carbohydrates in microalgae contribute to their antioxidant, antimicrobial, and immune-modulatory activity [18–20]. *Chlorella vulgaris* is a widely used microalgae species in aquaculture due to its low production cost, high content of protein and functional compounds including lutein, ß-carotene, phytosterols, and vitamin E [21]. In our previous study, we demonstrated that diets enriched with 8% *Chlorella vulgaris* induced anti-inflammatory responses (reduction of *saa5* in the liver, *nfkbia* in the

spleen, and *il10rb* and *il1r2* in the intestine) in Atlantic salmon [22]. Likewise, the activity of antioxidant enzymes (superoxide-dismutase, catalase, and glutathione peroxidase) and innate immune response (lysozyme, respiratory burst activity and total antibody counts) were increased in plasma of Nile tilapia when fed with diets supplemented with *Chlorella vulgaris* [23]. However, optimal inclusion rates and timing of application of *Chlorella vulgaris* in diets for Atlantic salmon are currently unknown.

Functional feeds not only directly affect the immune system of fish but also their intestinal microbiome, which is closely associated with animal health and performance [24,25]. Besides playing an important role in nutrient digestion, intestinal microbiota is involved in host immune responses as well as in the gut-brain axis [26–30]. The intestinal microbiota can be divided into transient (allochthonous) microbes associated with the digesta and resident (autochthonous) microbes residing within a complex matrix of mucin proteins [31]. The diet provides specific substrates for bacteria colonizing the digesta, shaping diet-dependent ecological niches. For example, plant-based diets have been associated with the occurrence of lactic acid bacteria of the phyla *Firmicutes* in the digesta of rainbow trout [32] and Atlantic salmon [33]. Bacteria residing within the intestinal mucus are thought to be less influenced by diet [34], but rather in intimate association with the host's immune system. Whether functional diets enriched with *Chlorella vulgaris* can modulate both the microbiota of digesta and intestinal mucosa, as well as immunity of Atlantic salmon, remains currently unknown.

This study aimed to investigate the use of *Chlorella vulgaris* as a functional feed additive in diets for Atlantic salmon. More specifically, we hypothesized that the inclusion rate (2% vs. 14%) and mode of application (daily vs. weekly) of *C. vulgaris* fed to Atlantic salmon would influence growth performance and the microbiota of the digesta as well as intestinal mucosa. Furthermore, feeding the functional enriched diets was hypothesized to counteract stress- and immune-related effects caused by treatment with PAA as an oxidative stressor.

2 Material and methods

Feed and experimental groups

Three different diets were formulated (on dry matter basis) for Atlantic salmon [35] containing different levels of the microalgae *Chlorella vulgaris*: a control diet (CD) without microalgae addition, a diet containing 2% *C. vulgaris* (CV2) and a diet containing 14% *C. vulgaris* (CV14). A fourth experimental group was established, which received 14% *C. vulgaris* only once per week (CV14w). The diets were designed to replace various plant-based components with

microalgae, as indicated in Table 1. The feed was pressed into pellets of 4 mm diameter (Type 14U175, Amandus Kahl, Hamburg, Germany) at temperatures below 60 °C. The produced feed was air-dried for 48 h and subsequently stored at 4 °C.

Experimental setup

Atlantic salmon post-smolts were sourced from Danish Salmon A/S (Hirtshals, Denmark) and allowed to acclimate for one month in a recirculating aquaculture system (RAS). The RAS (24 m^3, turnover rate 4 times h^{-1}) was equipped with a drum filter (mesh size 40 µm, type KTS 8-12, Kunststoff Spranger, Plauen, Germany), a moving bed biofilter (Kunststoff Spranger), a protein skimmer (FLOTOR, Kunststoff Spranger) and an UV disinfection system (Aqua medic, Cologne, Germany) for water treatment. Oxygen was supplied through an oxygen cone, with additional air supplied in the tanks. During the time of acclimation, the fish were fed twice daily, at ~ 1% of their bodyweight, a commercial salmon diet (Aller Aqua A/S, Christiansfeld, Denmark). Prior to the start of the experiment, Atlantic salmon (~ 126 g) were randomly allocated in groups of 20 individuals into 12 tanks (150 L) of the RAS. Triplicate tanks were randomly assigned to receive either the control diet (CD), the diet with 2% (CV2) or 14% (CV14) *C. vulgaris* on a daily basis, or 14% *C. vulgaris* once weekly (CV14w) while receiving the CD diet during the other days. Weekly feeding of CV14 occurred on day 4 of each experimental week during the trial. All fish were fed twice daily (9 a.m. and 2 p.m.) for 56 feeding days at 1% of their body weight. Daily feed rations were adapted based on growth performance acquired after group weighing in weeks two and five of the experiment, during which two days the fish were not fed.

PAA treatment and sampling

Samples were collected before the start (T0) and after eight weeks of feeding the experimental diets (T1). Following this sampling, all groups were subjected to acute oxidative stress induced by treatment with peracetic acid (PAA; WOFA-steril classic, Kesla, Germany) at 2.5 µL/L water, which is commonly applied for prophylactic treatment in RAS [12,36]. The product is a stabilized PAA solution with 40% PAA and 12% H_2O_2. For this purpose, the water inlet of the tank was closed, while continuous aeration in the tank ensured oxygen levels were maintained above 85% saturation. Then 375 µL of PAA solution was added to the tank, and the water inflow was opened again after 1 h. All groups were subsequently sampled 1 h after stress (T2) and 18 h after stress (T3).

For all three samplings, 12 fish per treatment (4 per tank) were netted from the experimental tanks and euthanized by buffered MS-222 (0.3 mg/L). Total length and weight were recorded for each fish and a 2 mL blood sample was collected from the caudal vein using heparinized syringes. The blood was centrifuged at 4000 *g* for 8 min and aliquots of the plasma were flash-frozen on dry ice and stored at −80°C.

During the samplings T1 and T3, the fish were laterally, opened and the organs were carefully removed. The liver and spleen were weighed for the calculation of organ-specific indices. A piece of the liver, as well as the second left gill arch, were removed and flash-frozen on dry ice for later protein extraction. The head kidney and first left gill arch were removed, placed in a RNAse-free tube and flash frozen in liquid nitrogen for subsequent gene expression analysis.

At T0 and T1, the intestine was removed from the abdominal cavity using sterile tools and digesta of the distal intestine were squeezed into 2 mL sterile collection tubes and flash-frozen on dry ice. Remaining digesta were removed by flushing the intestine with 5 mL of sterile PBS. Subsequently the intestine was longitudinally opened and mucus of the distal intestine was collected by scraping over the intestinal surface with a sterile spatula. Intestinal mucus samples, hereafter referred to as mucosa, were flash-frozen and all samples were stored at −80°C until DNA extraction.

At both sampling points T0 and T1 four fish per tank were pooled for the analysis of whole-body proximate composition.

Proximate composition of whole body and diets

The proximate composition was assessed in diets (Table 1) and whole-body homogenates in duplicate using identical procedures. The whole-body samples underwent freeze-drying (Alpha 1-2 LD plus and Alpha 1-4 LSC, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) until a consistent weight was achieved. Subsequently, the samples were homogenized with a knife mill (GM 200, Retsch GmbH, Haan, Germany). The analysis of nutrients and gross energy was performed following EU guideline (EC) 152/2009. The determination of dry matter content involved drying the samples at 103°C in a drying oven for 4 hours (ED 53, Binder GmbH, Tuttlingen, Germany). Ash content was established by subjecting the samples to combustion at 550°C (P300, Nabertherm, Lilienthal, Germany). The Kjeldahl method was used to assess crude protein content (InKjel 1225M, WD30, Behr, Düsseldorf, Germany). Crude lipids were extracted using petroleum ether within a Soxhlet extraction system (Soxtherm, Hydrotherm, Gerhardt Königswinter, Germany) and crude lipid

content was subsequently quantified gravimetrically. Lastly, gross energy was quantified using a bomb calorimeter (C 200, IKA, Staufen, Germany).

Plasma stress and health indicators

Plasma ions (Na^+, K^+, Cl^-) were determined potentiometrically using commercial dry chem slides on a Fuji Dry Chem NX500i (Fujifilm, Tokyo, Japan). Plasma glucose was determined on a Fuji Dry Chem NX500i (Fujifilm) using kits from the manufacturer. Free cortisol in plasma samples was measured using a commercial Enzyme-linked Immunosorbent Assay (ELISA) Kit (Demeditec Diagnostics GmbH, Kiel, Germany).

Protein quantification in liver and gill

Protein concentrations of catalase (Cat) and Cu, Zn superoxide dismutase (Sod1) were analysed in salmon liver and gills. The protein concentrations in the tissue samples were examined with SDS-PAGE and Western Blot. The cytoskeletal filament protein ß-actin served as the housekeeping protein for the loading control. A no template control (nuclease-free water) as well as a positive control per antibody (*Danio rerio* liver for Cat, bovine liver for Sod1 and human HEK-293 cells for ß-actin) were run (Figure S1). Tissue samples of four fish per tank were pooled for each organ and the total protein was extracted using radioimmunoprecipitation (RIPA) lysis buffer (RIPA Lysis Buffer System, Santa Cruz Biotechnology Inc., Dallas, TX, USA), according to the manufacturer's protocol. A concentration of 20 µg extracted total protein per pooled sample, adjusted to 13 µL with nuclease-free water, was incubated in reducing conditions with 5 µL 4x LDS sample buffer (mPAGE, EMD Millipore Corporation, Burlington, MA, USA) and 2 µL 1M 1,4-Dithiothreit at 70°C for 10 min. The SDS-PAGE was performed in an Xcell SureLock Mini-Cell (Thermo Fisher Scientific, Waltham, MA, USA) using precast 4 - 12 % Bis-Tris gradient gels and MOPS SDS running buffer (both mPAGE, EMD Millipore Corporation, Burlington, MA, USA). The electrophoresis was performed at 140V for 90 min. Afterwards, proteins were electro-transferred to a PVDF membrane in transfer buffer (mPAGE, EMD Millipore Corporation, Burlington, MA, USA) at 30V for 100 min. For parallel protein detection of Cat and Sod1 the membrane was dried at room temperature, horizontally cut just below the 35 kDa marker, reactivated with 100% methanol, washed and blocked for 90 min at room temperature with 150 rpm in PBS-T containing 5% skim milk powder. Primary antibody incubation, with Cat antibody (ab209721, Abcam, Cambridge, UK) and Sod1 antibody (NBP2-24915, Novus Biologicals, Bio-Techne Ltd., Abingdon, UK) both in 1:500 dilution in PBS-T containing 2.5% skim milk, was performed at 4°C at150 rpm overnight. Secondary antibody anti-rabbit IgG conjugated HRP (sc-2357, Santa Cruz Biotechnology Inc, Dallas, TX, USA)

was incubated in a 1:5000 dilution at 20°C for 90 min at 150 rpm. Detection was performed using ECL detection reagents (Amersham, Global Life Sciences Solutions Operations UK Ltd, Little Chalfont, UK) and chemiluminescence film (Amersham, GE Healthcare Ltd, UK) with exposure times of 5 min for Cat and 20 sec for Sod1. For the subsequent detection of ß-actin, the antibodies were stripped off the membrane using 100 mM Glycin buffer (pH 2.5) for 2 x 30 min at 20°C with 150 rpm shaking. Afterwards, the membrane was incubated in a 1:5000 dilution of ß-actin antibody (NB600-503, Novus Biologicals, Bio-Techne Ltd.) at 4°C with 150 rpm overnight. Quantification of protein abundance was done by densitometric analysis of the protein bands using GIMP and normalized to housekeeping protein abundance.

Microbiota in digesta and mucosa: DNA extraction, sequencing and bioinformatic analysis

To evaluate the digesta and mucosa-associated bacterial communities of the intestine of individual fish, DNA from the digesta and mucus samples was extracted using approximately 200 mg of digesta/mucus sample as input for the QIAmp Fast DNA Stool Mini Kit (Qiagen, Germany, Cat. No. 51604) and QIAmp DNA Microbiome (Qiagen, Germany, Cat. No. 51704), respectively. Digesta material was transferred to 0.70 mm Garnet Bead tubes prefilled with 1 mL InhibitEx lysis buffer and bead beating was performed 2 x 45 sec using a bead mill homogenizer (Fisherbrand™, Thermo Fisher Scientific, Waltham, Massachusetts, US). In addition, samples were incubated at 95°C for 5 min and subsequently centrifuged for 3 min at 20000 rcf. The following DNA extraction using the supernatant was conducted according to the manufacturer's instructions. Mucosa material was extracted according to manufacturer's instructions with no manual pre-processing of the samples. To exclude and verify potential contamination during DNA extraction, preparation blanks were included in the extraction process. For sequencing, all DNA samples were amplified by PCR targeting the 16S rRNA V3–V4 (341 F 'CCTACGGGAGGCAGCAG' and 805R 'GGACTACHVGGGTWTCTAAT' [37]. Multiplexed samples were finally loaded on an Illumina MiSeq v3 platform 2x300 bp. A detailed description of PCR cycling conditions and sequencing has been published previously [38,39].

The 16S rRNA gene sequences were analysed using the Linux-based version of Quantitative Insights Into Microbial Ecology 2 (Qiime2, v.2021.2.0; Bolyen et al., 2019). Leftover primers and spacers were removed from sequencing reads using Cutadapt and filtering for low-quality reads and chimeras as well as merging of paired-end reads was conducted using DADA2 [41] with truncation parameters of 277 and 221 for forward and reverse reads, respectively, and truncation quality cut-off of 2. ASVs with a frequency of >25 and an occurrence in at least two

samples were retained for taxonomic determination using the SILVA reference database version 138.1 [42]. Based on the taxonomic information, only ASVs that are of bacterial origin and have been identified at least up to the phylum level were kept for downstream analysis. In addition, ASVs from *Cyanobacteria* and mitochondrial origin were excluded from the dataset. To investigate the metabolic activity, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) was utilized to assess the functional profiles of the gut microbial community in Atlantic salmon, focusing on the composition of the 16S rRNA gene [43]. Metabolic potential of bacterial communities was inferred from the MetaCyc database.

Gene expression in the head kidney and gill

After extracting the total RNA with TRIzol (Thermo Fisher Scientific), the ISOLATE II RNA Micro Kit (Meridian Bioscience Inc., Cincinnati, Ohio, USA) was used for its purification. The concentration and quality of isolated RNA was measured using NanoDrop One (Thermo Fisher Scientific). Reverse transcription of high-quality RNA into cDNA was then performed with the Reverse Transcription Master Mix (Standard BioTools, South San Francisco, California, USA). Fluidigm PreAmp Master Mix was used to preamplify cDNA samples, followed by a treatment with exonuclease I (New England BioLabs, Frankfurt/Main, Germany). The manufacturer's instructions were followed for all procedures.

Salmon's immunocompetence was evaluated by profiling the expression of genes involved in key immunological and stress-related pathways [44]. The selected set of genes and their corresponding primers, accession codes and further details are described in Table S1. Multiplex quantitative Real-Time PCR (qPCR) was carried out on 48.48 Gene Expression biochips, which were initially primed in the MX IFC Controller (Standard BioTools). The inlets for samples and assays were filled with pre-amplified cDNA samples and primers. Then, following the manufacturer's thermal protocol "GE Fast 48 × 48 PCR+Melt v2.pcl" (application type: gene expression; passive reference: ROX; assay: single probe), Biomark HD was used to determine the concentrations of selected transcripts. Fluidigm real-time PCR analysis software v3.0.2 (Standard BioTools) was used to obtain the raw Cq data and with this, the relative expression of the target genes was calculated by the ΔCt method. To normalize the results, three reference genes encoding β-actin (*actb*), ribosomal protein L4 (*rpl4*) and ribosomal protein S20 (*rps20*) were utilized. The mean Cq for all samples of every gene per organ served as a calibrator during the calculations. The fold changes were calculated per gene by dividing the relative expression after PAA treatment of a diet group by the relative expression before PAA treatment of the

same diet group. The log2-transformed values of the calculated fold changes were used to construct the corresponding heatmaps.

Statistical analysis

Statistical analysis and data visualization were performed in R (version 4.3.1; R Core Team 2021, Vienna, Austria) within the environment R studio. The significance threshold was set at $\alpha = 0.05$. Models based on generalized least squares (gls) were used to evaluate the effect of the diet on the production parameters (e.g. growth performance) and protein abundance in the gill and liver, which did not include a random (tank) effect. Models for protein abundance (per organ) included the factor diet and timepoint. For alpha diversity parameters, Observed ASVs and Shannon diversity, within the respected sample type (digesta and intestinal mucus), a linear-mixed effect model (lme; [45]) with tank as a random factor was used for statistical evaluation. Residuals were assumed to be approximately normal distributed and heteroscedastic based on graphical residual analysis. Following a pseudo R^2 calculation, [46] a two-way ANOVA was calculated with diet as the main factor. For beta diversity, permutational multivariate analysis of variance (PERMANOVA) with Bray-Curtis distance as input was conducted using the *adonis2* function from the *vegan* package [47] to test for statistical differences in the community composition according to the factor diet. Pairwise comparisons were conducted using a pairwise PERMANOVA. In addition, multivariate homogeneity of group dispersion was tested with factor diet using *betadisper* function from *vegan*. To infer key metabolic microbial pathways which were enriched in a specific diet PICRUSt analysis was performed, comparing enriched pathways between fish fed CV14 and CD in both the digesta and mucosa [43].

Mixed effect models (lme), which included a random tank effect, were further employed to evaluate the effect of diet and timepoint (before vs. after stress) on health indicators including plasma stress indicators as well as gene expression in the head kidney and gill. Relative gene expression values obtained from the multigene expression assays were log2 transformed before statistical analysis. Evaluation of the models followed the procedure described above.

Ethics statement

The experiment has been approved by the Ministry of Agriculture, Rural Areas, European Affairs and Consumer Protection (MLLEV, Kiel, Germany) under project number V 244 – 86776/2021). All guidelines of the EU Directive 2010/63/EU for animal experiments and the national regulations for animal welfare (TierSchVersV) were followed. The experiment was conducted in compliance with the ARRIVE guidelines.

3 Results

Fish performance and proximate body composition

FCR was significantly affected by the diet (*F(3,8) = 10.36, p = 0.004*) and increased FCR in fish fed CV14 resulted in a lowered SGR for this group (Table 2). FCR and SGR did not differ among the other diet treatments. PER was significantly influenced by the diet F*(3,8) = 10.74, p = 0.004*, where CV14 had a significantly lower PER than CV2 and CV14w. The diet furthermore influenced PRE (*F(3,8) = 6.81, p = 0.014*) and feeding CV2 and CV14w improved PRE compared to the control and CV14 group. Fish fed CV14 showed significantly higher whole-body ash content than all other treatments (*F(3,8) = 7.65, p = 0.01*) and exhibited the lowest energy, fat and protein content among all diets, albeit not significant (Table 2). Condition factor, hepatosomatic and spleen somatic index were not influenced by the diet (Table 2).

Intestinal microbiota in response to the diet

A total of 50 digesta and 51 mucosa samples (including initial samples) were obtained after downstream processing. The average read count for digesta samples was 19850 reads per sample, while it was 3660 reads per sample for mucosa samples. Alpha-diversity (observed and Shannon Diversity) was higher in digesta than in mucosa samples (Wilcoxon, $p<0.001$; Figure 1A,B) but in both sample types, not influenced by the diet (Figure 1A,B). Beta-diversity, displayed as phylogenetically weighed Bray-Curtis distances using non-metric multi-dimensional scaling (NMDS) indicated a structure based on diet in both digesta (Figure 1C) and mucosa samples (Figure 1D). Permutational analysis of variance (PERMANOVA) revealed for both sample types a significant effect of diet ($p = 0.001$; Table S2). Beta diversity in the digesta of fish fed CV14 was significantly different from CD and CV14w ($p = 0.001$; Table S2). Similarly, beta diversity in the mucosa of fish fed CV14 was significantly different to fish fed CD and CV14w ($p = 0.001$), as well as to CV2 ($p < 0.05$). Furthermore, microbial beta diversity in both the digesta and mucosa was significantly different between initial samples and samples taken after feeding the experimental diets (Figure S2; PERMANOVA, $p = 0.001$ for digesta and $p = 0.003$ for mucosa).

The bacterial community structure of the digesta was comprised of multiple taxa and no single taxa dominated overall (Figure 2A). However, phyla that were most abundant in the digesta were *Firmicutes*, *Proteobacteria* and *Actinobacteria* (Figure S3). On the genus level, the most abundant taxa in the digesta were *Floricoccus* (21.8 % relative abundance), *Vibrio* (19 %), *Lactococuccus* (12.1 %) and *Streptococccus* (7 %; Figure 2A).

In the digesta, several taxa were enriched in fish fed CV14 compared to the control (CD) diet (Figure 2C). These included *Microbacterium* ($p < 0.001$) and *Neochlamydia* ($p < 0.001$), as well as *Paenarthrobacter* and *Trichococcus* (Figure 2C) which were only present in CV2 and CV14. In contrast, *Peptostreptococcus* ($p = 0.03$), *Weissella* ($p < 0.001$), *Corynebacterium* ($p = 0.03$), *Romboutsia* ($p = 0.008$) and *Lactobacillus* ($p = 0.006$) were reduced in fish fed CV14 compared to CD. Linear discriminant analysis of effect size (LEfSe) analysis furthermore revealed that *Paenarthrobacter* was significantly associated with the CV14 diet (LDA score > 4.0), whereas *Vibrio* was associated with the diet CV2 and *Lactobacillus* with the control diet (CD; Figure 2B).

Similar to the digesta, the majority of bacteria in the intestinal mucosa belong to either *Firmicutes*, *Proteobacteria* or *Actinobacteria* (Figure S4). The genus *Vibrio* (15 %) and *Floricoccus* (15.6 %) were the two most abundant across all diets in the mucosa (Figure 3A). Interestingly, we did not detect the genus *Mycoplasma* in the mucosa samples. *Mycoplasma* was also not detected in initial samples of intestinal mucosa and digesta, during which the fish received a standard commercial diet (Figure S5.)

In the mucosa *Neochlamydia*, *Paenarthrobacter* and *Trichococcus* were enriched in fish fed CV14 compared to CD, where they were not detected (Figure 3A), while *Floricoccus* was reduced ($p = 0.025$). These results were furthermore reflected in the LEfSe analysis, which revealed that the same genera were associated with a specific diet as found for the digesta (Figure 3B).

A total of 152 genera were identified as core microbiota shared between digesta and mucosa samples of the intestine (Figure S6). These included *Vibrio*, *Floricoccus*, *Streptococcus* and *Lactococcus*.

To gain a deeper understanding of potential microbial pathways that were enriched in fish fed CV14 as compared to CD, we conducted PICRUSt analysis based on MetaCyc metabolic pathway information for microbiota in the digesta and mucosa. Several predicted microbial pathways were enriched in the digesta of fish fed CV14 compared to CD (Figure S7). These included the pathways aerobic respiration (cytochrome c), TCA cycle and glucose, xylose and inositol degradation (Figure S7). In the mucosa pathways associated with nitrogen metabolism such as purine degradation and adenine salvage, as well as biosynthesis pathways of adenosine and guanosine, were enriched in fish fed CD (Figure S8). Pathways associated with aerobic respiration I and TCA cycle were particularly enriched in fish fed CV14.

Response to oxidative stress

Plasma stress indicators (ions, glucose, cortisol)

The stress response was investigated regarding classical blood plasma indicators such as glucose and cortisol, as well as the primary ions of the electrolytes sodium (Na^+), potassium (K^+) and chloride (Cl^-). Glucose levels were significantly altered across timepoints ($F(2, 124) = 11.36$, $p < 0.001$) and slightly increased one hour after the introduction of PAA (T2) as observed for all treatments with no diet-specific effects (Figure 4A). After 18 hours (T3), the glucose concentrations returned to their initial values. Cortisol increased following exposure to PAA ($p < 0.001$) and was not different among diets 1 h after stress exposure (Figure 4B). Sodium and chloride concentrations remained unchanged in the plasma 1 h after introduction of PAA (Figure 4C,D).

Protein concentration in response to diet and stress

Catalase protein concentrations in the liver increased by 3-fold (Tukey; $p < 0.001$; Figure 5A) 18 h after exposure to PAA in CD and CV14w groups. In fish fed CV14, this increase was only modest (Tukey, $p = 0.18$). In the gills, no major changes occurred, except for fish fed CV2 where Cat increased significantly after PAA exposure (Tukey; $p < 0.01$; Figure 5B) and was significantly higher compared to CV14w (Tukey; $p = 0.047$).

The concentration of Sod1 in the liver was 50% lower in CV14w compared to CV2 before stress (Tukey; $p = 0.034$; Figure 5C) and decreased by 50% in CV14 ($p = 0.001$) and 25% in CV2 ($p = 0.045$) after stress exposure. Sod1 protein concentrations in the gill were slightly increased before stress in a dose-dependent manner, albeit not significant. Sod1 levels were reduced after stress exposure in all groups and most strongly in CV2 (Tukey; $p = 0.045$; Figure 5D) and CV14 ($p = 0.0014$).

Gene expression in head kidney and gill related to the stressor PAA

When comparing immune- and stress-regulated gene expression, no significant differences between the different diets within one timepoint nor an overall diet effect was detected (Table S3, S4). The only exception is an overall diet effect for *ier2-2*, with significantly induced transcript levels ($p = 0.04$) in the head kidney of fish fed CV14w compared to fish fed CD (Table S3). Overall, PAA treatment affected the expression of a large proportion of selected genes involved in different immune- and stress-related pathways (17/44 genes in the head kidney and 24/44 genes in the gill), as indicated by a significant timepoint effect (Table S3, S4). Additionally, significant interactions in the model indicate that the diet influenced the response to the oxidative stressor in both head kidney and gill (Table S3, S4).

In the head kidney of fish fed CD, the oxidative stressor PAA had only a minor effect on immune- and stress-related gene expression. 18 hours after PAA exposure, the transcript levels of five genes (*hspa5, ier2-2, ifn2a, il18* and *cd28*) were slightly (-0.30 to -0.55 log2 fold) but significantly reduced ($p < 0.05$). Additionally, a stronger reduction (-1.85 log2 fold) was observed for *ccl4* transcript numbers ($p < 0.05$). In comparison to the head kidney, exposure to PAA affected stress- and immune-related gene expression in the gill of fish fed CD more substantially. Transcript levels of 14 immune- and stress-related genes were enhanced in the gill ($p < 0.05$) including *arg2* (0.58 log2 fold), *b2m* (0.63 log2 fold), *c1ql2* (0.91 log2 fold), *c4b* (0.62 log2 fold), *cat* (0.35 log2 fold), *cd4* (0.43 log2 fold), *cebpb* (0.73 log2 fold), *clra* (1.02 log2 fold), *drtp1* (0.78 log2 fold), *ier2-2* (0.77 log2 fold), *ifit5* (0.79 log2 fold), *ikba* (0.81 log2 fold), *il18* (0.51 log2 fold) and *tnfrsf14* (0.99 log2 fold).

Diets supplemented with *Chlorella vulgaris* had only a modest effect on the PAA-induced expression modulation of selected genes. In the head kidney of fish fed CV14, the transcript concentrations of seven genes were significantly reduced after PAA exposure, including *arg2* (-0.46 log2 fold), *fcgr1a* (-0.57 log2 fold), *hspa5* (-0.65 log2 fold), *ifit5* (-0.80 log2 fold), *il18* (-0.32 log2 fold), *mpo* (-0.35 log2 fold) and *tnfrsf14* (-0.58 log2 fold) ($p < 0.05$). Fish fed with CV14w had -0.33 log2 fold reduced transcript levels of *il18* and -0.68 log2 fold reduced transcript levels of *sod1* ($p < 0.05$) after occurrence of the oxidative stressor. None of the selected genes was significantly induced in the head kidney of salmon from both diet groups upon PAA exposure.

In the gill of fish fed with CV14 and CV14w, the expression of selected genes was modulated only slightly after PAA treatment. In gill of fish fed CV14, the transcript levels of eight genes were significantly induced after the oxidative stressor, including *b2m* (0.82 log2 fold), *cat* (0.32 log2 fold), *cebpb* (0.47 log2 fold), *clra* (0.63 log2 fold), *drtp1* (1.37 log2 fold), *ikba* (0.90 log2 fold), *lao1* (0.68 log2 fold) and *marco* (1.76 log2 fold) ($p < 0.05$). Only *hsp1a1* level was significantly reduced by -0.58 log2 fold after PAA exposure in fish fed with CV14 ($p < 0.05$). In the gill of fish fed with CV14w, only *rsad2* level was significantly induced by 0.66 log2 fold and none of the selected genes were reduced after the oxidative stressor.

4 Discussion

In this study we show that the amount of microalgae *Chlorella vulgaris* included in a functional diet as well as the mode of application differently affects growth, the intestinal microbiota and response to an acute oxidative stressor in Atlantic salmon. Our data sheds new light on how a

functional diet enriched with microalgae contributes to safeguarding health of Atlantic salmon under challenging conditions in recirculating aquaculture systems.

Feed conversion is impaired at high Chlorella inclusion

Feeding a diet with 14% *C. vulgaris* daily to Atlantic salmon post-smolts impaired growth via increasing feed conversion efficiency in our study. Contrasting results were reported for diets containing 10 – 20% *C. vulgaris* that improved feed conversion efficiency of gilthead seabream [48], African catfish [49] and Nile tilapia [50]. However, in rainbow trout, inclusion rates of 5 – 10% of *Chlorella sorokiniana* did not change feed conversion [51], which seems to be related to the anatomy and function of the digestive system. Omnivorous and herbivorous fish species are able to digest plant-based material, including complex carbohydrates, much more efficiently than carnivorous fish. Salmon that received a diet containing only 2% *C. vulgaris* daily or 14% once weekly, however, showed no tendency towards reduced feed conversion and protein retention efficiency was even improved. Hence, a threshold in the microalgae inclusion rate beyond which potential positive effects turn into negative effects that reduce growth of Atlantic salmon seem to exist. This was further reflected in the proximate body composition, which in line with our previous findings with an inclusion of 8% *C. vulgaris* [22], the inclusion of 14% *C. vulgaris* in this study also increased whole-body ash content. A likely explanation for this phenomenon could be an increased bone-to-flesh ratio, as growth was also impaired.

Microbiota in digesta and mucosa are modulated in response to dietary Chlorella

The intestinal microbiome plays an important role in nutrient digestion and was generally found in fish to be less complex than in mammals, dominated by often very few taxa (Wang et al., 2018). In case of salmonids, these belong to the phyla *Fusobacteria*, *Proteobacteria* and *Firmicutes* [26,27,53]. The two habitats within the intestine, namely digesta and mucosa, harbour distinct microbial communities. In line with what has been found earlier, our results confirm a significantly lower relative abundance and diversity of bacteria residing within the mucosa as compared to bacteria associated with the digesta of fish [34,54]. However, 152 genera were identified as core microbiota shared between digesta and mucosa samples, less than the sum of unique genera of both tissues. This contrasts previous studies in rainbow trout [39] and Atlantic salmon [55] which have revealed a smaller core microbiota and less similar microbiota composition among digesta and mucosa. We can only speculate why we found a generally larger overlap between the microbial composition of digesta and mucosa, but it might be related to the type of experimental feed used in the trial. While almost all other studies in salmonids

have used extruded feeds, we used pelletized feeds in our study, which generally differ in their water stability and thus retention time within the intestine compared to extruded feeds. Hence, the physical properties of the digesta could have resulted in a microenvironment, that was overall more similar to that of the mucosa when comparing it to that of extruded feeds. The physical aspect of fish feeds should be considered when investigating dietary effects on the microbiome in future studies.

At the phylum level, the dominance of bacteria belonging to *Firmicutes, Proteobacteria* and *Actinobacteria* is in line with what has been previously found in the intestine of salmonids [29,54,56]. Strikingly, *Mycoplasma*, a bacterium that has been observed to be particularly enriched in the intestinal mucosa of salmonids and thought to be tightly linked to host-health [25,57] was not detected in either the mucosa nor in digesta samples. Our results on the microbial composition of digesta and mucosa at the start of the experiment furthermore confirm that even before the application of the experimental feeds and while the fish were fed a standard commercial type diet, the *Mycoplasma* genus was not present in the intestine of the salmon. It has been found that the abundance of *Mycoplasma* in the intestine of Atlantic salmon is life-stage dependent and increases later in life in seawater, while it was absent in fish held in freshwater RAS [58]. Colonization of the intestine with the genus *Mycoplasma* was found to be dependent on the host environment [59] and the absence of *Mycoplasma* in this study suggest that fish haven't been exposed to *Mycoplasma* before. As the salmon were sourced from a fully closed RAS facility importing sterilized eggs, it seems likely that the fish haven't been exposed and colonized by *Mycoplasma* earlier in life. The overall microbial composition in both digesta and mucosa seems to be more complex than reported in many other studies, however, care must be undertaken in these comparisons, as different DNA extraction, sampling- and sequencing methods introduce different biases.

Diet is a main factor shaping the intestinal microbiota in fish and mammals, as its composition selects for a particular microbial community. A particular function of the microbiota associated with the digesta is the conversion of complex (indigestible) carbohydrates into short-chain fatty acids. The cell wall of *C. vulgaris* is built up of a multitude of complex cellulose- and chitin-like carbohydrates [60] and when included in fish diets, can provide a specific substrate for bacteria able to breakdown such complex carbohydrates. Interestingly, 14% inclusion of *Chlorella vulgaris* in this study increased the relative abundance of *Trichococcus* in the digesta and metabolic pathway prediction based on the microbial composition revealed an enrichment of carbohydrate degradation pathways for fish fed this diet. *Trichococcus* species are known

for their ability to utilize various carbohydrate sources including cellobiose, sucrose and glucose [61–64] and their presence is likely related to the availability of complex carbohydrates. Although it could generally be beneficial if the bacterial metabolites from the degradation process are available for the fish, in many cases, these are used as energy substrates by the bacteria themselves. This was likely the case in our study, as feed conversion efficiency in fish fed 14% *C. vulgaris* was impaired. The genus *Paenarthrobacter* was furthermore particularly associated with fish fed *C. vulgaris* in the digesta and mucosa but absent in fish fed the control diet. Although this genus was found to exhibit metabolic capacity to degrade herbicides [65] and iprodione [66] it seems unlikely that pesticide contents in the microalgae are the reason for their occurrence. The microalgae were analysed for a variety of pesticides and cultured in closed bioreactors, minimizing the risk of contamination. It seems more likely that the microalgae provide a specific yet unknown substrate, which is preferably used by *Paenarthrobacter*. Interestingly, *Paenarthrobacter* was found to be associated with growth and carotenoid production in the microalgae *Haematococcus lacustris* [67] and thus carotenoids from *C. vulgaris* might explain why *Paenarthrobacter* was found exclusively in fish fed these diets. *Lactobacillus*, which is considered a probiotic in fish, was reduced in the digesta of fish fed 14% *C. vulgaris* and slightly reduced in fish fed 2% *C. vulgaris*. *Lactobacillus* was found to protect against the development of bacterial infection in various fish species [68–70]. However, the relative abundance of lactic acid bacteria in the intestine of Atlantic salmon has been found to increase in diets containing fermented soybean meal [71]. During diet formulation for this experiment, inclusion of *C. vulgaris* was done in exchange for soy protein, which could explain the reduced abundance of lactic acid bacteria and thus reduced abundance of *Lactobacillus* may not be detrimental to host health but rather a consequence of a slightly different diet composition.

Response to acute oxidative stress is slightly modulated by functional diets

Stress activates the release of stress hormones such as glucocorticoids, which then trigger physiological changes on tissue level, allowing fish to cope with the stressor [72,73]. Acute stress affects the immune response mainly by inducing distinct innate immune pathways and suppressing components of the adaptive immune response. This crosstalk of stress and immune pathways is reflected in challenged teleost by the activation of Th1 immune response, including pro-inflammatory cytokines and increased numbers of neutrophils and granulocytes and concomitantly, less lymphocytes in blood [74,75].

In previous studies, the oxidative stressor PAA triggered a systemic stress response, which did not lead to chronic manifestations due to an adaptive response of the salmon [13,76]. We also observed a systemic stress response in the present study, reflected by an elevation of cortisol and glucose in the plasma of all diet groups one hour after PAA treatment with a subsequent return to initial values of glucose after 18 hours. As an oxidative biocide, PAA induces ROS production, which decreased in previous studies the activity of the antioxidative enzymes Sod1 and Cat in both the liver and gill of grass carp [77] and in the muscle, liver and heart tissues of rainbow trout [78]. In our study, PAA treatment increased Cat abundance in the liver. The liver is the main detoxifying and metabolizing organ [79,80] and it is plausible that ROS scavenging was enhanced by Cat-generated antioxidants.

The head kidney consists of endocrine, hematopoietic and immune cell populations and is central in the systemic immune and stress response [81,82]. The gill is a key mucosal organ that plays a crucial role in sensing stressors and potential pathogenic threats [83]. Independent of the different diet groups, PAA as an oxidative stressor significantly affected the expression of many genes, indicating that PAA *per se* has an effect on the immune and stress response in Atlantic salmon. However, to assess the mitigation potential of the different diets, in this study we focused on the effect of the diet on the immune and stress response.

In our study, marker genes indicating the activation of Th1-directed immunity (*il18*; interleukin 18, *ccl4*; chemokine C-C motif ligands 4*)* and the presence of T cells (*cd28*; cluster of differentiation 28) were significantly reduced in the head kidney of fish fed CD, indicating the migration of immune cells, including APCs and CD28-positive T cells, from the head kidney to the periphery. In the gill, *il18* and *cd4* (cluster of differentiation 4) were induced, which suggests the immigration of immune cells, specifically (CD4-positive) T cells, to the gill. This is consistent with Gomez et al. (2013), where in mucosal tissues such as the gill, large numbers of CD4- and CD28-positive T cells were found. We assume that a slight innate immune reaction occurred in the gill of fish fed CD upon PAA exposure, based on our observation of the induced expression of genes coding for receptors such as Clra (c-type lectin receptor A) and Tnfrsf14 (TNF receptor superfamily member 14). The pathogen receptor encoded by *clra* binds lectins, which are carbohydrate-binding proteins recognizing pathogens [85]. The TNFRSF14 protein is expressed on different cells and upon binding with its ligand, activates the NF-κB pathway, resulting in expression of cell survival genes [86] and manages T-cell immune responses [87], suggesting a migration of myeloid cells to the gill. Furthermore, downstream-signalling genes such as *cebpb* and *ikba* were induced in the gill after PAA exposure. *Cebpb* encodes the

transcription factor CCAAT/enhancer-binding protein β, which regulates cell cycle, hematopoiesis and immunity [88] and *ikba* (encoding for NF-κB inhibitor α) controls the NF-κB transcription factor, which is an important driver of immune and stress responses in salmonid fish [89]. Moreover, in line with the findings of Carletto et al. (2022), the transcript levels of *cat* (catalase) were enhanced in the gill, suggesting a counteracting reaction in response to PAA. Stress-related genes (*drtp1;* differentially regulated trout protein 1, *ier2*-2; immediate early response 2-2) were increased in the gill, whereas in the head kidney, *ier2-2* and *hspa5* (heat shock protein family A (Hsp70) member 5) were reduced after PAA exposure. The exact functions of *drtp1* and *ier2-2* are not yet fully understood [90], but like *hspa5,* they seem to respond to external stimuli such as stress. This implies that PAA primarily activates a local stress response in the gill, which also activates mechanisms in the head kidney of fish fed CD.

Overall, our gene expression data indicate the exit of immune cells from the head kidney to the mucosal tissue in the gill where a local immune and stress response has been activated by PAA treatment in fish fed CD. As a consequence of exposure to the chemical oxidant PAA, an increase in antioxidants and proteins to repair damaged tissue was anticipated. However, a strong immune response was not expected due to the absence of visual injuries at the gill of salmon, and in addition, the fish were maintained in a protected RAS environment.

One main aspect of this study was to assess the mitigation potential of *C. vulgaris* on the effects of acute oxidative stress in salmon. Antioxidant activity reflected by catalase was significantly enhanced after oxidative stress in liver of fish fed CV2 and CV14w and in the gills of fish fed CV2. In both the liver and gill, *Chlorella vulgaris* dose-dependently increased the anti-oxidative enzyme Sod1 before PAA exposure. Interestingly, after PAA exposure, comparable Sod1 levels are measured in all treatments, with significantly reduced Sod1 levels in both liver and gill of fish fed CV2 and CV14. Possibly the excessive release of superoxide radicals by PAA treatment lowers Sod1 concentration to basal levels that are the same for each treatment group, and *C. vulgaris* cannot exert an inducing effect on Sod1 activity. The enzymes Sod1 and Cat are closely related to each other, in which Sod1 is the first line of defense against ROS and catalyzes superoxide anions $O_2^{\bullet-}$ to H_2O_2 and O_2. Next, Cat catalyzes H_2O_2 to harmless molecules H_2O and O_2 [91]. Interestingly, in this study, Sod1 concentration was stable or decreased, while Cat concentration in some treatment groups increased after PAA treatment. Since the used PAA solution contains 12% H_2O_2, it may induce specifically Cat to convert the excess of H_2O_2.

PAA treatment affected the immune and stress response of fish fed CV14, which was assessed by a significantly differential expression of several selected genes. In the head kidney, we

profiled a reduced expression of genes involved in Th1-focused immunity (*il18*) and stress response (*hspa5*). Since a decreased expression after PAA treatment was observed for *il18* in all diet groups, and for *hspa5* in fish fed with CD and CV14, this reduction appears to be primarily driven by the oxidative stressor. Furthermore, PAA treatment suppressed in fish fed with CV14 the expression of genes involved in immune functions, namely T-cell immune responses (*tnfrsf14*), binding of host antibodies presenting, for example, antigens (*fcgr1a*; Fc gamma receptor Ia), antiviral defense (*ifit5*), anti-inflammatory response (*arg2*) and anti-microbial defense (*mpo;* myeloperoxidase). The reduction of these gene products suggests diminished immune protection in the head kidney. In the gill of fish fed CV14, PAA induced the expression of genes associated with stress response (*drtp1*), pathogen recognition (*clra*), antioxidant activity (*cat*), antigen presentation (*b2m*) and regulation of gene transcription (*ikba, cebpb).* Since the same effect was observed in fish fed CD, this induced expression is probably caused by the oxidative stressor and not strongly altered by *Chlorella* supplementation. However, *marco* and *lao1* (l-amino acid oxidase 1) were also significantly enhanced in fish fed with CV14 after PAA exposure. As *marco* encodes a pathogen recognition receptor and *lao1* encodes an enzyme with antibacterial and antiparasitic activity, the induction of these genes could be due to the bioactive and immune-modulating properties of *Chlorella vulgaris*. *Chlorella vulgaris* has been reported recently to exert antibacterial activity, which has been attributed to its polysaccharides, chlorophyllin, and lipid content [92–94]. Interestingly, we also detected in the gill reduced transcript level of *hsp1a1* (Heat shock 70 kDa protein) and an increase in *drtp1* expression after PAA treatment, while both genes are involved in the stress response [95].

While differential expression of several selected immune- and stress-related genes was observed in fish fed CD and CV14, exposure to PAA had negligible effects on the expression of selected genes in fish fed CV14w. In the head kidney, next to a reduction of *il18* expression, PAA reduced the expression of *sod1*, suggesting a decreased antioxidant activity. In the gill, only the antiviral-related gene *rsad2* was significantly enhanced upon PAA exposure in fish fed CV14w. Furthermore, independently of the oxidative stressor, induced transcript levels of *ier2-2* were measured in fish fed CV14w compared to fish fed CD, indicating an activated immediate early response.

On the transcriptional level, in fish fed the control diet and a diet enriched with 14% *C. vulgaris*, an immune and stress response upon exposure to PAA was apparent, whereas in fish receiving 14% *C. vulgaris* once weekly, these markers did not show an induced immune and

stress response. Due to a comparable growth performance and observed primary adaptive stress response in all dietary groups, it does not appear that fish fed CV14w have an impaired immune and stress status, but rather an enhanced resilience against PAA. Further, due to the limited impact of PAA as a stressor, *C. vulgaris* may not reveal its full dietary potential on the immune and stress status of the fish. Overall, this study revealed that the health of Atlantic salmon can be influenced by dietary application of *Chlorella vulgaris* but the intestinal microbiota and stress resilience are influenced differently by both inclusion level and mode of application.

Data availability statement

The raw sequences that support the findings of this study were deposited in the sequence read archive (SRA) database from NCBI under the BioProject PRJNA1083322.

Ethics statement

The experiment was approved by the animal welfare officer of the "Fraunhofer IMTE Büsum" and the local authority of Schleswig-Holstein, according to the German animal welfare law (V 244 – 86776/2021).

Authors contribution

CS and TG conceived the project and together with EM secured funding. JM designed the experiment with input from CS and HS. JM and JE performed the experiment. JM, JE, DvM, MW and TS conducted laboratory work and acquired data. SH and CB took care of the microbiota extraction and sequencing. JM, MS and DvM analysed the data, performed statistical evaluation and prepared figures, supervised by AR, HS and SH. JM and DvM wrote the manuscript with input from all co-authors. All authors read and approved the final version of the submitted manuscript.

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930 **Tables**

931 Table 1. Feed formulation and proximate composition of experimental feeds in g/100g dry
932 matter (DM).

Ingredients (g/100g DM)	**CD**	**CV2**	**CV14**
Fish meal[1]	15	15	15
Microalgae	0	2	14
Blood meal[2]	6	6	6
Gelatine[3]	5	5	5
Pea protein isolate[4]	6	6	6
Soy protein concentrate[5]	18	18	10.65
Wheat gluten[6]	11.75	10.50	8.60
Wheat starch[6]	15	14.45	13
Canola oil[7]	7	7	7
Fish oil[1]	5	5	5
Palm oil	7	7	6.3
Biolysine	0.6	0.6	0.5
Methionine[8]	0.15	0.15	0.15
Vitamin & mineral premix[4]	0.5	0.5	0.5
$CaHPO_4$[9]	2	2	2
Filler[10]	1.0	0.8	0.3
Chemical comp. (g/ 100g DM)			
Dry matter			
Crude protein	49.8	49.9	50.1
Lipid	22.4	22.4	22.6
Ash	6.4	6.7	7.4
Crude energy (MJ/kg)	24.2	24.2	24.2

933 [1] Bioceval GmBH & Co. KG, Cuxhaven; Germany; [2] Saria SE & Co. KG, Selm, Germany; [3] Gustav Ehlert
934 GmbH & Co. KG, Verl, Germany; [4] Emsland-Aller Aqua GmbH, Golßen, Germany; [5] EURODUNA Rohstoffe
935 GmbH, Barmstedt, Germany, [6] Kröner-Stärke GmbH, Ibbenbüren, Germany; [7] Cargill GmbH, Riesa, Germany; [8]
936 Evonik Industries AG, Esssen, Germany, [9] Lehmann & Voss & Co. KG, Hamburg, Germany [10] Mikro-Technik
937 GmbH & Co. KG, Bürgstadt am Main, Germany

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939

940 Table 2. Growth performance, organ indices and proximate composition (OS) of Atlantic
941 salmon after eight weeks of feeding the experimental diets.

	CD	**CV2**	**CV14w**	**CV14**	**ANOVA**
IBW [g]	125.47 ± 3.56	125.97 ± 5.41	127.25 ± 2.19	125.73 ± 3.46	ns
FBW [g]	199.70 ± 4.09	202.65 ± 9.79	205.73 ± 5.14	190.99 ± 4.03	ns
WG [g]	74.23 ± 1.72	76.69 ± 4.41	78.48 ± 4.79	65.26 ± 0.65	ns
SGR	0.83 ± 0.02^{a}	0.85 ± 0.01^{a}	0.86 ± 0.04^{a}	0.75 ± 0.01^{b}	0.003
FCR	1.24 ± 0.04^{b}	1.21 ± 0.02^{b}	1.21 ± 0.07^{b}	1.39 ± 0.03^{a}	0.004
PER	1.75 ± 0.06^{ab}	1.87 ± 0.03^{a}	1.88 ± 0.11^{a}	1.61 ± 0.04^{b}	0.004
PRE	32.52 ± 0.81^{ab}	34.25 ± 1.01^{a}	35.13 ± 2.32^{a}	30.14 ± 1.22^{b}	0.014
CF	0.93 ± 0.07	0.89 ± 0.05	0.89 ± 0.04	0.89 ± 0.05	ns
HSI [%]	1.30 ± 0.13	1.21 ± 0.09	1.24 ± 0.09	1.29± 0.08	ns
SSI [%]	0.10 ± 0.04	0.08 ± 0.02	0.10 ± 0.02	0.09 ± 0.02	ns
Ash	6.47 ± 0.13^{b}	6.65 ± 0.34^{b}	6.74 ± 0.2^{b}	7.52 ± 0.15^{a}	0.01
Crude protein	59.50 ± 0.33	59.35 ± 1.54	60.48 ± 0.59	60.95 ± 1.17	ns
Crude lipid	34.86 ± 0.38	35.52 ± 1.56	33.87 ± 1.2	33.42 ± 0.95	ns
Energy [MJ/kg]	27.37 ± 0.08	27.42 ± 0.46	27.24 ± 0.19	26.97 ± 0.34	ns

942
943 Data is displayed as mean ± SD, with n = 3 tanks per treatment for performance parameters and proximate body
944 composition and n = 12 individuals for organ specific indices. A significant difference ($p < 0.05$) among the
945 different diet groups is indicated by different superscript letters and based on Tukey´s multiple comparisons.
946 IBW (initial body weight); FBW (final body weight); WG (weight gain); SGR (specific growth rate) = (ln
947 (FBW) – ln (IBW))/experimental days * 100; DFI(daily feed intake) = daily feed intake in % body weight; FCR
948 (feed conversion ratio) = total feed intake (g)/weight gain (g); PER (protein efficiency ratio) = weight gain
949 (g)/crude protein intake (g); PRE (protein retention efficiency) = crude protein gained (g)/crude protein intake
950 (g) * 100; CF (Fulton´s condition factor) = weight/fish length3 *100; HSI (hepatosomatic index) = liver weight
951 (g)/fish weight * 100; SSI (spleen somatic index) = spleen weight (g)/fish weight (g) * 100; SW seawater.

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Figures

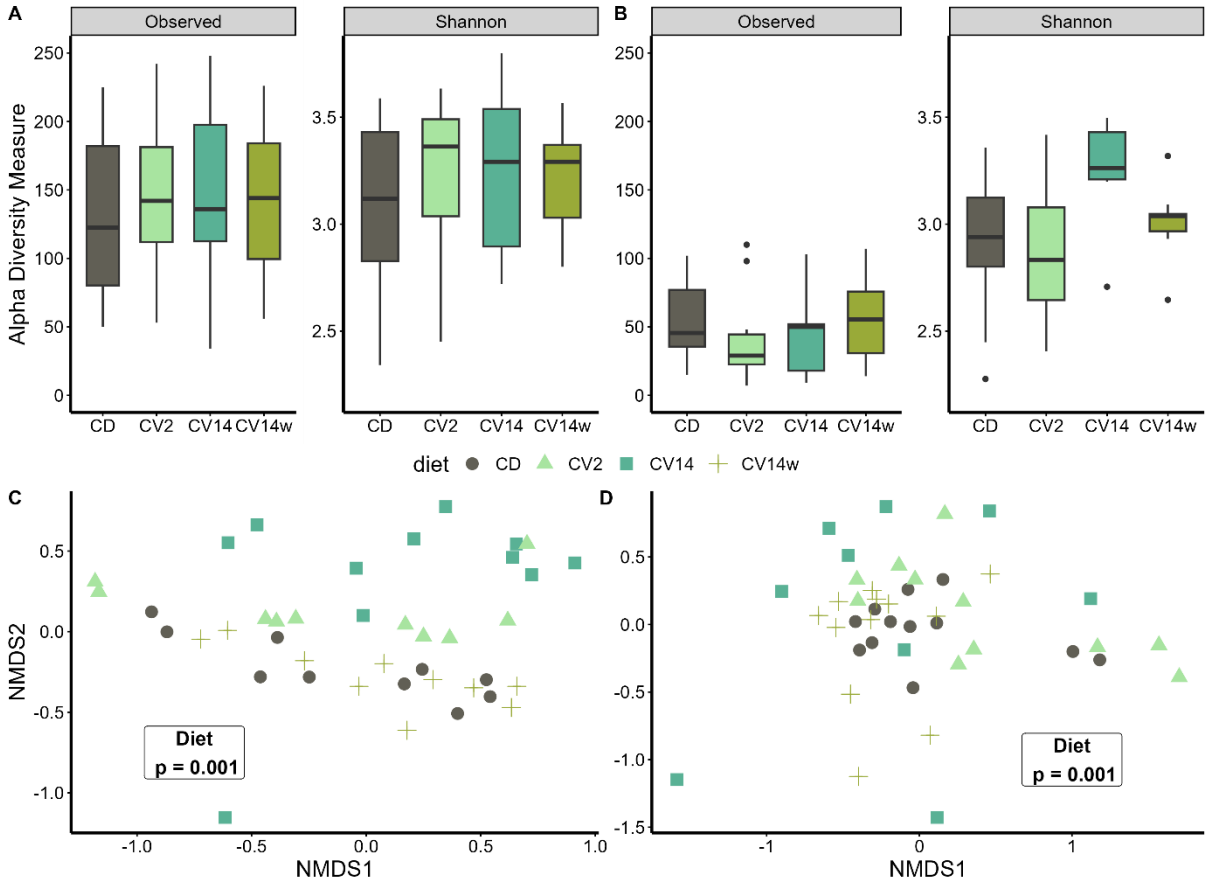

Figure 1. Alpha and beta diversity of the digesta and intestinal mucosa of the hind-gut of Atlantic salmon fed with *C. vulgaris* enriched diets for eight weeks. Observed ASVs and Shannon diversity index of digesta (**A**) and mucosa (**B**) do not differ among diets tested by one-way ANOVA. Non-metric multidimensional scaling on Bray-Curtis distances based on weighed genus-level data revealed significant differences between dietary treatments in both digesta (**C**) und intestinal mucosa (**D**) according to PERMANOVA; n = 10 – 11 per diet.

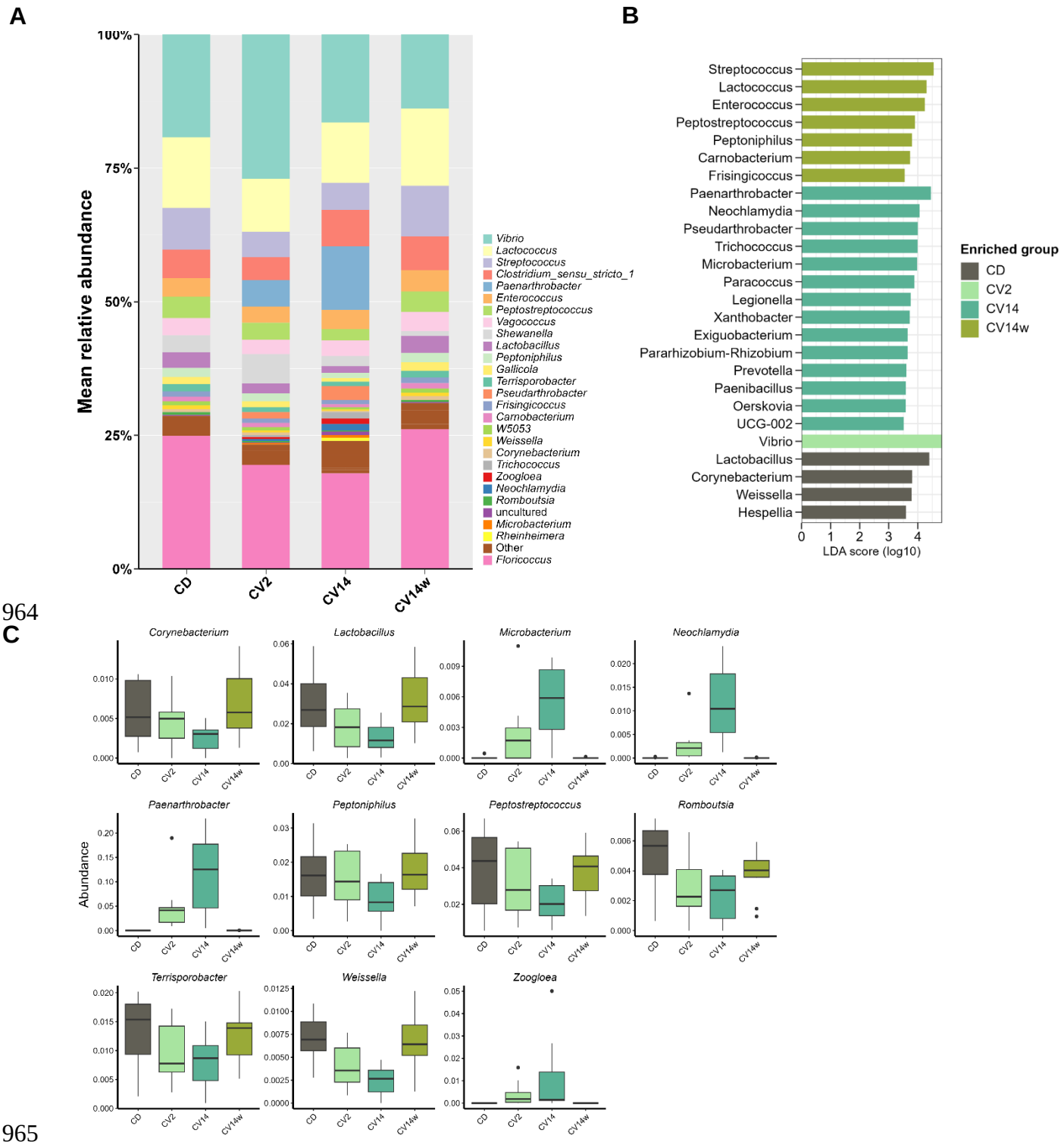

Figure 2. Microbial composition of the digesta of Atlantic salmon fed diets enriched with *C. vulgaris* for eight weeks. Relative abundance of microbiota on genus level per diet are displayed in a stacked bar plot (A). Note that category 'Other' includes taxonomical clades with an overall abundance of < 0.5%. Significantly enriched genera revealed by LEfSe analysis (B). Boxplots display relative abundance of main genera between the different diets (C).

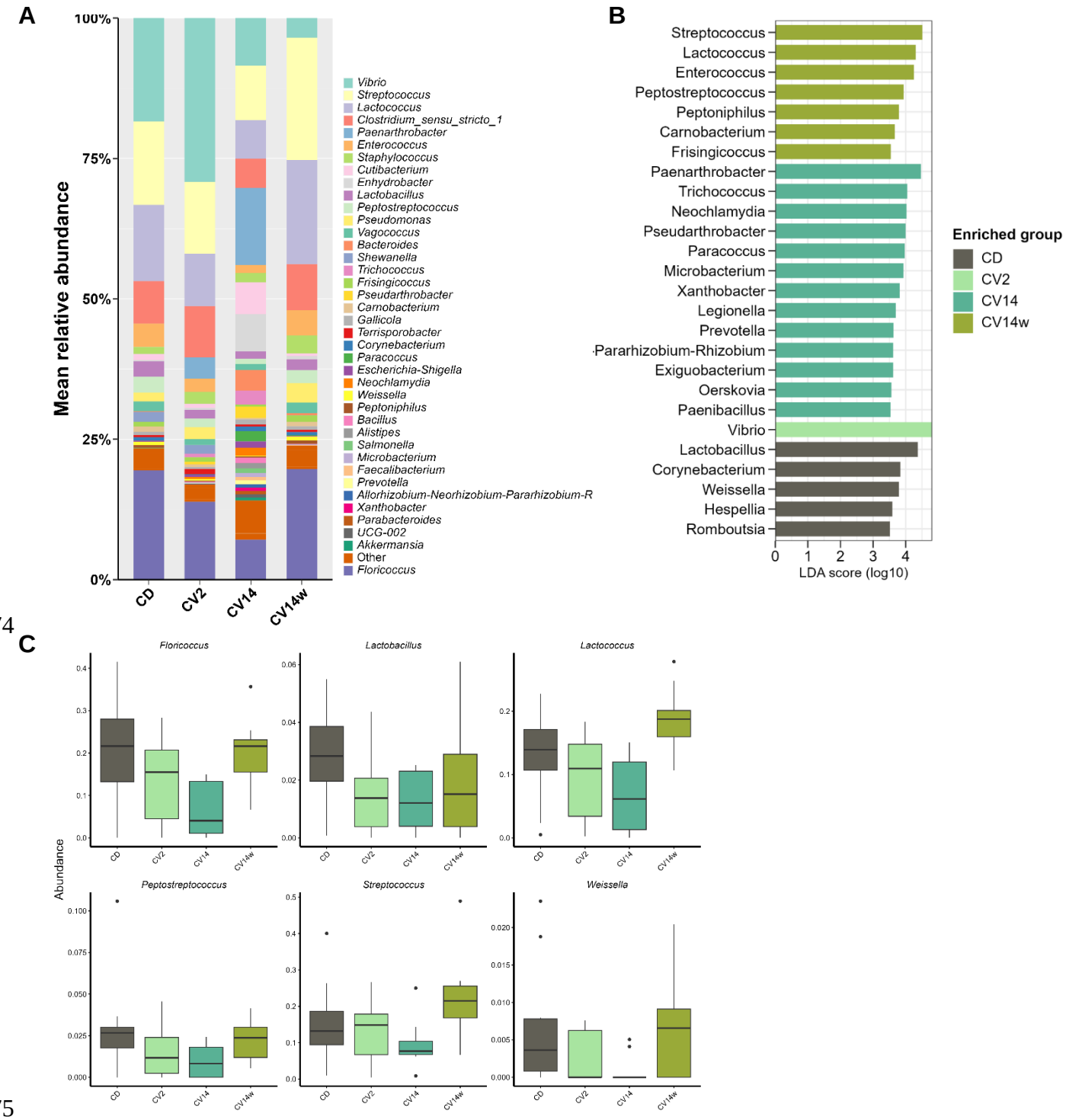

Figure 3. Microbial composition of the intestinal mucosa of Atlantic salmon fed diets enriched with *C. vulgaris* for eight weeks. Relative abundance of microbiota on genus level per diet are displayed in a stacked bar plot (A). Note that category 'Other' includes taxonomical clades with an overall abundance of < 0.5%. Significantly enriched genera revealed by LEfSe analysis (B). Boxplots display relative abundance of main genera between the different diets (C).

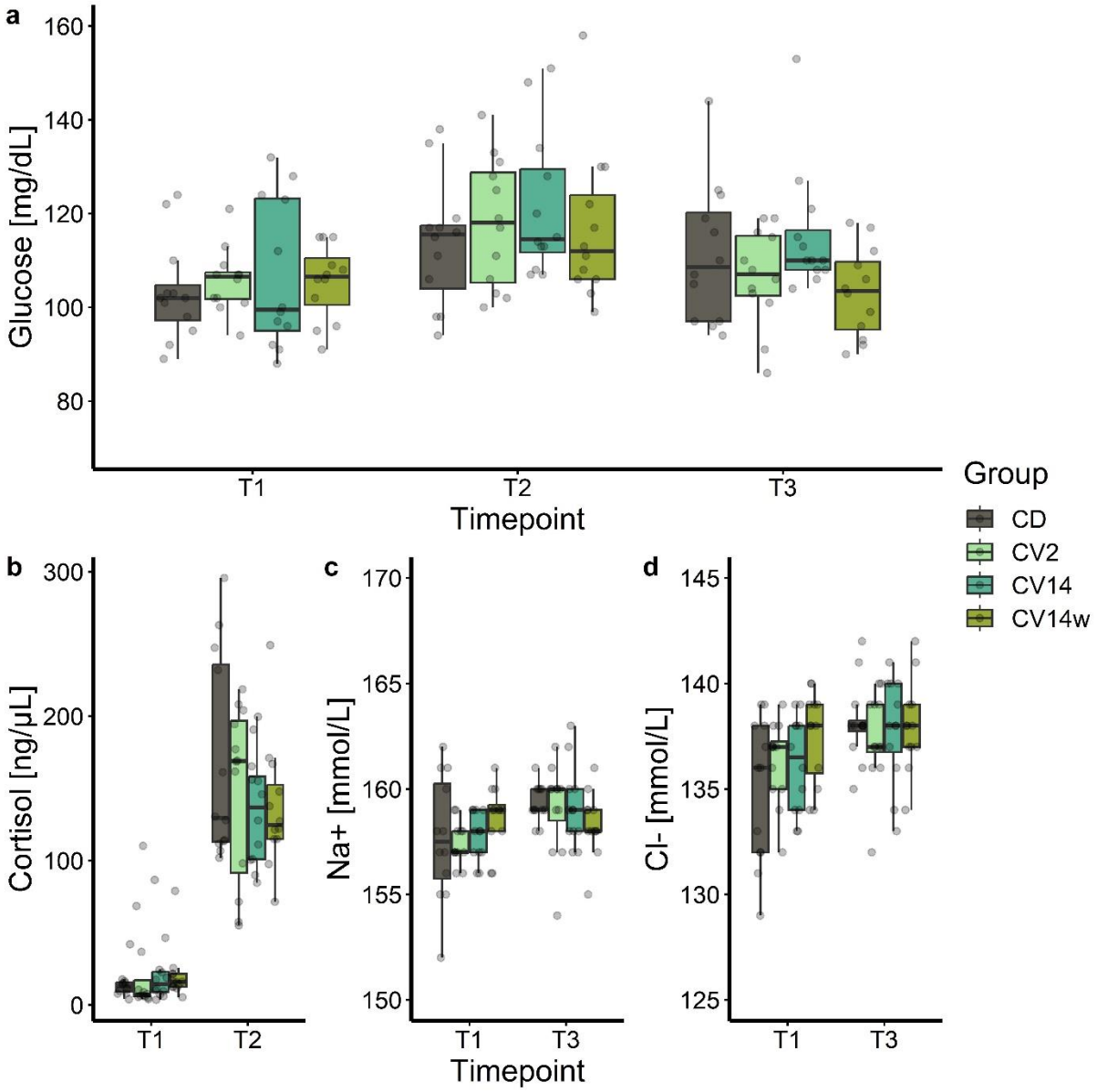

Figure 4. Plasma glucose (A), cortisol (B), sodium (C) and chloride ions (D) of Atlantic salmon fed with *C. vulgaris* enriched diets for eight weeks and sampled before (T1), 1 h after (T2) and 18 h after (T3) exposure to peracetic acid-based disinfectant. The salmon received either a control diet (CD), a diet with 2% (CV2) or 14% *C. vulgaris* (CV14) on a daily basis, or a diet containing 14% *C. vulgaris* once weekly. Data is presented as boxplots with median, n = 12. Statistical significance ($p < 0.05$) was assessed by Tukey multiple comparisons.

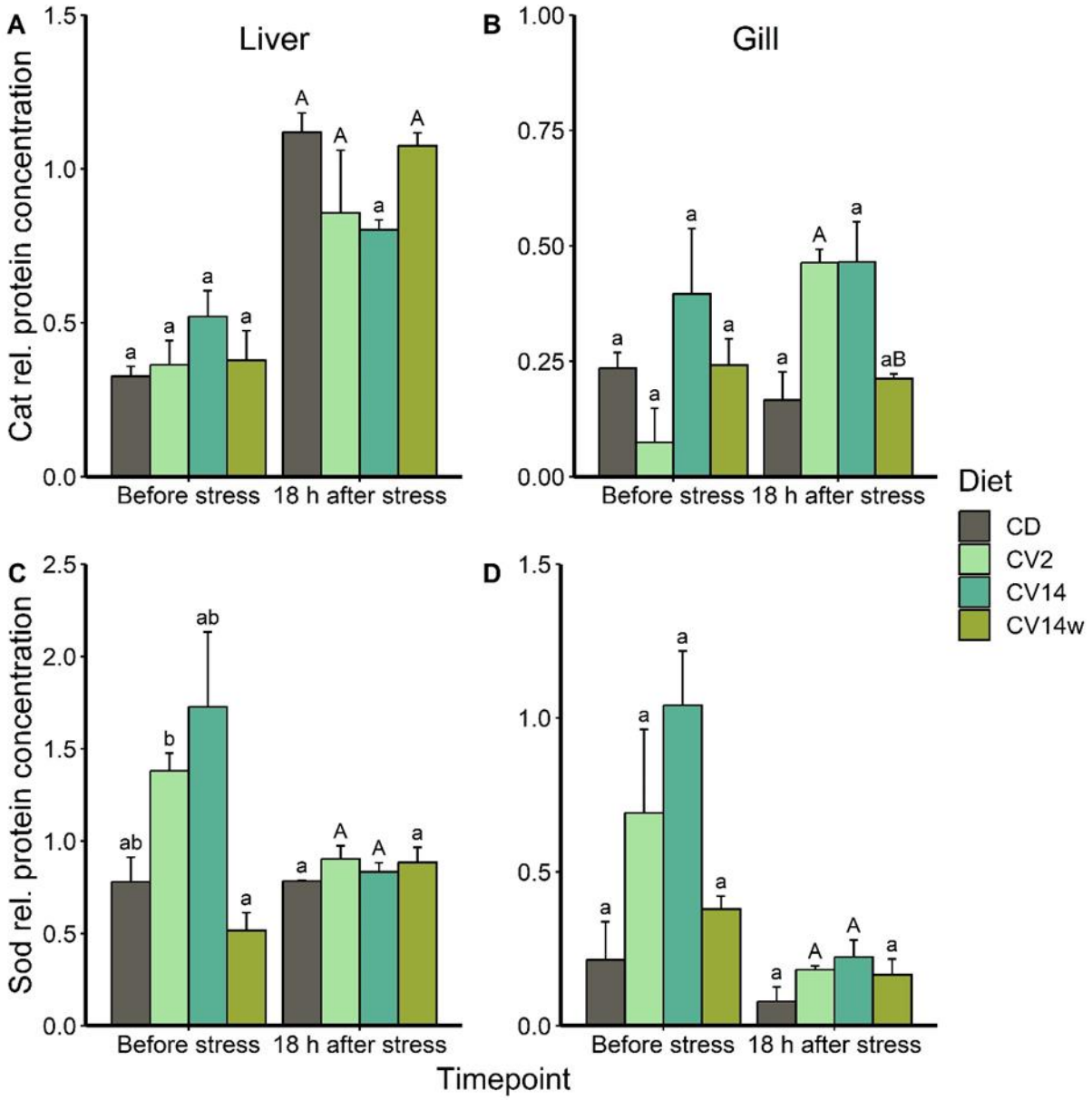

Figure 5. Protein abundance of catalase in liver (A) and gill (B), as well as superoxide-dismutase 1 in gill (C) and liver (D) of Atlantic salmon fed with *C. vulgaris* enriched diets for eight weeks. The fish were subsequently exposed to an oxidative stressor via treatment with peracetic acid-based disinfectant and sampled 18 h following stress treatment. The salmon received either a control diet (CD), a diet with 2% (CV2) or 14% *C. vulgaris* (CV14) on a daily basis, or a diet containing 14% *C. vulgaris* once weekly. Data is presented as mean + SEM, n = 3 (pooled on tank level). Statistical significance ($p < 0.05$) was assessed by Tukey multiple comparisons; bars not sharing similar letters within each timepoint are statistically different, while a significant effect with a diet group is indicated with an upper/lowercase letter.

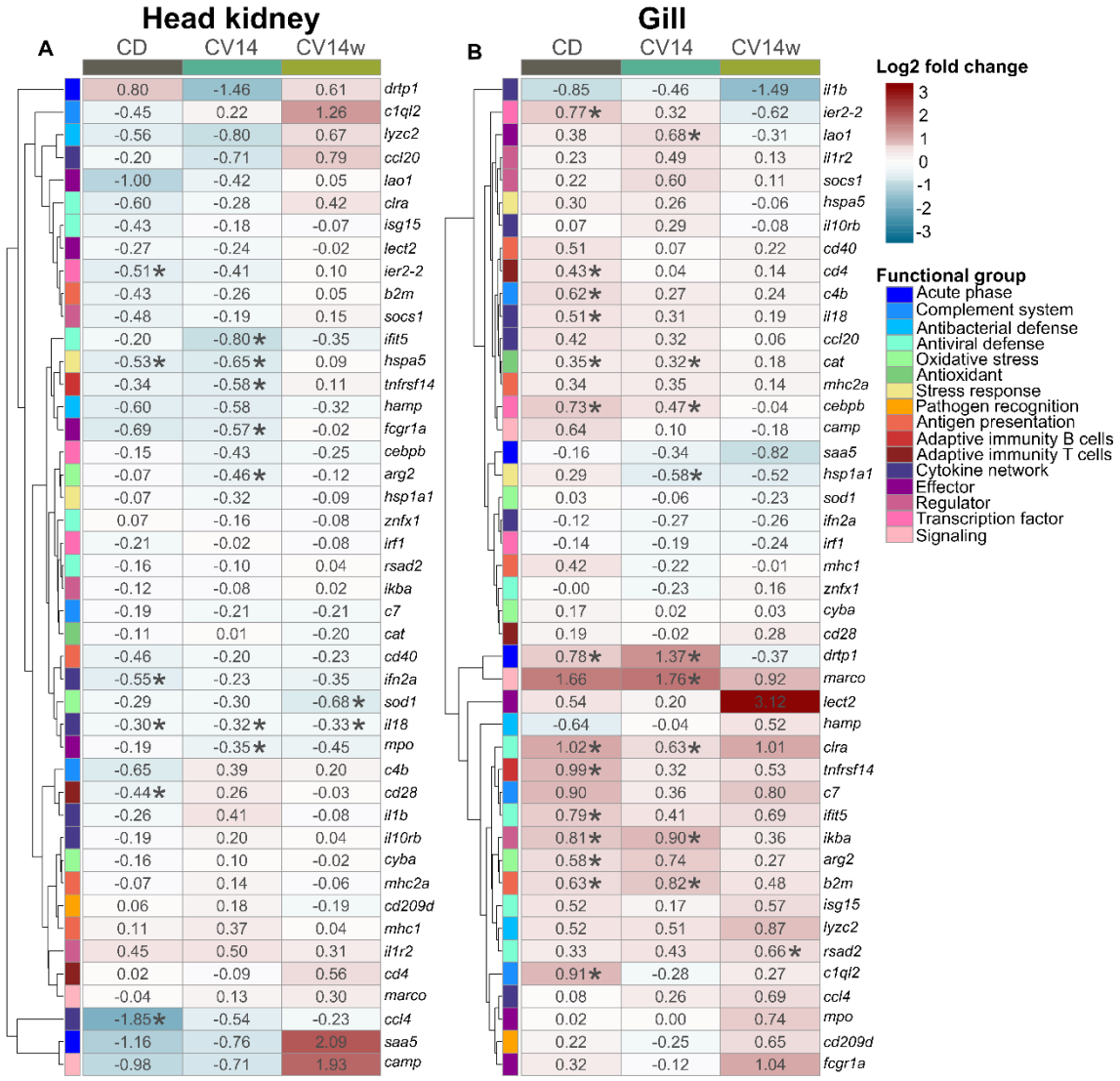

Figure 6 Hierarchical clustering of log-2 fold changes of immune- and stress regulated genes in the head kidney (A) and gill (B) of Atlantic salmon fed with *C. vulgaris* enriched diets for eight weeks, sampled after exposure to oxidative stress and relative to initial gene expression values of selected genes based on their relative transcript levels in (A) head kidney and (B) gills. Statistical significance compared to before stress values of its corresponding diet group was assessed using mixed effect models (*, $p < 0.05$). The rows represent different genes categorized into functional groups as illustrated in the legend on the right. The columns display fish fed control diet (CD), a diet with 14% *C. vulgaris* (CV14) on a daily basis, or a diet containing 14% *C. vulgaris* once weekly. Each cell is colorized based on the fold change of that gene, as visualized in the legend on the right. For both figure panels (A, B) n = 11–12 per timepoint per diet group.

5.3. Publication III: Salinity change evokes stress and immune responses in Atlantic salmon with microalgae showing limited potential for dietary mitigation

The seawater transfer is a critical period for salmon due to their suppressed immune system and high pathogen pressure in the marine environment. Retaining salmon longer as post-smolts in brackish water in RAS may make the transition to full-salinity seawater more gradual and improve fish health and performance. Microalgae, as a functional feed ingredient, can play a valuable role here by mitigating stress and the immune-related effects of salinity change. In this study, salmon were fed microalgae-enriched diets for eight weeks, after which they were transferred to seawater and fed for two more weeks with their corresponding diet. Local and systemic effects were analyzed by taking samples from the head kidney, gills, plasma, and liver.

Highlights

- Salmon had hypo-osmoregulatory capacity in brackish water, which further strengthened after seawater transfer.
- Salinity change has *per se* an effect on antiviral immunity, as demonstrated by a reduction in transcript levels of antiviral-related genes in both the gill and head kidney two weeks after seawater transfer.
- Reduced expression of several immune-related genes 20 h and two weeks after seawater transfer in the gill.
- An acute response to salinity change was apparent from increased plasma stress indicators and induced transcript levels of genes involved in acute-phase response, antimicrobial defense, and stress response in the head kidney.
- The diets enriched with microalgae did not influence the primary stress response.
- Supplementation with *A. platensis* reduced expression of genes involved in antimicrobial defense and pathogen recognition in the gill.

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EDITED BY
Carlo C. Lazado,
Norwegian Institute of Food, Fisheries and Aquaculture Research, Norway

REVIEWED BY
Benjamin Costas,
University of Porto, Portugal
Brian D. Ott,
United States Department of Agriculture (USDA), United States

*CORRESPONDENCE
Jonas Mueller,
jmueller@tierzucht.uni-kiel.de
Tom Goldammer,
tomgoldammer@fbn-dummerstorf.de

†These authors have contributed equally to this work and share first authorship

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Salinity change evokes stress and immune responses in Atlantic salmon with microalgae showing limited potential for dietary mitigation

Doret R. van Muilekom[1†], Jonas Mueller[2,3*†], Jacqueline Lindemeyer[4], Thekla Schultheiß[4], Edmund Maser[4], Henrike Seibel[3], Alexander Rebl[1], Carsten Schulz[2,3] and Tom Goldammer[1,5*]

[1]Fish Genetics Unit, Institute of Genome Biology, Research Institute for Farm Animal Biology (FBN), Dummerstorf, Germany, [2]Department for Marine Aquaculture, Institute of Animal Breeding and Husbandry, Kiel University, Kiel, Germany, [3]Fraunhofer Research Institution for Individualized and Cell-Based Medical Engineering IMTE, Aquaculture and Aquatic Resources, Büsum, Germany, [4]Institute of Toxicology and Pharmacology for Natural Scientists, University Medical School Schleswig-Holstein, Kiel, Germany, [5]Faculty of Agriculture and Environmental Sciences, University of Rostock, Rostock, Germany

Smoltification was found to impact both immune and stress responses of farmed Atlantic salmon (*Salmo salar*), but little is known about how salinity change affects salmon months after completed smoltification. Here, we examined (1) the effect of salinity change from brackish water to seawater on the stress and immune responses in Atlantic salmon and (2) evaluated if functional diets enriched with microalgae can mitigate stress- and immune-related changes. Groups of Atlantic salmon were fed for 8 weeks with different microalgae-enriched diets in brackish water and were then transferred into seawater. Samples of the head kidney, gill, liver and plasma were taken before seawater transfer (SWT), 20 h after SWT, and 2 weeks after SWT for gene-expression analysis, plasma biochemistry and protein quantification. The salmon showed full osmoregulatory ability upon transfer to seawater reflected by high *nka⍺1b* levels in the gill and tight plasma ion regulation. In the gill, one-third of 44 investigated genes were reduced at either 20 h or 2 weeks in seawater, including genes involved in cytokine signaling (*il1b*) and antiviral defense (*isg15, rsad2, ifit5*). In contrast, an acute response after 20 h in SW was apparent in the head kidney reflected by increased plasma stress indicators and induced expression of genes involved in acute-phase response (*drtp1*), antimicrobial defense (*camp*) and stress response (*hspa5*). However, after 2 weeks in seawater, the expression of antiviral genes (*isg15, rsad2, znfx1*) was reduced in the head kidney. Few genes (*camp, clra, c1ql2*) in the gill were downregulated by a diet with 8% inclusion of *Athrospira platensis*. The results of the present study indicate that salinity change months after smoltification evokes molecular stress- and immune responses in Atlantic salmon. However, microalgae-enriched functional diets seem to have only limited potential to mitigate the related changes.

KEYWORDS

microalgae, salmon, smoltification, salinity, immunity, functional feed

1 Introduction

Atlantic salmon (*Salmo salar)* is an anadromous salmonid of high economic value (Forseth et al., 2017). Global salmon production of approximately ~2.6 million tons has stagnated over the last years (Glover et al., 2020) as high mortalities and increasing sea lice infestations of salmon as well as negative environmental impacts caused by nutrient pollution and escapes have hampered further growth of the industry (McGinnity et al., 2003; Quiñones et al., 2019). Traditionally a large part of the production cycle of Atlantic salmon takes place in sea cages, while more recently many producers turn towards using land-based recirculating aquaculture systems (RAS; Dalsgaard et al., 2013). Before Atlantic salmon can be transferred into seawater they have to undergo different physiological and morphological changes known as smoltification to prepare for life in seawater (Hoar, 1988; McCormick et al., 1998). Smoltification not only affects the physiology, but also the immune system of salmon, which manifests in decreased levels of total serum protein and immunoglobulin M (IgM) (Melingen et al., 1995) as well as reduced expression of multiple immune genes in smolts (Johansson et al., 2016). The persistence of these effects after transfer to seawater, coinciding with an increased abundance of pathogens in the marine environment, likely explain some of the high disease-related mortalities seen during the first weeks in seawater (Soares et al., 2011; Bang Jensen et al., 2020). Nowadays, salmon are often reared as larger smolts or post-smolts in RAS on land to shorten the overall time they spend in the sea. As the RAS environment can be fully controlled, many environmental factors such as salinity and water velocity can be adapted for optimal growth and welfare (Ytrestøyl et al., 2020; Mortensen et al., 2022). After their smoltification in freshwater, an acclimation period in brackish water could alleviate the salmon's transition to salt water and improve their overall health and performance (Ytrestøyl et al., 2020; Ytrestøyl et al., 2022).

Besides changes in the rearing environment, the use of functional feeds can improve the fish's ability to cope with environmental stressors, stimulate immunity and eventually improve production performance (Dawood et al., 2018; Marimuthu et al., 2022). Microalgae, which have known immune-stimulatory properties in different fish species (Morris et al., 2007; Abdel-Tawwab and Ahmad, 2009; Cerezuela et al., 2012; Zhang et al., 2014; An et al., 2016; Luo et al., 2018), are promising candidates for functional feeds for Atlantic salmon. For example, the addition of *Chlorella* sp. to diets increased the levels of IgM and IgD antibodies in blood, and the cytokine levels of interleukin-22 (Il-22) and C-C motif chemokine ligand 5 (Ccl-5) in the kidney and liver of Gibel carp *Carassius gibelio* (Zhang et al., 2014). Supplementation with the microalgae *Phaeodactylum tricornutum* increased phagocytic activity and *Nannochloropsis gaditana* enhanced the expression of β-defensin in the head kidney of gilthead seabream *Sparus aurata* (Cerezuela et al., 2012). Furthermore, it has been shown that diets containing *Tetraselmis* sp. lowered cortisol levels after confinement stress in gilthead seabream (Pereira et al., 2020) and diets containing *Arthrospira platensis* counteracted the increasing glucose concentrations after air exposure and enhanced antioxidant activity in gilthead seabream (de Mattos et al., 2019).

A variety of phenotypic and molecular markers are used to assess the health and welfare of farmed fish (Barreto et al., 2022). Gene expression profiling allows the detection of both systemic and local immune responses in key immune organs. Recent studies on Atlantic salmon have revealed a variety of genes that can be used to assess the salmon's immune status (Krasnov et al., 2020; Lund et al., 2022). Accordingly, expression analysis of stress- and immune-related genes can complement traditional indicators such as growth performance, organ health and plasma indicators. In this study, three key immune organs with varying functions in stress and immune response were analyzed. The head kidney is a major lymphoid and myeloid organ that controls the systemic stress and immune response of fish (Press et al., 1994; Press and Evensen, 1999). The gill is part of the mucosa-associated lymphoid tissue and plays a crucial role in the local immune response, as it is in direct contact with the environment (Haugarvoll et al., 2008; Rességuier et al., 2020). Lastly, the liver was included as the main metabolizing organ to gain insight into the fish's oxidative status (Birnie-Gauvin et al., 2017; Hussein et al., 2023).

Given the lack of knowledge on how salinity change affects stress physiology and immunity of Atlantic salmon and its potential dietary mitigation, the aim of our study was twofold: (1) assessing the effect of salinity change to seawater following acclimation of Atlantic salmon to brackish water on the stress and immune response and (2) evaluating whether microalgae enriched functional diets can mitigate stress and immune-related changes following salinity change.

2 Material and methods

2.1 Experiment and sampling

The experimental setup has been described in detail in Mueller et al. (2023). Briefly, two identical RAS (7.6 m^3, turnover rate 4 times h^{-1}) at the facilities of the Fraunhofer IMTE, Büsum, Germany were used for the trial, where one was operated on brackish water (BW; 13.0 ± 0.8 psu, 13.5 ± 0.4°C, 7.3 ± 0.1 pH, 10.3 ± 0.2 mg/L O_2, 0.12 ± 0.07 mg N/L total ammonia 0.06 ± 0.01 mg N/L NO_2^-) while the other was operated on full marine conditions (SW; 31.8 ± 0.5 psu, 13.4 ± 0.3°C, 7.2 ± 0.1 pH, 10.4 ± 0.3 mg/L O_2, 0.12 ± 0.07 mg N/L total ammonia 0.07 ± 0.02 mg N/L NO_2^-). Atlantic salmon smolts, raised in freshwater, were obtained from Jurassic Salmon, Karnice, Poland and acclimated in brackish water for 2 months in the RAS prior to the start of the experiment. Following acclimation, the fish were fed six different functional diets for 8 weeks in brackish water until 2 weeks after transfer to seawater. This time-interval was chosen as one of the most critical phases during the production of Atlantic salmon (Bang Jensen et al., 2020; Sommerset et al., 2020). The fish were hand-fed twice daily (8 a.m. and 2 p.m.) until apparent satiation. The functional diets were designed to be isonitrogenous and isoenergetic (dry matter basis) and were enriched with one of the following microalgae: *Chlorella vulgaris* (either intact cell wall, CVI or broken cell wall, CVB), *Tetraselmis chuii* (TC), *Athrospira platensis* (AP) or *Schizochytrium limacinum* (SL) at an inclusion level of 8%. A diet without added microalgae served as a control (CD; Table 1). The microalgae were chosen based on their production volume, allowing for industrial

TABLE 1 Formulation of experimental diets in g/100 g dry matter (DM) as well as crude composition in % DM.

Ingredients (g/100 g DM)	CD	CVI	CVB	TC	AP	SL
Fish meal[a]	15.0	15.0	15.0	15.0	15.0	15.0
Microalgae	0.0	8.0	8.0	8.0	8.0	8.0
Blood meal[b]	6.0	6.0	6.0	6.0	6.0	6.0
Gelatine[c]	5.0	5.0	5.0	5.0	5.0	5.0
Pea protein isolate[d]	14.0	14.0	14.0	14.0	14.0	14.0
Soy protein concentrate[e]	11.0	11.0	11.0	11.0	11.0	11.0
Wheat gluten[f]	12.0	7.2	6.5	8.4	5.3	9.4
Wheat starch[f]	21.4	18.4	18.9	17.9	20.2	19.3
Canola oil[g]	5.5	5.5	5.5	6.1	5.5	2.5
Fish oil[a]	6.0	6.0	6.0	6.0	6.0	6.0
Methionine[h]	0.1	0.1	0.1	0.1	0.1	0.1
Vitamin and mineral premix[d]	0.5	0.5	0.5	0.5	0.5	0.5
CaHPO4[i]	2.0	2.0	2.0	2.0	2.0	2.0
Bentonite[j]	1.5	1.3	1.5	0	1.5	1.2
Crude composition (% DM)						
Dry matter (%)	90.03	91.52	91.59	92.90	92.08	93.02
Protein (%)	51.04	51.26	51.00	50.93	51.11	51.38
Fat (%)	16.18	16.38	16.60	16.87	16.19	15.11
Ash (%)	7.47	8.15	8.28	8.61	8.07	7.72
Crude energy (MJ/kg)	22.72	22.71	22.68	22.72	22.69	22.70

CD, control diet, CVI *C. vulgaris* intact, CVB *C. vulgaris* broken, TC *T chuii*, AP *A. platensis*, SL *S. limacinuum*.
[a]Bioceval GmBH & Co. KG, Cuxhaven; Germany.
[b]Saria SE & Co. KG, Selm, Germany.
[c]Gustav Ehlert GmbH & Co. KG, Verl, Germany.
[d]Emsland-Aller Aqua GmbH, Golßen, Germany.
[e]EURODUNA Rohstoffe GmbH, Barmstedt, Germany.
[f]Kröner-Stärke GmbH, Ibbenbüren, Germany.
[g]Cargill GmbH, Riesa, Germany.
[h]Evonik Industries AG, Essen, Germany.
[i]Lehmann & Voss & Co. KG, Hamburg, Germany.
[j]Del Lago Bentonite, Castiglioni Pes y Cía., Buenos Aires, Argentina.

upscaling, as well as containing a diversity of functional compounds. The inclusion level of 8% reflected a compromise of formulating diets without making major adjustments to a variety of ingredients within the feed formulation, as well as making sure sufficient amounts of functional components are available for the fish. At the start of the experiment 28 fish were stocked into each tank (with three tanks assigned to each treatment), but due to serial samplings, 19 fish per tank were transferred to seawater.

Samples were collected after 8 weeks of feeding the experimental diets in brackish water (T1), 20 h following transfer to seawater (T2) and 2 weeks in seawater (T3). These timepoints include both the acute stress and immune response (T2) as well as adaptive immune response and further adaptive responses (T3) to salinity change. Each time nine fish per diet (three per tank) were randomly sampled. The salmon were netted from the tanks and euthanized with MS-222 (0.3 mg/L). Length and weight were recorded and a 2 mL blood sample was withdrawn in heparinized syringes by caudal vein puncture. Blood samples were immediately centrifuged at 4000 *g* for 8 min and plasma aliquots flash-frozen at −80°C, to be used for the determination of plasma metabolites. The liver and spleen were removed, weighed and a small piece of the liver was flash-frozen on dry ice for determination of protein concentrations. The first left gill arch and the head kidney were sampled for gene-expression analysis and flash-frozen in a RNase-free tube in liquid nitrogen. Somatic indices were calculated as follows:

CF (Fulton´s condition factor) = weight/fish length^3 *100.

HSI (hepatosomatic index) = liver weight /fish weight * 100.

SSI (spleen somatic index) = spleen weight /fish weight * 100.

2.2 Plasma enzymes and metabolites

Plasma glucose, Na^+ and Cl^--ions in plasma samples were measured on a Fuji Dry Chem NX500i (Fujifilm, Tokyo, Japan)

using kits of the manufacturer. Plasma cortisol was determined by an enzyme-linked immunosorbent assay (ELISA) Kit (Demeditec Diagnostics GmbH, Kiel, Germany) following the manufacturer's instructions.

2.3 Western blots

Liver samples (pooled per tank) were processed to extract total protein based on radioimmunoprecipitation (RIPA) following the manufacturer's instructions (RIPA Lysis Buffer System, Santa Cruz Biotechnology, Dallas, Texas, United States). Cu, Zn superoxide dismutase (Sod1) and myeloperoxidase (Mpo) proteins were analyzed using SDS-PAGE and Western blot techniques. The protein ß-actin (Actb) was used as a loading control. A no template control and a positive control per antibody were included, with *Danio rerio* liver used for Mpo, bovine liver for Sod1, and HEK-293 cells for ß-actin. To process 20 µg of total protein, reducing conditions were applied with SDS sample and reducing buffer (both TruPAGE, Sigma-Aldrich, Schnelldorf, Germany) for 10 min at 70°C. SDS-PAGE was performed with precast 4%–12% gradient gels, TruPAGE running buffer, and antioxidant (Sigma-Aldrich) in an Xcell SureLock Mini-Cell (Thermo Fisher Scientific, Waltham, Massachusetts, United States). The proteins were then electro-transferred to a PVDF membrane. The membrane was cut horizontally for parallel protein detection of Mpo and Sod1. Primary antibody incubation was carried out overnight at 4°C using Mpo antibody (ab210563, Abcam, 1:5000 dilution in PBS-T containing 2.5% skim milk) and Sod1 antibody (NBP2-24915, Novus Biologicals, 1:500 dilution in PBS-T containing 2.5% skim milk). Using a dilution of 1:5000, the secondary antibody anti-rabbit IgG conjugated HRP (sc-2357, Santa Cruz Biotechnology) was incubated at room temperature for 90 min. ECL detection reagents and chemiluminescence film (both GE Healthcare, Amersham, United Kingdom) were used for detection, with 40 s of exposure time for both Mpo and Sod1. To detect Actb, the antibodies were stripped in 100 mM glycin buffer (pH 2.5). The membrane was then incubated overnight at 4°C in a 1:5000 dilution of Actb antibody (NB600-503, Novus Biologicals, Wiesbaden, Germany). Protein abundance was quantified by densitometric analysis of the protein bands using GIMP and normalized to the concentration of the reference protein Actb.

2.4 RNA isolation and multiplex gene-expression profiling

TRIzol (Thermo Fisher Scientific) was used to extract total RNA, which was then purified with the ISOLATE II RNA Micro Kit (Meridian Bioscience Inc., Cincinnati, Ohio, United States). NanoDrop One (Thermo Fisher Scientific) was used to determine the concentration of the isolated RNA. High-quality RNA was then reverse-transcribed into cDNA utilizing the Reverse Transcription Master Mix (Standard BioTools, South San Francisco, California, United States). cDNA samples were preamplified with the Fluidigm PreAmp Master Mix before purification with exonuclease I (New England BioLabs, Frankfurt/Main, Germany). All procedures were conducted according to the manufacturer's instructions.

Exon-skipping oligonucleotide primers for the smoltification markers *nkaα1a* (homologous to mammalian *atp1a1*) and *nkaα1b* were designed using the Pyrosequencing Assay Design software v.1.0.6 (Biotage, Uppsala, Sweden; Supplementary Table S1). To evaluate salmon immunocompetence, Krasnov et al. (2020) designed a set of genes involved in important immunological and stress-relevant pathways and functions, such as antigen presentation, T cell activity, oxidative stress, antiviral and antibacterial defense as well as stress-related pathways such as nf-κb pathway, acute phase response and heat shock protein (HSP) signaling pathway. In addition to 41 of those target genes and three reference genes (Supplementary Table S1), three more immune-specific genes were included (Pyrosequencing Assay Design software v.1.0.6) for *hamp*, *saa5*, and *sod1* (Supplementary Table S1). The LightCycler-96 system (Roche Diagnostics International AG, Rotkreuz, Switzerland) was utilized to assess the smoltification status using the *nkaα1a* and *nkaα1b* primers (1 µL) together with 6 µL SensiFAST SYBR No-ROX Mix (Meridian Bioscience Inc.) and 5 µL cDNA in a 12-µL-reaction volume. The transcripts were amplified according to following program: initial denaturation at 95°C for 300 s, then 40 cycles including a denaturation step at 95°C for 30 s, primer annealing at 60°C for 15 s and at last, elongation step at 72°C for 15 s followed by fluorescence measurement at 72°C for 10 s. Afterwards, gel electrophoresis and melting-curve analysis were conducted to check the quality of the amplicons. Finally, the qPCR results were obtained using the LightCycler-96 analysis software v. 1.1.0.1320 (Roche Diagnostics International AG) and normalized using the geometric mean of two reference genes ribosomal protein L4 (*rpl4*) and ribosomal protein S20 (*rps20*). Standard curves of *nkaα1a* and *nkaα1b* oligonucleotides were generated (10^8–10^3 copies per 5 µL; $R^2 > 0.99$) to calculate the individual copy numbers. Multiplex immunogene-expression profiling was conducted on 48.48 Gene Expression biochips. The pre-amplified cDNA samples and primers were transferred into the sample and assay inlets of these biochips that were first primed in the MX IFC Controller (Standard BioTools). Subsequently, the concentrations of the target transcripts were quantified using the Biomark HD (Standard BioTools) according to the manufacturer's thermal protocol "GE Fast 48 × 48 PCR + Melt v2.pcl" (application type: gene expression; passive reference: ROX; assay: single probe). By use of Fluidigm real-time PCR analysis software v3.0.2 (Standard BioTools), the raw Cq values were gathered and used for calculation of the relative expression of the target genes based on the ΔCt method. Three reference genes *actb*, *rpl4* and *rps20* were employed as internal normalizers. Throughout the calculation, the mean Cq per gene for all samples per organ served as a calibrator. The data collected for four individuals were excluded from the dataset because they exhibited signs of sickness (for example, liver cysts and/or irregular spleen) and subsequently showed abnormal immune-gene expression.

2.5 Statistical analysis

All statistical analyses were performed using R (v4.1.2; R Core Team 2021; Vienna, Austria) in the environment R studio. The relative gene expression values obtained from the multigene expression assays as well as the actual copy numbers from the

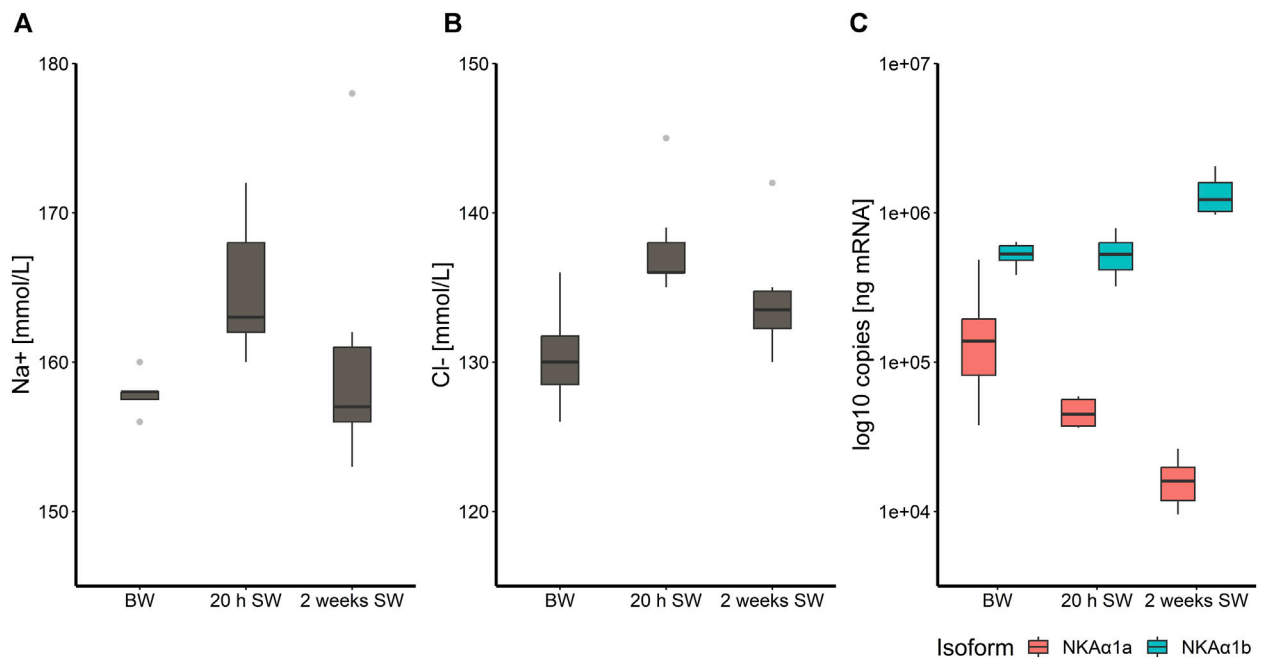

FIGURE 1
Boxplot with median and 1.5 x interquartile range whiskers showing sodium-ion **(A)** and chloride-ion **(B)** concentration in plasma of Atlantic salmon fed the control diet (CD) after 8 weeks experimental period in brackish water (T1), 20 h in seawater (T2) and 2 weeks in seawater (T3), as well as log-10-transformed copy numbers of *nkaα1a/b* transcripts for the three timepoints **(C)**; n = 6–8. Note that the y-axis in panel **(A)**, **(B)** and **(C)** does not start at zero.

qPCR evaluation of the smoltification markers (*nkaα1a/b*) were log2-transformed before statistical analysis. However, the copy numbers of the smoltification markers were displayed in a boxplot on a log10 scale to guide visual interpretation. Mixed effect models were defined for the response variable expression of gene of interest, plasma parameter or organ health index. For testing the expression of the smoltification markers, the model included isoform (*nkaα1a/b*) and timepoint as fixed factors and tank was included as a random factor. For the other gene expression data as well as plasma parameter the model included diet, timepoint and their interaction as fixed factors and the tank as a random factor. In case of testing the organ health index, as well as plasma ion concentrations among the diet groups, timepoint was not included in the model. Model residuals were inspected graphically and found to be normally distributed and heteroscedastic. Analysis of variance (ANOVA) was conducted followed by multiple contrast tests for heteroscedastic data (Hasler and Hothorn, 2008). This was done to compare the microalgae-enriched diets to the control within a given time point as well as within each diet for effects over time. Comparisons were considered significant at $p < 0.05$. In case an interaction effect of diet and timepoint was absent, subsequent contrasts were pooled over the remaining factor. For the analysis of liver protein concentrations (pooled on tank level), a model based on generalized least squares (GLS) was defined, which included only the fixed factors diet and timepoint and subsequently model evaluation followed the procedure described above.

To evaluate the overall effect of seawater transfer on gene expression data, principal component analysis (PCA) with time point as grouping was conducted on log2-transformed relative expression values for the 44 target genes investigated per tissue, as implemented in the ade4 package in R (Bougeard and Dray, 2018). The individual contribution of each gene to the overall separation of the time points in the PCA was visualized using factor maps implemented in the facto extra package in R (Kassambara and Mundt, 2020). Fold changes of each gene across the factor time, relative to the timepoint in brackish water (T2), were calculated from relative gene expression values. Subsequently fold changes were visualized in a hierarchically clustered heatmap for both organs separately using the ppheatmap package (Kolde, 2022). A range of 0.33 to 3-fold change based on the relative gene expression values was considered as basal levels. The relative expression levels of three differentially expressed genes in at least one microalgae group (*clra*, *c1ql2*, and *camp*) in the gill were visualized as log2-transformed relative expression in boxplots.

3 Results

3.1 Smoltification status and osmoregulation

Salmon acclimated to BW and transferred to full strength SW had a slight but significant increase in plasma Na^+ and Cl^- concentrations (Figures 1A,B) following 20 h in SW ($p < 0.05$) in the control group. However, after 2 weeks in SW, plasma ion levels returned to levels prior to transfer. This was furthermore reflected in the expression of the two NKA isoforms in the gill. The transcripts of the "seawater isoform" *nkaα1b* were 5.1 times more abundant in the gill by the end of the brackish water phase

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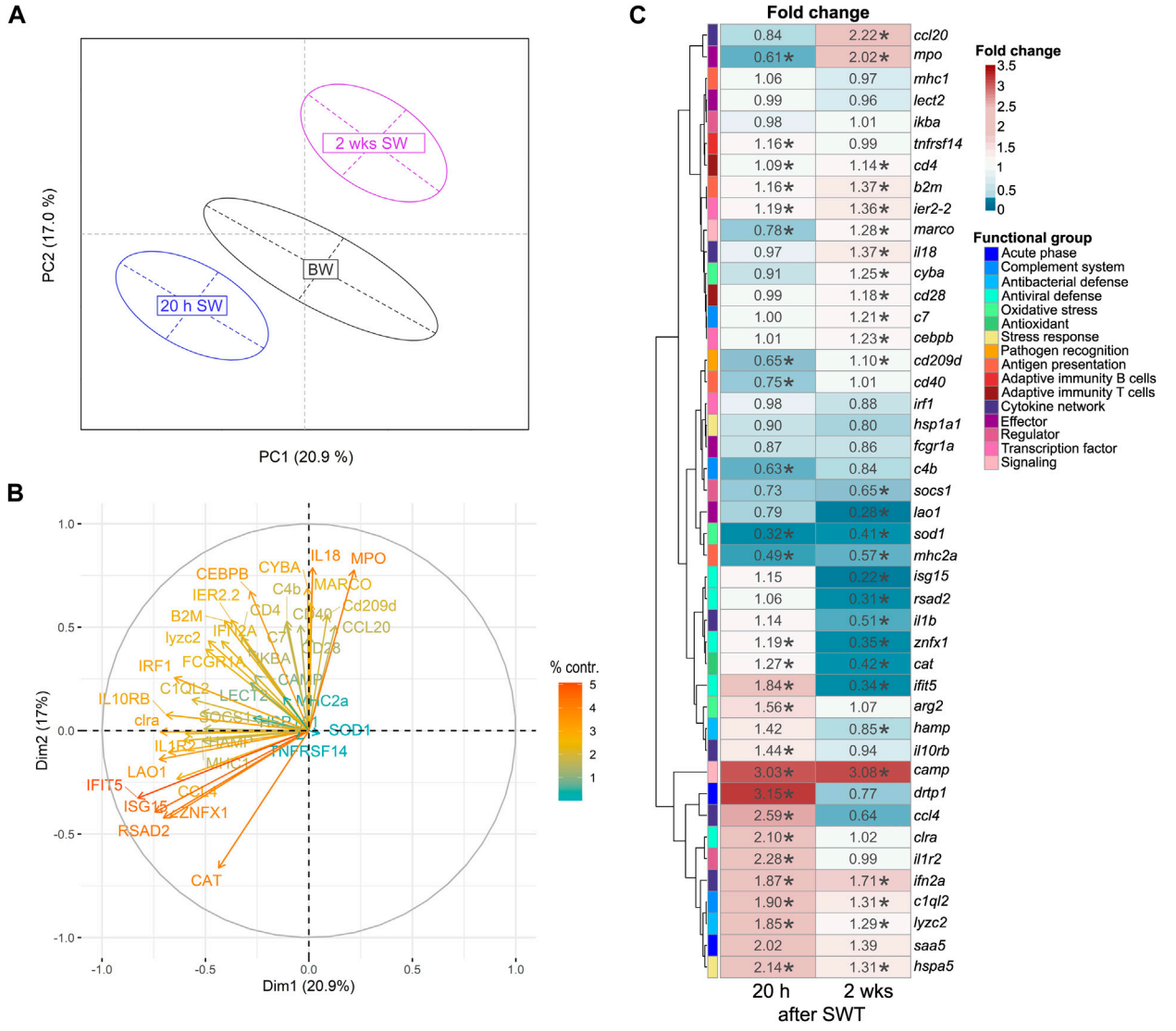

FIGURE 2
Principal component analysis **(A)** of 44 stress-and immune-regulated genes in the head kidney of Atlantic salmon after 8 weeks experimental period in brackish water (BW, T1), 20 h in seawater (T2) and 2 weeks in seawater (T3). Factor map **(B)** explaining the contribution of individual genes to the overall separation of the PCA. Length and color of the arrows indicate percent contribution of each gene. Heatmap **(C)** of fold changes for the genes investigated in relation to timepoint T1 (BW) based on relative gene expression values. Statistical significance compared to timepoint T1 was assessed using multiple contrast tests (*, $p < 0.05$). The rows represent different genes categorized into functional groups as illustrated in the legend on the right. The columns display the time points 20 h in seawater (T2) and 2 weeks in seawater (T3). Each cell is colorized based on the fold change of that gene, as visualized in the legend on the right. For each figure panel **(A–C)** n = 35–50 per timepoint.

than the "freshwater isoform" *nkaα1a* ($p < 0.001$) and this ratio increased even more 20 h and 2 weeks in SW where *nkaα1a* transcript abundance was significantly reduced further ($p < 0.05$) while *nkaα1b* transcript abundance increased ($p < 0.001$; Figure 1C).

3.2 Effects of salinity change on expression of immune and stress-related genes

In the head kidney, when salinity changed from BW to SW, levels of immune- and stress-related genes were significantly modulated in a time-dependent manner (Figure 2A). Principal component analysis (PCA) revealed a 21% share of variability of the first principal component for the conditions timepoint/salinity. The overall gene-expression pattern of salmon from brackish water (T1) grouped between early (T2) and the later (T3) timepoint in seawater. This pattern was driven by the differential transcript concentrations of *ifit5* (interferon-induced protein with tetratricopeptide repeats 5), *isg15* (interferon-stimulated gene 15), *rsad2* (viperin alias radical S-adenosyl methionine domain containing 2), *znfx1* (NFX1-type zinc finger containing protein), *cat* (catalase), *mpo* (myeloperoxidase) and *il18* (interleukin 18; Figure 2B).

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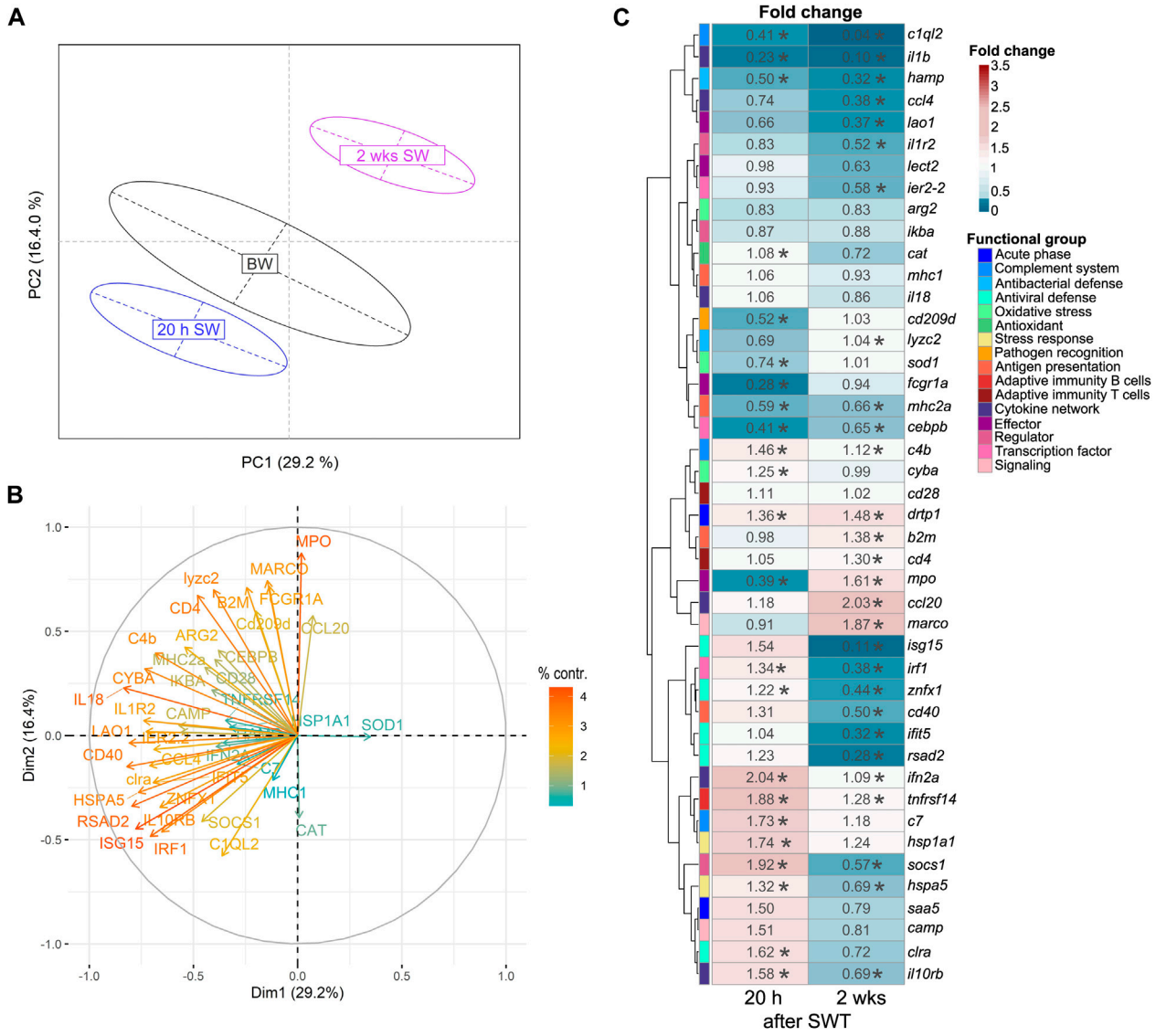

FIGURE 3
Principal component analysis **(A)** of 44 stress-and immune-regulated genes in the gill of Atlantic salmon after 8 weeks experimental period in brackish water (BW, T1), 20 h in seawater (T2) and 2 weeks in seawater (T3). Factor map **(B)** explaining the contribution of individual genes to the overall separation of the PCA. Length and color of the arrows indicate percent contribution of each gene. Heatmap **(C)** of fold changes for the genes investigated in relation to timepoint T1 (BW) based on relative gene expression values. Statistical significance compared to timepoint T1 was assessed using multiple contrast tests (*, $p < 0.05$). The rows represent different genes categorized into functional groups as illustrated in the legend on the right. The columns display the time points 20 h in seawater (T2) and 2 weeks in seawater (T3). Each cell is colorized based on the fold change of that gene, as visualized in the legend on the right. For each figure panel **(A–C)** n = 36–50 per timepoint.

Similarly to the head kidney, the overall expression pattern in the gill was modulated in a time-dependent manner (Figure 3A). In this case, the first principal component explained 29.2% of the variance and this effect was primarily driven by *cd40* (cluster of differentiation 40), *irf1* (interferon regulatory factor 1) and, like in the head kidney, *isg15*, *rsad2*, *mpo* and *il18* (Figure 3B).

After seawater transfer, none of the selected genes was increased in the gill above the defined threshold for basal levels (fc > 3). In contrast, the levels of several genes were significantly reduced (fc < 0.33) in the gill after SWT, such as the reduced (0.28-fold; $p < 0.001$) transcript level of *fcgr1a* (Fc gamma receptor Ia) shortly after SWT (Figure 3C). Several genes were reduced at both 20 h and 2 weeks after SWT (Figure 3C), including *c1ql2* (complement C1q-like 2; 0.41- and 0.04-fold; $p < 0.001$); *il1b* (interleukin 1β; 0.23- and 0.10-fold; $p < 0.001$); and *hamp* (hepcidin; 0.5- and 0.32-fold; $p < 0.001$). The antiviral genes *isg15*, *rsad2* and *ifit5* were only found significantly reduced (up to 0.11-fold; $p < 0.001$) at 2 weeks after salinity change.

The transcript profile in the head kidney differed from that in the gill. Most notably, the transcript levels of *camp* (cathelicidin) were enhanced by > 3-fold 20 h and 2 weeks after seawater transfer ($p < 0.001$, Figure 2C). Furthermore, *drtp1* (differentially

TABLE 2 Organ indices (n = 9, mean ± sem) of Atlantic salmon fed with six microalgae enriched diets sampled 2 weeks in seawater (T3).

	CD	CVI	CVB	TC	AP	SL	ANOVA
CF	0.90 ± 0.01	0.86 ± 0.01	0.86 ± 0.01	0.89 ± 0.01	0.93 ± 0.01	0.87 ± 0.01	0.049
HSI (%)	1.31 ± 0.09	1.31 ± 0.1	1.51 ± 0.08	1.37 ± 0.09	1.28 ± 0.04	1.46 ± 0.07	0.635
SSI (%)	0.15 ± 0.02	0.13 ± 0.01	0.15 ± 0.01	0.13 ± 0.02	0.12 ± 0.01	0.11 ± 0.01	0.477

CD, control diet, CVI *C. vulgaris* intact, CVB *C. vulgaris* broken, TC *T chuii*, AP *A. platensis*, SL *S. limacinum*; CF (Fulton´s condition factor); HSI (hepatosomatic index); SSI (spleen somatic index).

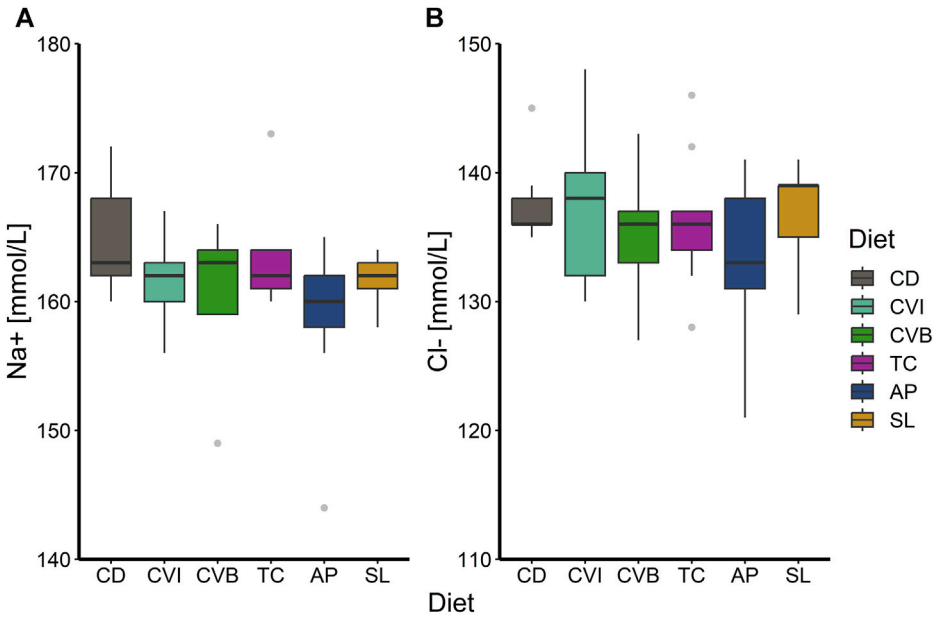

FIGURE 4
Boxplot with median and 1.5 x interquartile range whiskers showing sodium-ion **(A)** and chloride-ion **(B)** concentration in plasma of Atlantic salmon 20 h in seawater (T2) fed with the microalgae enriched diets *C. vulgaris* intact (CVI), *C. vulgaris* broken (CVB), *T. chuii* (TC), *A. platensis* (AP) or *S. limacinum* (SL) at 8% inclusion or a control diet (CD); n = 9. Note that the y-axis in panel **(A)** and **(B)** does not start at zero.

regulated trout protein 1) and *saa5* (serum amyloid A-5 protein) were 2.01- to 3.15-fold induced 20 h after seawater transfer (p < 0.001). After salinity change, also reduced levels of genes involved in different immune pathways were observed in the head kidney. *Sod1* (superoxide dismutase 1) was reduced 20 h (0.32-fold) and 2 weeks (0.41-fold) after seawater transfer (p < 0.001). Furthermore, *lao1* was reduced (0.28-fold; p < 0.001) 2 weeks after seawater transfer. Noteworthy, the genes *isg15*, *rsad2*, *znfx1* and *ifit5* which are all involved in antiviral defense, were reduced (0.22- to 0.35-fold; p < 0.001) 2 weeks after seawater transfer.

3.3 Mitigation potential of microalgae on immune and stress responses

At the end of the trial in seawater, the condition of the salmon, their hepatosomatic index (HSI) and spleen somatic index (SSI) across all diet groups was similar (Table 2). Moreover, no diet-dependent effects were observed in plasma concentrations of sodium and chloride after 20 h in seawater (Figure 4). Plasma cortisol levels decreased significantly (p < 0.001) between timepoints in seawater for fish fed CD but remained unchanged in SW for the other diet groups (Figure 5A). Furthermore, cortisol levels after 20 h in seawater were elevated as compared to control fish in brackish water (Supplementary Figure S1). Glucose levels increased over time in seawater, which was significant for fish fed AP (p < 0.01) and SL (p < 0.001; Figure 5B). The protein levels of myeloperoxidase increased over time in seawater for fish fed CD (p < 0.05), CVB (p < 0.001) and AP (p < 0.01; Figure 6A). At both timepoints, the concentration of Sod1 was not significantly different in the microalgae-fed groups as compared to the control group (Figure 6B). However, for fish fed CVI and TC levels decreased over time in seawater (p < 0.05; Figure 6B).

The different diet groups followed a similar trend of gene expression over the three timepoints corresponding to brackish water phase, acute seawater transfer and 2 weeks in seawater (Supplementary Figure S2, S3). None of the investigated genes in the head kidney were differentially expressed comparing microalgae fed fish to control fish, although a diet effect in the main model was detected (Supplementary Table S2). A few genes were found to be

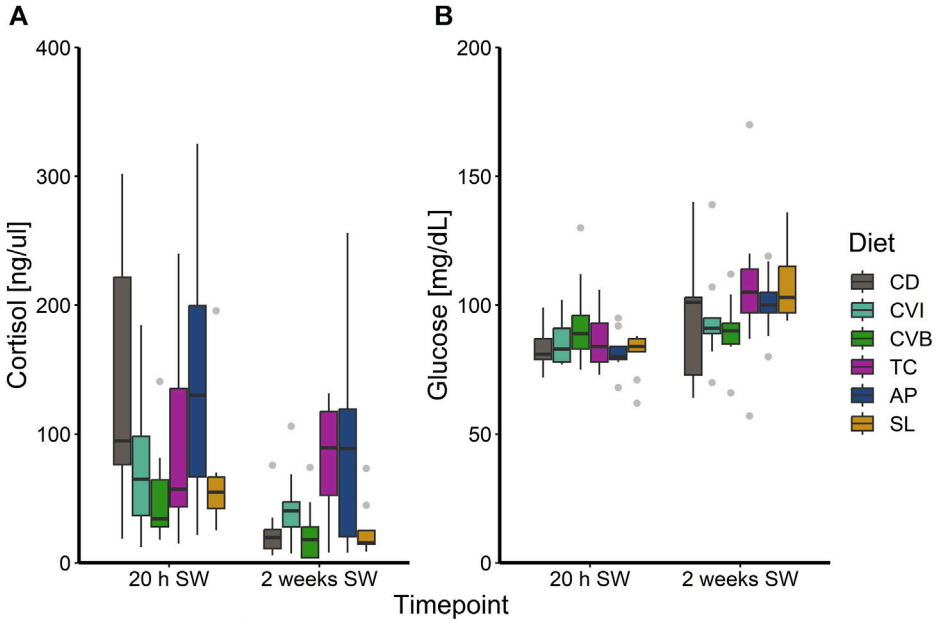

FIGURE 5
Boxplot with median and 1.5 x interquartile range whiskers showing plasma cortisol **(A)** and glucose **(B)** of Atlantic salmon 20 h in seawater (T2) and 2 weeks in seawater (T3) fed with the microalgae enriched diets *C. vulgaris* intact (CVI), *C. vulgaris* broken (CVB), *T. chuii* (TC), *A. platensis* (AP) or *S. limacinum* (SL) at 8% inclusion or a control diet (CD); $n = 9$.

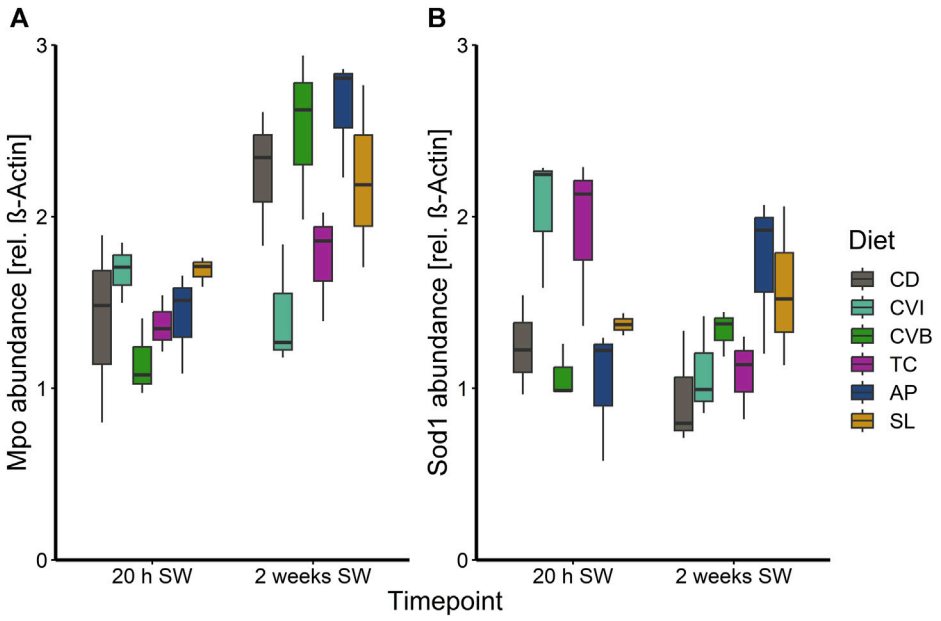

FIGURE 6
Boxplot with median and 1.5 x interquartile range whiskers showing protein abundance of myeloperoxidase **(A)** and superoxide dismutase 1 **(B)** in liver tissue of Atlantic salmon 20 h in seawater (T2) and 2 weeks in seawater (T3) fed with the microalgae enriched diets *C. vulgaris* intact (CVI), *C. vulgaris* broken (CVB), *T. chuii* (TC), *A. platensis* (AP) or *S. limacinum* (SL) at 8% inclusion or a control diet (CD); $n = 3$ pool of 3 fish per tank.

differentially regulated in the gill of salmon fed particular microalgae as compared to the control (Supplementary Table S3; Figure 7). *Clra*, *c1ql2* and *camp* were reduced in fish fed AP 20 h after seawater transfer (Figures 7A–C). While expression of clra was reduced only modest as compared to the control (0.43-fold, $p < 0.05$), *c1ql2* (0.10-fold, $p < 0.01$) and *camp* (0.23-fold, $p = 0.077$) were more strongly reduced.

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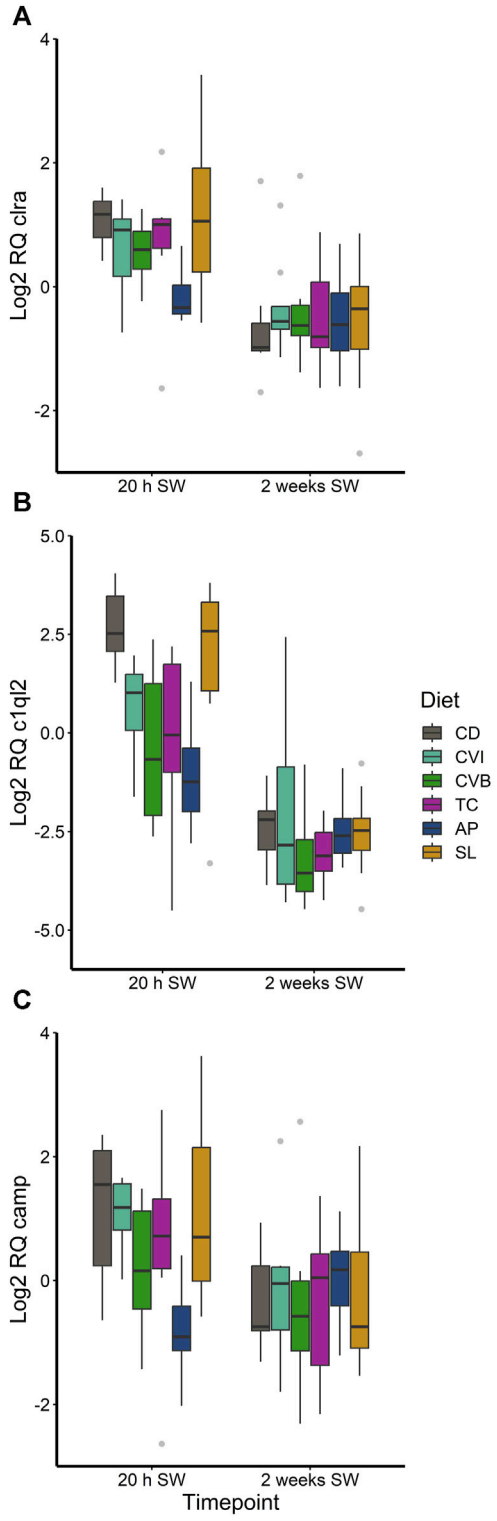

FIGURE 7
Boxplot with median and 1.5 x interquartile range whiskers showing gene expression of *clra* **(A)**, *c1ql2* **(B)** and *camp* **(C)** in the gill of Atlantic salmon 20 h in seawater (SW) and 2 weeks in seawater fed with the microalgae enriched diets *C. vulgaris* intact (CVI), *C. vulgaris* broken (CVB), *T. chuii* (TC), *A. platensis* (AP) or *S. limacinum* (SL) at 8% inclusion or a control diet (CD), *n* = 6-9.

4 Discussion

4.1 Salinity change following BW acclimation does not impair the osmoregulatory ability

Upon completed smoltification salmon smolts show hypo-osmoregulatory capacity and are ready for entering seawater. However, it is generally accepted that holding salmon smolts for longer periods after smoltification in freshwater or brackish water can induce a process known as desmoltification, where the physiological ability to tolerate seawater is transiently lost (Mortensen and Damsgård, 1998; Stefansson et al., 1998). Although the salmon in the present study were reared in brackish water for several months, the fish were able to regulate their ion balance well within physiological limits upon transfer to seawater. The slight increase in plasma Na^+ and Cl^- concentrations 20 h after SWT returned to basal levels after 2 weeks in SW, similar to findings from McCormick et al. (2013). In the gill, the sodium-potassium pump Na^+/K^+-ATPase (NKA), plays a crucial role in regulating osmolality and ion homeostasis: the NKA regulates the absorption of ions in freshwater and their secretion in seawater (Zaugg and Wagner, 1973; Evans et al., 2005). While the NKA isoform NKA α1a dominates in pre-smolts in freshwater, levels of the seawater isoform NKA α1b increase during smoltification and dominate in seawater (McCormick et al., 2009). The ability to regulate osmolality properly in seawater was also reflected by a greatly increased *nkaα1b*/*nkaα1a* ratio in the gill. In line with previous findings (Nilsen et al., 2007; McCormick et al., 2013) *nkaα1b* expression increased further in SW, while expression of *nkaα1a* decreased strongly.

4.2 Acute and prolonged salinity exposure induce divergent responses in the head kidney and gill of Atlantic salmon

Seawater transfer is a critical process during the life-cycle of Atlantic salmon in the wild, as well as under production. To cope with the associated altered environmental conditions, a stress response is triggered that leads to the activation of the hypothalamic-pituitary-interrenal (HPI) axis (Wendelaar Bonga, 1997). Hereby, stress hormones (such as cortisol and adrenaline) are released that induce a variety of physiological changes, allowing the fish to adapt to new the environment and return to internal homeostasis (Wendelaar Bonga, 1997). In addition to changes in, for example, oxygen uptake, metabolism or hydromineral balance, the immune system is also affected, mainly with regard to lymphocyte activities and the levels of circulating cytokines (Tort, 2011). Due to the wide range of stressors and the duration of their exposure, the stress response and also the interlinked immune response adapts accordingly, at the systemic but also at the local tissue level.

In this study, we examined how salinity change affects the expression of immune- and stress-related genes in the head kidney and gill, to get an understanding of the systemic and local immune and stress response after seawater transfer. We found that in the head kidney, the genes dominating the overall gene-expression pattern were mainly involved in antiviral defense (*ifit5*, *isg15*, *rsad2*, *znfx1*), and additionally in anti-

oxidant activity (*cat*), antimicrobial defense (*mpo*) and Th1 immunity (*il18*). Transcriptional suppression of immune genes, particularly related to antiviral defense, were found during smoltification, and these effects continued after seawater transfer (Johansson et al., 2016). Interestingly, our data indicates that salinity change *per se* has an effect on antiviral immunity. This might be an evolutionary conserved response resulting from an energy-allocation trade-off upon acclimation to seawater. Concomitantly, the modulated expression of antiviral genes may be directly connected to the abundance of different viruses in freshwater and marine habitats (Maranger and Bird, 1995; Clasen et al., 2008) which requires further investigation.

Similar to the head kidney, the overall expression pattern in the gill was dominated by the antimicrobial gene *mpo* and antiviral-related genes *isg15* and *rsad2*. In addition, the antiviral gene *irf1* and the transmembrane protein-encoding gene *cd40* (alias *tnfrsf5*) majorly influenced the expression pattern of the gill. CD40 is a marker for antigen-presenting cells including neutrophilic granulocytes, dendritic cells and macrophages (Lagos et al., 2012) and could thus indicate the remodeling of the cellular immunity in the gill after salinity change, which has been found to occur in Atlantic salmon during smoltification and following seawater transfer from freshwater (West et al., 2021).

The gill is in direct contact with the environment and is therefore responsible for a local immune response. In our study, salinity change caused a suppression of several genes in the gill. Shortly after SWT, *fcgr1a* encoding an immunoglobulin Fc receptor (FcRs) was reduced. These receptors are present on the surface of phagocytes (Stafford et al., 2006). The reduction of *fcgr1a* likely indicate a decreased number of phagocytes in the gill maybe associated with an impaired antigen recognition capability. This effect is probably related to the acute stress resulting from acute salinity change, as 2 weeks later *fcgr1a* returned to the level similar to before seawater transfer. Furthermore, we observed decreased transcript levels of *c1ql2* 20 h and 2 weeks after salinity change. *C1ql2* encodes a protein which is structurally related to C1q (Köbis et al., 2017) and although its precise role is not fully defined in salmonids yet, c1ql2 might serve as a carbohydrate-binding protein (known as lectins) and bridge between innate and adaptive immunity (Köbis et al., 2017; Hillestad et al., 2020). Johansson et al. (2016) described a similar pattern in the gill of Atlantic salmon one and 3 weeks after seawater transfer, with *c1ql2* transcript numbers in gill half those relative to pre-smolts. Additionally, two genes (*il1b*, *hamp*) involved in early immune-related pathways were reduced 20 h after SWT, and still 2 weeks later. *Il1b* encodes the pro-inflammatory cytokine il-1β (Secombes et al., 2011) and *hamp* encodes for the antimicrobial peptide hepcidin (Douglas et al., 2003). Lastly, the transcript levels of the antiviral defense genes *isg15, rsad2* and *ifit5* were reduced 2 weeks after SWT and this observation may suggest that alternative defense strategies may be more beneficial in salmon exposed to salinity change. In this context it may be noted that *isg15* and *rsad2* were downregulated both in the head kidney and gill during acclimation to seawater and could thus indicate a systemic reaction.

Several genes in the head kidney were activated in our study after salinity change. Especially *camp* was greatly enhanced at both timepoints in seawater. *Camp* encodes an antimicrobial peptide with immunomodulatory activities (Scocchi et al., 2009). Furthermore, two biomarker genes (*drtp1*, *saa5*) of the acute-phase and stress response (Talbot et al., 2009; Lee et al., 2017) in fish were induced shortly after SWT. In parallel, several genes were suppressed in the head kidney after salinity change, similar as observed in the gill. *Sod1* is one of the genes, which was downregulated 20 h and 2 weeks after SWT. Since this biomarker gene of oxidative stress encodes a potent antioxidant, the reduction of *sod1* therefore suggests a diminished antioxidant defense or decreased amount of reactive oxygen species (ROS) in the gill after salinity change. In previous research, a change in salinity has been found to induce oxidative stress in fish (Liu et al., 2007; Lushchak, 2011). Blood samples of the freshwater fish Nile tilapia *Oreochromus niloticus* had a lower superoxide dismutase and catalase activity when fish were held in higher salinity (4 or 12 psu, respectively) than in the control group for 2 weeks (Elarabany, 2017). In contrast, in liver of anadromous chum salmon *Oncorhynchus keta,* superoxide dismutase and catalase activity was significantly enhanced in fish held for 42 days in 8, 16 and 24 psu compared to fish kept in 0 psu (Li et al., 2022). Moreover, in addition to antiviral genes (*isg15*, *rsad2*, *znfx1*, *ifit5*), *lao1* was also reduced merely 2 weeks after SWT. *Lao1* encodes for L-amino acid oxidases with antibacterial and antiparasitic activity (Kitani and Nagashima, 2020). The downregulation of these genes with important roles in immune defense may be due to the low pathogen pressure in RAS and therefore, energy could be rather directed to cope with salinity change than to maintain increased immune barriers.

In this study, expression of several immune-genes (*c1ql2, fcgr1a, hamp, ifit5, il1b, isg15, rsad2*) was reduced in the gill while in the head kidney both an induction (*camp, drtp1, saa5*) and reduction (*ifit5, isg15, lao1, sod1, znfx1*) of genes was observed after salinity change. However, these alterations in transcript levels were rather modest, possibly due to the transfer within the protected RAS environment with a lower pathogen pressure than the open marine environment. Due to different experimental setups, it is difficult to compare our results with other studies investigating freshwater to seawater transfer (Johansson et al., 2016; Karlsen et al., 2018; Lund et al., 2022), but the general pattern of gene expression is similar.

4.3 Microalgae have limited potential for modulating the response to changing salinity

Microalgae could contribute to improve the fish's ability to cope with salinity, as their immune-stimulatory and stress-modulatory properties have previously been demonstrated (Cerezuela et al., 2012; Zhang et al., 2014; Raji et al., 2018). Since each of the microalgae species have their own beneficial characteristics (based on various functional compounds), this study evaluated the effects of different microalgae-enriched functional diets on the stress and immune status of salmon

after salinity change. The overall growth performance among the different diet groups was not different over the 10-week trial period (Mueller et al., 2023) and furthermore similar condition and organ indices among the diet groups indicate that the incorporated microalgae did not impair growth nor energy reserves of the fish.

Cortisol is the primary stress hormone in fish and rapidly elevated in response to acute stress (Wendelaar Bonga, 1997; Barton, 2002) and alongside with glucose used as the primary stress indicator in fishes (Sopinka et al., 2016). Moreover, high cortisol levels induce hypo-osmoregulatory ability during smoltification (Langhorne and Simpson, 1986). We also observed increased levels of cortisol in post-smolts after 20 h in seawater, probably resulting from a response to salinity change rather than to handling, as cortisol in similarly handled fish that had been transferred back into brackish water, remained on a basal level. After 2 weeks in seawater, the levels of cortisol decreased, most notable for the control group and likely due to adaptation to the new environment of the fish. Notably, the functional diets did not influence this primary stress response. Interestingly glucose levels showed an opposite trend and we can only speculate why glucose levels were not elevated initially but increased slightly over time in seawater. The slightly lower glucose levels at 20 h in SW might have resulted from the usage of glucose as an energy substrate.

In addition to the transcriptional profiles in the head kidney and gills, we determined the protein levels of myeloperoxidase in the liver. Myeloperoxidase is an antimicrobial enzyme characteristic for neutrophilic granulocytes (Castro et al., 2008; Aratani, 2018; Chen et al., 2019). Fish fed with the control diet, or diets enriched with *C. vulgaris* broken and *A. platensis* showed an increase of Mpo levels over time in seawater, suggesting an increase in antimicrobial activity in the liver of these fish. The hepatic abundance of Sod1 can give insights into the antioxidant activity in the liver of the fish. The high Sod1 concentration in liver of fish fed *C. vulgaris* intact and *T. chuii* acutely after salinity change (20 h in seawater) may provide a better oxidative state during this time period but protein levels potentially decreased 2 weeks in seawater because lower ROS levels were subsequently present. In line with these results, previous research on salmon fed with a pre-extruded *Tetraselmis* diet also revealed significantly induced *sod1* expression in the liver (Sørensen et al., 2021).

Overall, microalgae enriched diets only induced subtle changes in immune-gene expression. This can be likely explained by the fact that feed intake in seawater was drastically reduced (Mueller et al., 2023), which has limited the uptake of functional compounds and their immune modulatory effects. The functional diet supplemented with *A. platensis* caused a reduction of genes in the gill involved in antimicrobial defense (*camp*) and pathogen recognition (*clra*, *c1ql2*) and we can only speculate if these modest reductions in gene expressions are reflected at the protein level. However, extracts of *A. platensis* showed strong antimicrobial activity (Metekia and Ulusoy, 2023) and this could have reduced the need to produce antimicrobial peptides as well as pathogen recognition receptors, explaining its reduced expression.

Altogether, the transfer from brackish water to seawater had a more pronounced effect on the immune status of the salmon than the diet, which caused subtle changes in immune-gene expression. This is an important finding for the development of future stress and immune-mitigation strategies in aquaculture.

5 Conclusion

The current study evaluated the effect of salinity change from brackish water to seawater on Atlantic salmon. Although the fish showed full osmoregulatory ability after rearing in brackish water, salinity change caused a reduction of immune genes in the head kidney and gill even several months after smoltification. The herein-investigated microalgae had only marginal potential to modulate the observed responses. Future research should explore whether other functional feed additives, e.g., beta-glucans can stimulate the immune system of salmon during this critical period. Furthermore, different salinity adaptation strategies and their effect on the stress and immune status of Atlantic salmon need to be evaluated experimentally.

Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by the animal welfare officer of the "Fraunhofer IMTE Büsum" and the local authority of Schleswig-Holstein, according to the German animal welfare law (NTP—ID 00043858-1-0). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

DM: Data curation, Visualization, Writing–original draft, Investigation, Formal Analysis. JM: Writing–original draft, Data curation, Investigation, Methodology, Visualization, Conceptualization, Formal Analysis. JL: Data curation, Writing–review and editing. TS: Data curation, Writing–review and editing. EM: Funding acquisition, Writing–review and editing. HS: Supervision, Writing–review and editing, Project administration. AR: Supervision, Writing–review and editing. CS: Conceptualization, Funding acquisition, Writing–review and editing, Project administration. TG: Conceptualization, Funding acquisition, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2024.1338858/full#supplementary-material

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5.4. Publication IV: Lost and found: The family of NF-κB inhibitors is larger than assumed in salmonid fish

NF-κB inhibitors are potentially useful bioindicators. They control NF-κB proteins that regulate immune and stress responses following activation by various environmental and endogenous signals. In this study, *nfkbi* genes in the salmonid fish rainbow trout *Oncorhynchus mykiss* were identified and compared through synteny. The transcript levels of the *nkfbi* genes were measured in different tissues and immune cell fractions. Through alignments and phylogenetic and structural analyses, the different iκb proteins were characterized. I further investigated the regulation of NF-κB pathways in salmonids by localizing iκbα and iκbε proteins in a salmonid cell line and assessing their influence on NF-κB activity. Finally, the expression of NF-κB-dependent immune genes in the same salmonid cell line was analyzed upon overexpression of iκbα and iκbε.

Highlights

- All *nfkbi* genes from rainbow trout are present as pairs of ohnologs.
- It is likely that iκbβ is not lost in the genome of salmonids, but is currently misnamed as iκbα.
- The transcript levels of *nfkbia-a* and *nfkbie* were significantly higher in immune-relevant tissues, as well as *nfkbia-a* transcript levels in a cell fraction enriched with granulocytes, monocytes/macrophages, and dendritic cells from the head kidney of rainbow trout.
- The full protein version of iκbα was mostly localized in the cytoplasm, while iκbε proteins were equally distributed in the nucleus and cytoplasm.
- Overexpression of both iκb proteins blocked basal and stimulated NF-κB activity in a salmonid fish cell line.
- The inflammatory markers il1b, cxcl8a, and also nfkbia were increased after zymosan stimulation of the salmonid fish cell line.

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Lost and Found: The Family of NF-κB Inhibitors Is Larger than Assumed in Salmonid Fish

Doret R. van Muilekom [1], Bertrand Collet [2], Henrike Rebl [3], Kristina Zlatina [4], Fabio Sarais [1], Tom Goldammer [1,5,*] and Alexander Rebl [1,*]

[1] Institute of Genome Biology, Research Institute for Farm Animal Biology (FBN), 18196 Dummerstorf, Germany; muilekom@fbn-dummerstorf.de (D.R.v.M.); sarais@fbn-dummerstorf.de (F.S.)
[2] VIM, UVSQ, INRAE, Université Paris-Saclay, 78350 Jouy-en-Josas, France; bertrand.collet@inrae.fr
[3] Department of Cell Biology, Rostock University Medical Center, 18057 Rostock, Germany; henrike.rebl@med.uni-rostock.de
[4] Institute of Reproductive Biology, Research Institute for Farm Animal Biology (FBN), 18196 Dummerstorf, Germany; zlatina@fbn-dummerstorf.de
[5] Faculty of Agriculture and Environmental Sciences, University of Rostock, 18059 Rostock, Germany
* Correspondence: tomgoldammer@fbn-dummerstorf.de (T.G.); rebl@fbn-dummerstorf.de (A.R.)

Abstract: NF-κB signalling is largely controlled by the family of 'inhibitors of NF-κB' (IκB). The relevant databases indicate that the genome of rainbow trout contains multiple gene copies coding for iκbα (*nfkbia*), iκbε (*nfkbie*), iκbδ (*nkfbid*), iκbζ (*nfkbiz*), and *bcl3*, but it lacks iκbβ (*nfkbib*) and iκbη (*ankrd42*). Strikingly, three *nfkbia* paralogs are apparently present in salmonid fish, two of which share a high sequence identity, while the third putative *nfkbia* gene is significantly less like its two paralogs. This particular *nfkbia* gene product, iκbα, clusters with the human IκBβ in a phylogenetic analysis, while the other two iκbα proteins from trout associate with their human IκBα counterpart. The transcript concentrations were significantly higher for the structurally more closely related *nfkbia* paralogs than for the structurally less similar paralog, suggesting that iκbβ probably has not been lost from the salmonid genomes but has been incorrectly designated as iκbα. In the present study, two gene variants coding for iκbα (*nfkbia*) and iκbε (*nfkbie*) were prominently expressed in the immune tissues and, particularly, in a cell fraction enriched with granulocytes, monocytes/macrophages, and dendritic cells from the head kidney of rainbow trout. Stimulation of salmonid CHSE-214 cells with zymosan significantly upregulated the iκbα-encoding gene while elevating the copy numbers of the inflammatory markers interleukin-1-beta and interleukin-8. Overexpression of iκbα and iκbε in CHSE-214 cells dose-dependently quenched both the basal and stimulated activity of an NF-κB promoter suggesting their involvement in immune-regulatory processes. This study provides the first functional data on iκbε—versus the well-researched iκbα factor—in a non-mammalian model species.

Keywords: IκB; innate immunity; immune regulation; NF-κB; nfkbia; nfkbie

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1. Introduction

The family of NF-κB (nuclear factor kappa-light chain-enhancer of activated B cells)/Rel transcription factors is activated by a broad range of environmental and endogenous cues, including viral and bacterial pathogen-associated molecular patterns (PAMPs) and cytokines [1,2]. The activated NF-κB pathways strongly drive immune and stress responses [3,4], as they direct inflammatory processes and cell growth, differentiation, and survival [5–9].

NF-κB activity is primarily controlled by a dynamic interplay between the inhibitors of NF-κB (IκB) and their opponents, IκB kinases (IKK) [10,11], thereby enabling adaptation to the prevailing circumstances and preventing excessive immune responses [12]. The family of NF-κB inhibitors comprises nine members in mammals [10,13–22], each with different or mutual affinities for various combinations of NF-κB/Rel dimers [23–25]. Similarly, the IκB

family is subdivided according to the structural and functional properties of its members. IκBα, IκBβ, and IκBε are considered canonical IκB proteins, as they retain NF-κB/Rel factors in the cytosol by masking their nuclear translocation signals (NLS) [10,15,26,27] or by capturing free NF-κB/Rel factors in the nucleus and exporting them back to the cytoplasm [28]. The atypical IκB proteins IκBζ, IκBδ (aka IκBNS), IκBη, and BCL3 control the transcriptional activity of NF-κB in the nucleus [18,19,25,29]. IκBδ is a repressive regulator [19,30,31], whereas IκBη is an activating regulator [20], and IκBζ and BCL3 act as both repressors and activators of NF-κB-driven gene transcription [25,32,33]. The NF-κB precursor proteins p105/NF-κB1 and p100/NF-κB2 also share sequence similarities with Iκb factors, thereby allowing them to bind and retain preformed NF-κB/Rel proteins in the cytoplasm [14,21].

The ankyrin-repeat motif is the evolutionarily best conserved feature of all IκB proteins [34]. The number of ankyrin repeats determines the binding specificity of IκBs to the Rel homology domain (RHD) of NF-κB/Rel proteins [23,26,34–38], including p105/NF-κB1 and p100/NF-κB2. Mammalian IκB proteins contain 5 to 7 ankyrin repeats about 30 to 33 amino acids in length [39]. In addition to the ankyrin-repeat motifs, the mammalian IκB proteins IκBα and IκBβ contain a C-terminal PEST sequence [40,41], rich in proline (P), glutamate (E), serine (S), and threonine (T) residues. These PEST sequences prevent the NF-κB factors from binding to their response elements, thereby regulating the basal turnover of IκBα and IκBβ [23,40,42,43]. Moreover, all mammalian canonical IκB proteins possess signal-responsive serine residues at their N-termini [27,44,45] that are phosphorylated by the ternary IKK complex (IKKα, IKKβ, and NEMO/IKKγ) following an appropriate stimulus [11]. This phosphorylation triggers the polyubiquitination and proteasomal degradation of the IκB proteins [45]. The NF-κB/Rel subunits then migrate into the nucleus, where they dimerise and induce the transcription of predominantly immuno-relevant target genes [46]. Included in this panel of target genes is the IκBα-encoding *NFKBIA* gene, which serves as part of an autocrine loop [47] to safeguard the oscillation of NF-κB between the nucleus and cytoplasm [47]. In general, the degradation and resynthesis of the various mammalian NF-κB inhibitors depend on adequate stimuli [48–50] and the presence of associated factors, such as the NF-κB-inhibitor-interacting Ras-like proteins (NKIRAS) [51,52].

Several members of the IκB family have been well conserved during evolution, as orthologs have been identified in birds [23], fishes [53], and even insects [54]. However, a detailed characterisation of the iκb proteins from lower vertebrates is still pending. Iκbα is the best researched iκb ortholog and has been characterised in different fish species [53,55–64], whereas its iκbβ paralog has apparently been lost in various bony fish families. According to the gene database of the National Center for Biotechnology Information (NCBI), the iκbβ-encoding *nfkbib* gene is absent from the sequenced genomes of the Salmonidae, Percidae, Gadidae, Carangidae, and Oryziinae, although it is still present in other teleost fish species from the Cyprinidae or Ictaluridae (as of December 2022). Iκbη encoded by the *ankrd42* gene is apparently absent from most of the teleostean genomes sequenced so far.

In this report, we provide evidence that iκbβ has not been lost from salmonid genomes, and we characterise this putative iκbβ together with the canonical iκbα and iκbε proteins, the nuclear iκbδ and iκbζ proteins, and bcl3 from the rainbow trout salmonid fish (*Oncorhynchus mykiss*). The findings of the present study, therefore, provide a comprehensive overview of the structural and functional diversity of nf-κb inhibitors in a non-mammalian model species while also offering starting points for further research into the inflammatory signalling processes in bony fish.

2. Results

2.1. Iκb Proteins from Rainbow Trout Are Encoded on 14 Distinct Genes

Our search of the NCBI gene database for *NFKBI* orthologs in the rainbow trout *Oncorhynchus mykiss* (assembly USDA_OmykA_1.1) yielded six *nfkbia*, two *nfkbie*, two *nfkbid*, two *nfkbiz*, and two *bcl3* genes (Table 1). By contrast, the orthologs of *NFKBIB* and *ANKRD42* seemed absent not only in salmonids but in many other teleost fishes. The same

number of *nfkbi* genes was present in the closely related Chinook salmon, *Oncorhynchus tshawytscha* (assembly Otsh_v2.0) (Table 1, last column).

Table 1. *Nfkbi* genes identified in rainbow trout *O. mykiss*.

Gene	Chromosome	LOC Symbol	CDS Length [nt]	UTR Length [bp] 5′	UTR Length [bp] 3′	Polyadenylation Motifs [a]	Instability Motifs	Prot. NCBI Acc.#	Protein Length [aa]	Ankyrin Repeats	Orthologs in Chinook Salmon
nfkbia-a1	4	LOC110522049	942	91	368	4	4	XP_021455792	313	6	LOC112217446
nfkbia-a2	8	LOC100136058	945	49	407	4	2	NP_001117840	314	6	LOC112249975
nfkbia-b1	19	LOC110497729	1005	66	454	3	8	XP_021429724	334	5	LOC112244922
nfkbia-b2	25	LOC110505735	963	75	438	4	8	XP_021440813	320	5	LOC112256803
nfkbia-c1	10	LOC110533787	1176	403	1408	9	8	XP_021473979	391	6	LOC112250205
nfkbia-c2	12	LOC110537332	1191	425	1420	9	13	XP_021478957	396	6	LOC112257706
nfkbie-a1	4	LOC110522047	1059	392	885	8	16	XP_021455790	352	6	LOC112217444
nfkbie-a2	8	LOC110529432	1065	449	1421	5	14	XP_021467277	354	6	LOC112249971
nfkbid-a1	2	LOC110538540	1461	117	721	2	2	XP_021481102	486	6	LOC112245876
nfkbid-a2	3	LOC118936618	1707	536	797	1	2	XP_036826041	568	6	LOC112227108
nfkbiz-a1	7	LOC110527232	1605	106	1369	4	8	XP_036838138	534	6	LOC112247547
nfkbiz-a2	18	LOC110495544	1605	108	1369	4	8	XP_036807230	534	6	LOC112266936
bcl3-a1	2	LOC110537869	2061	444	380	7	9	XP_021479984	686	6	LOC112235280
bcl3-a2	3	LOC110510160	2010	1728	414	8	11	XP_021447196	669	6	LOC112226570

[a] AATAAA and ATTAAA.

All *nfkbi* genes from rainbow trout are present as pairs of ohnologs that most likely arose from a whole-genome duplication, which is also reflected by their location on separate chromosomes [65,66]. Additionally, the *nfkbia* genes clearly exist as three pairs of duplicated paralogs (Figure 1): (a) one pair of ohnologous *nfkbia* genes (a1 and a2 on chromosomes 4 and 8, respectively) is located in the immediate vicinity of the genes *hsp90b* and *psma6*; (b) a second *nfkbia* pair (*b1* and *b2* on chromosome 19 and 25) both neighbour the gene *insm2* and lie in the vicinity of the gene *fam177a1*; and (c) the third *nfkbia* gene pair (on chromosomes 10 and 12) has been annotated adjacent to the genes *gjd2* and *zscan21*. The ohnologous *nfkbie* genes *a1* and *a2* are both in direct proximity to the genes *tmem15b* and *slc35b2*, located on the same chromosomes as the *nfkbia* genes *a1* and *a2*. The ohnologous *nfkbid* genes *a1* and *a2* on chromosomes 2 and 3 share *fxyd6* in their spatial vicinity, and the *bcl3* ohnologs on the same chromosomes are flanked by the genes *cbl* and *tom40*. The two *nfkbiz* ohnologs *a1* and *a2* on chromosomes 7 and 18 are adjacent to the genes *eed*, *znf*, and *epr1*.

Figure 1. Synteny between the *nfkbi* genes in rainbow trout, *O. mykiss*, determined using the NCBI gene database. Arrows represent the reading direction of genes found in synteny; the same colours indicate orthologous/ohnologous genes. Numbers indicate the chromosomal location in nucleotides. Not characterised genes are represented by hatched boxes.

The shorter coding sequences of *nfkbia* and *nfbkie* (both between 942 bp and 1191 bp in length) are distributed across 5 to 6 exons, while the coding sequences of *nfkbid* and *nfkbiz* are significantly longer (between 1461 bp and 1707 bp in length) and are divided over 9 to 12 exons. The *bcl3* ohnologs represent the longest *nfkbi* sequences (>2000 bp) in rainbow trout and are distributed across 12 exons. Based on automated computational analyses, the latest USDA_OmykA_1.1 assembly of the rainbow trout transcriptome includes one

shorter *nfkbia* transcript variant and four shorter *nfkbiz* transcript variants, two of which are non-sense mRNAs (Table 2). These variants most likely arose from exon skipping during splice events, but neither this assumption nor the existence of the predicted transcript isoforms has been experimentally validated.

Table 2. Predicted *nfkbi*-transcript isoforms from *O. mykiss* recorded in the NCBI database.

Nfkbi Transcript Isoform	Chromosome	Nucleotide NCBI Acc.#	LOC Symbol	CDS Length [nt]	UTR Length [bp]		Polyadenylation Motifs [a]	Instability Motifs	Prot. NCBI Acc.#	Protein Length [aa]	Ankyrin Repeats
					5′	3′					
nfkbia-c2.2	12	XM_021623283	LOC110537332	1101	425	1420	9	13	XP_021478958	366	6
nfkbiz-a1.2	7	XR_005052489	LOC110527232				None	0	No CDS		
nfkbiz-a1.3	7	XM_036982244	LOC110527232	1407	107	555	None	0	XP_036838139	468	5
nfkbiz-a1.4	7	XM_036982245	LOC110527232	1407	107	524	None	0	XP_036838140	468	5
nfkbiz-a2.2	18	XR_005037322	LOC110495544				None	0	No CDS		

[a] AATAAA and ATTAAA.

The sequence identity of the ohnologous *nfkbi*-encoded iκb proteins from rainbow trout (including bcl3) ranges from 82% to 100% (Table S1). The two pairs of the iκbα ohnologs a1/a2 and b1/b2 still share about 60% identity. However, comparison of the iκbα paralogs a1/a2 or b1/b2 versus c1/c2 reveals a sequence identity below 30%. This is a similarly low identity to that shared, for instance, by the paralogs iκbα and iκbε.

A phylogenetic analysis across the amino acid sequences of all IκB proteins from humans and fishes revealed that the ohnologous iκbδ, iκbε, and bcl3 sequences from rainbow trout form separate clusters with their counterparts in the other salmonid fishes, including Chinook salmon *O. tshawytscha*, Atlantic salmon *Salmo salar*, and brown trout *Salmo trutta* (Figure 2a), whereas the iκbζ isoforms instead cluster in a species-specific fashion.

Unexpectedly, a pair of ohnologous iκbα sequences (c1 and c2) cluster with the human IκBβ factor (dark blue section on the left side of the dendrogram in Figure 2a), while the other two pairs of iκbα ohnologs (a1 and a2, as well as b1 and b2) cluster—as previously supposed—with the human IκBα factor. The homology of the iκbα ohnologs c1 and c2 with iκbβ from other teleost species is also reflected by 79 amino acid residues between positions 171 and 390 that are shared with the iκbβ sequences from at least two other fish species, but different in one or both iκbα-a and iκbα-b paralogous pairs (Figure 2b).

The overall structural differences of the iκb proteins from rainbow trout are depicted with three-dimensional models highlighting the well-conserved ankyrin-repeat motifs (Figure 3a–g). A defined number of ankyrin-repeat motifs is a characteristic of all IκB proteins. The iκb proteins listed in the NCBI database for rainbow trout contain six ankyrin repeats, except for two iκbα isoforms (nfkbia-b1, nfkbia-b2) and two iκbζ variants (nfkbiz-a1.3, nfkbiz-a1.4) (Figure 3h). The iκbζ variants a1.3 and a1.4 lack the 6th ankyrin repeat, while the amino acid sequence of the 6th ankyrin repeat of the iκbα isoforms b1 and b2 differs from the canonical motif. In addition, the sequences of the 4th ankyrin repeats of both the iκbδ and iκbζ proteins differ from their counterpart sequences in iκbα and iκbε as well as bcl3.

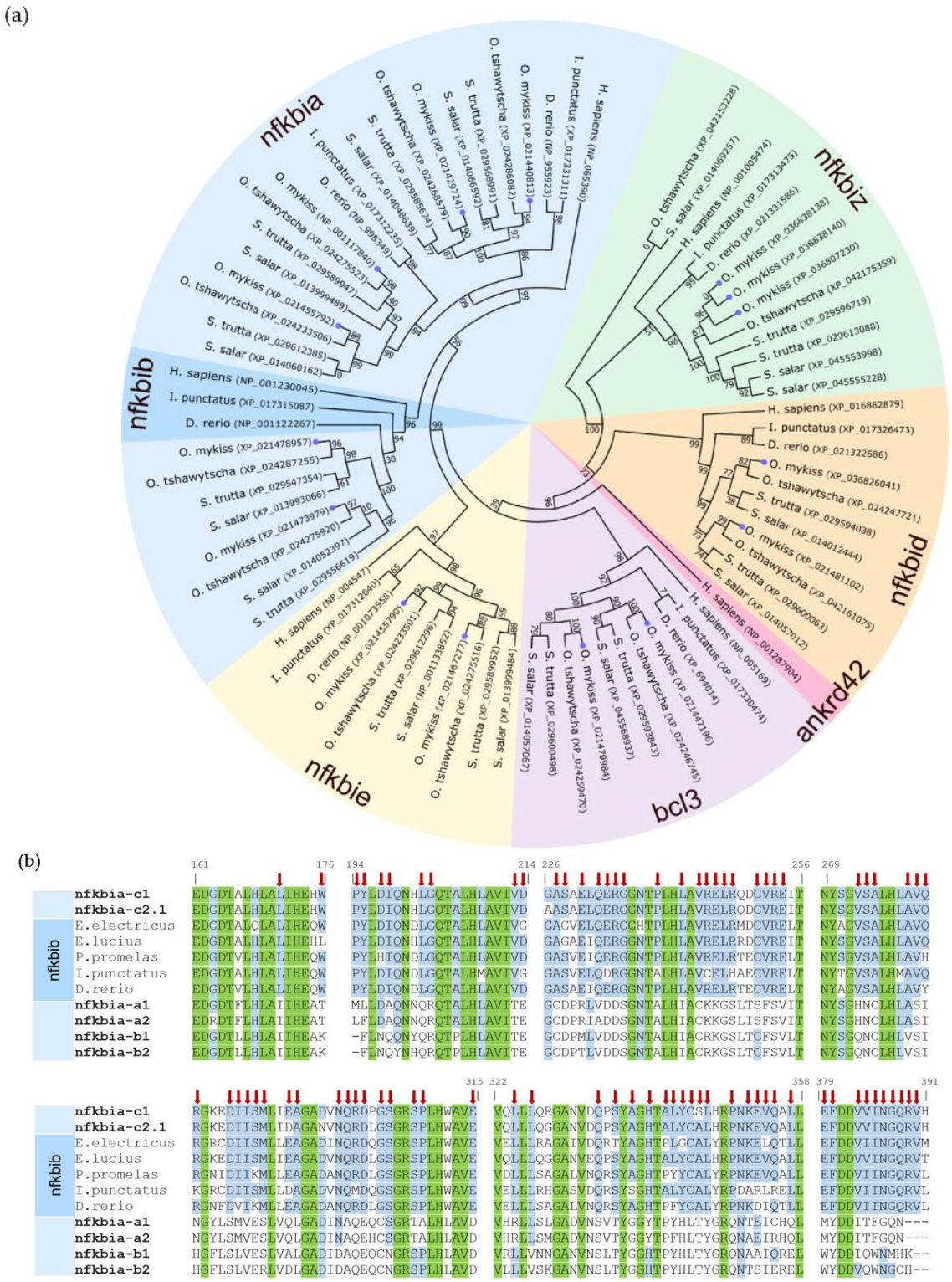

Figure 2. (**a**) Circular phylogenetic tree of selected IκB amino acid sequences from humans (*Homo sapiens*) and their orthologs in rainbow trout (*O. mykiss*; labelled with a purple dot at the branch end), Chinook salmon (*O. tshawytscha*), Atlantic salmon (*S. salar*), brown trout (*S. trutta*), channel catfish (*Ictalurus punctatus*) and zebrafish (*Danio rerio*); the NCBI accession codes are given in brackets. This neighbour-joining tree was constructed using the Poisson-correction distance model; bootstrap values are given at the nodes of each clade. The shaded underlays label the assignment to the IκB subfamilies iκbα/nfkbia (light blue), iκbβ/nfkbib (darker blue) iκbε/nfkbie (yellow), iκbδ/nkfbid

(orange), iκbζ/nfkbiz (green), iκbη/ankrd42 (pink), and bcl3 (purple). (**b**) Alignment of various amino acid sections of the six putative iκbα/nfkbia sequences from rainbow trout and five selected iκbβ/nfkbib orthologs from electric eel (*Electrophorus electricus*; XP_026884418), northern pike (*Esox lucius*; XP_010862954), fathead minnow (*Pimephales promelas*; XP_039505967), channel catfish, and zebrafish. Green underlay marks amino acid residues that are identical across all selected sequences; blue underlay denotes those residues conserved between nfkbia-c sequences and at least two nfkbib sequences. Red arrows denote residues identical in at least one of the nfkbia-c ohnologs from trout and at least two nfkbib sequences from the other fish species, but different in at least two of the nfkbia-a and nfkbia-b sequences. Amino acid positions given above the alignment refer to the nfkbiac1 sequence.

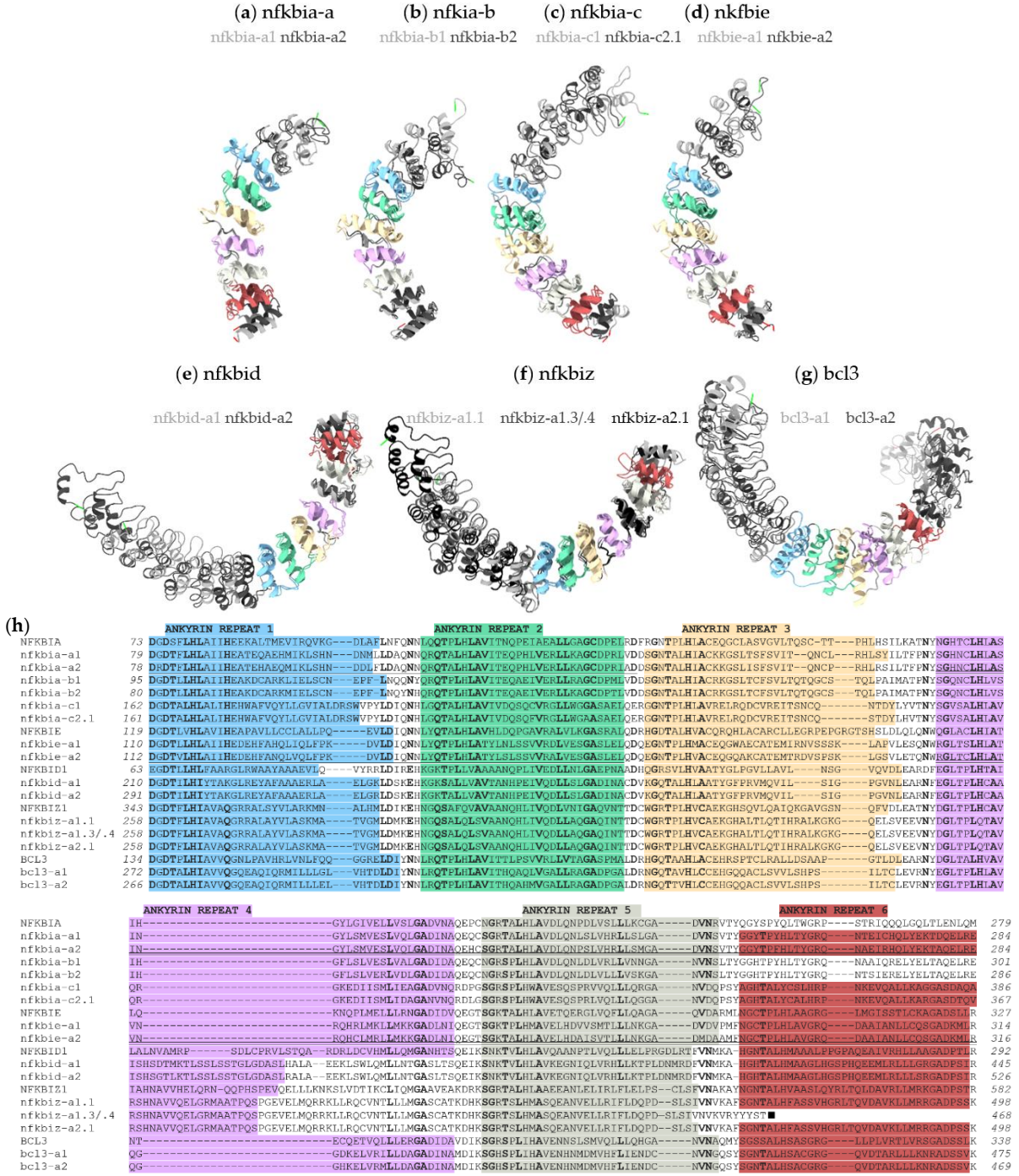

Figure 3. (**a**–**g**) Tertiary structures of the ohnologous iκb proteins from the rainbow trout (*O. mykiss*). One ohnolog is coloured in light grey, and the other one is coloured in dark grey (or black for nfkbiza-2.1)

to indicate structural (dis)similarities. The individual ankyrin-repeat domains are each shown in different colours. The N- and C-termini of each protein are indicated by green and red stretches, respectively. (**h**) Alignment of the ankyrin-repeat domains of the iκb proteins from rainbow trout and their human orthologs. The six ankyrin-repeat domains are coloured according to the above 3D structures (**a**–**g**). Bold characters mark amino acid residues that are well conserved across the aligned IκB sequences. A single underline indicates the sequence used for the expression constructs 'nfkbia-AR12' and 'nfkbie-AR12', while a double underline indicates the sequence used for the expression constructs 'nfkbia-AR456' and 'nfkbie-AR456'. The black square indicates the end of the protein sequences of nfkbiza-1.3 and nfkbiz-a1.4. (For the NCBI accession codes see Figure 2).

2.2. IκBα-Encoding *nfkbia-a* Transcripts Are Most Strongly Expressed in Immune Tissues and Immune-Cell Fractions

Fifteen genes from rainbow trout produce (at least) seventeen transcript variants coding for iκbα, iκbε, iκbδ, iκbζ, or bcl3 (Tables 1 and 2), as identified in the relevant gene database. We determined the transcript levels of the individual *nfkbi* genes in nine selected tissues and two sorted immune-cell fractions by designing primer pairs common to ohnologs of the *nfkbia-a*, *nfkbia-b*, *nfkbia-c*, and *bcl3* genes as well as primer pairs discriminating between the ohnologs of *nfkbie*, *nfkbid*, and *nfkbiz* (Tables 3 and S2). Across the quantified *nfkbia* genes, the *nfkbia-a* transcripts had the highest levels in the spleen (4.1×10^6 copies), gills (3.6×10^6 copies), head kidney, and trunk kidney (1.1 to 1.7×10^6 copies) and exceeded the levels of the *nfkbia-b* ohnologs by 2.6- to 3.7-fold and the *nfkbia-c* ohnologs by 37- to 66-fold in the same four tissues (with $p < 0.05$) (Figure 4a).

Table 3. Primers used in this study for quantitative PCR analysis.

Gene	Primer Sequence 5′→3′ (Sense, Antisense)	NCBI-Nucleotide Accession Code	Fragment Length [bp]
nfkbia-a	GCATGTCTGATGATGAACAGATG, GAACTCCAGGTCCCAGAAGCC	XM_021600117, NM_001124368 [b]	149
nfkbia-b	ACCCAGCTCCCAGCCATTATG, GACATCGATGCACAGGAGCAG	XM_021574049 [a], XM_021585138	135
nfkbia-c	GGGAGCTGAGGCAGGACTGT, CAACTACTCGGGGGTGAGTGC	XM_021618304, XM_021623282 [a], XM_021623283 [a]	91
nfkbid-a1	AGTCAGCCGTATCATCTATGTTTT, CTTTATGTAGGCCGTTTGTGATC	XM_036970146	153
nfkbid-a2	AGGTTGAATCCAGACATCTGTAC, AATAATGGCTAGCTAGTAATGAGC	XM_021625427	191
nfkbie-a1	CTGTAGGGTTATTTATCGTTGTTG, ATTCTCTGCTAGCAAAGTGGTAC	XM_021600115	107
nfkbie-a2	GCAACCGCTACCTTTGGTTTCA, CGGTCAAGACTACCTGGAGTG	XM_021611602	140
nfkbiz-a1.1, *nfkbiz-a2.1*	TCGTCAATGTCAAGGCATTCAGT, AAGAACCTGGAGAATGAGCAGC	XM_036982243, XM_036951335	144
nfkbiz-a1.3, *nfkbiz-a1.4*	TTCTGAGCTGACAAACAGTGTTC, ACTAAACCCTCTAACATGAGTTCT	XM_036982244, XM_036982245	86
bcl3-a	GCCAGTCGTACAGTGGGAACA, CAAGAACAAGAWGGTAACAGATGT	XM_021624309, XM_021591521	160
cxcl8a	CCATTACTGAGGGGATGAGTCTG, GAGACACTGAGATCATTGCCACTC	XM_021625342 [a], XM_024415648	153
cxcl8b	CTACATGATACAAGGGAGAGG, GGAAGAAGTCATTGTCACAC	XM_036989276, XM_024434566	146
il1b	GCTGTGGAAGAACATATAGTGTTGG, GCTACCACAAAGTGCATTTG	XM_036979104, XM_024418276	198
il10	ATGAACAACAGAACACAGAACAACA, CCAATGTAGGAACTACTTCTCCTG	NM_001245099 [a], NM_001246350 [c], XM_042324963	113

Table 3. *Cont.*

Gene	Primer Sequence 5′→3′ (Sense, Antisense)	NCBI-Nucleotide Accession Code	Fragment Length [bp]
nfkbia	GCACAGGAACAATGTAGCG, GATGAACAGATGTACGATGACATTAC	XM_021600117, XM_024377738	281
nfkbie	AGGAGCGGTTGGATTCTGCTTAT, CCTTCTCACCACCATCACTGAA	XM_021600115 [b], XM_021611602, XM_024419748, XM_024377733 [a]	158
tgfb	CATTCCAAGGTGCTAGGTCTGT, ACATCGGCAAGACCCCCAAGA	XM_024386204, XM_024402049 [a], XM_021563342, XM_021596503 [a]	121
tnf	TTTACCTGGCACTCCAAGGATC, GCATACCCTGAGACAACTCTCT	XM_024407165, XM_021572765 [c], XM_036971683 [c]	93

[a] Indicates one mismatch of the primer sequence in comparison with the CDS sequence. [b] Indicates two mismatches of the primer sequence in comparison with the CDS sequence. [c] Indicates at least two mismatches of the primer sequence in comparison with the CDS sequence.

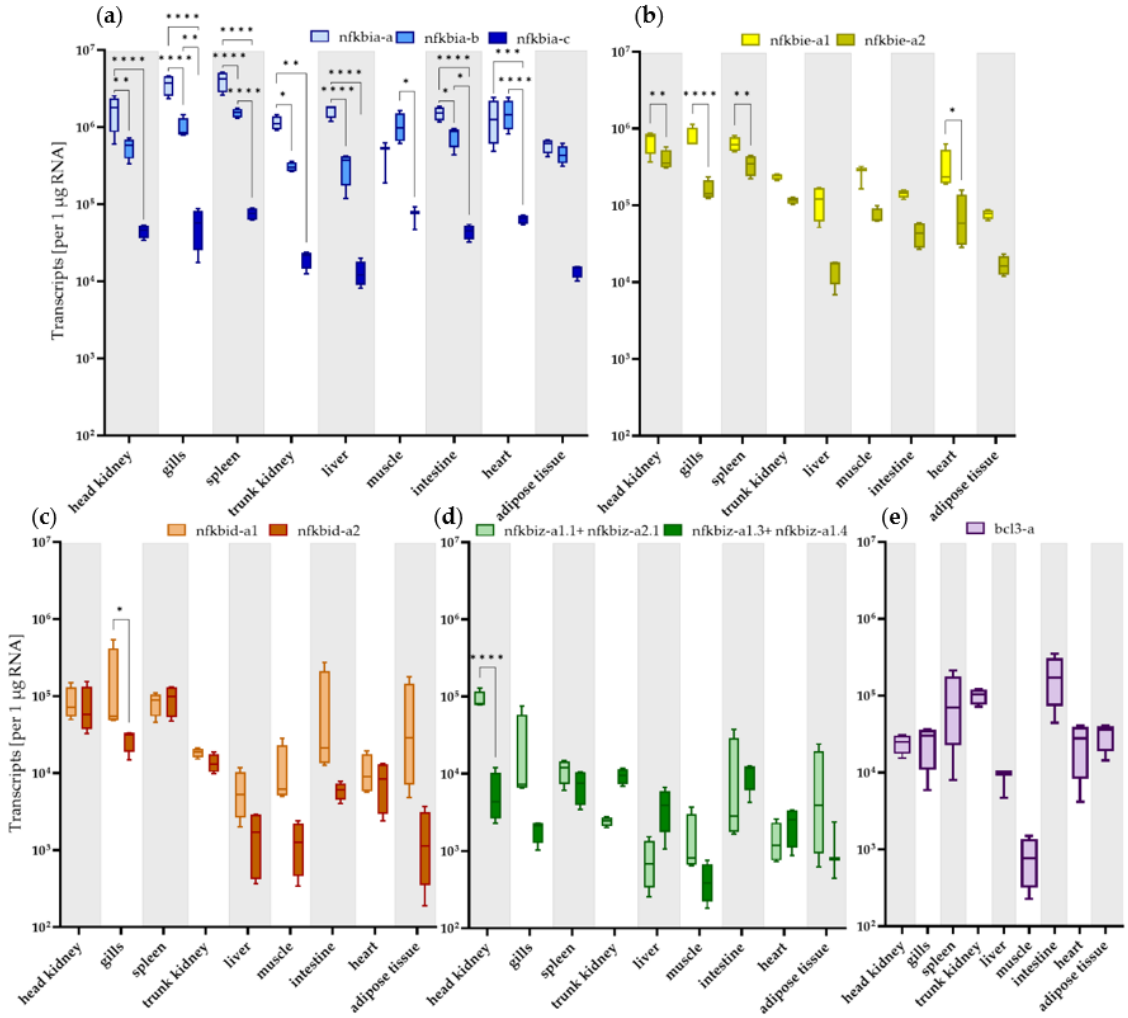

Figure 4. Levels of *nfkbi* transcripts per µg RNA in (**a**–**e**) various tissues (as listed on the abscissa) from the rainbow trout *O. mykiss*. Bars represent the averaged copy numbers (n = 3) normalised against the reference genes *eef1a1* and *rps5*; error bars represent the standard error of the mean (SEM). Asterisks represent significantly different transcript levels across transcript isoforms (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$).

In the liver, muscle, intestine, heart, and adipose tissue, the *nfkbia* expression differences were less pronounced and, in part, not significant. High transcript levels were also recorded for the *a1* ohnoloe of the *nfkbie* gene in the gills (7.6×10^5 copies), head kidney (7.1×10^5 copies), and spleen (6.3×10^5 copies), and these levels exceeded those of the *nfkbie-a2* ohnologs by a factor of about 2 to 5 (with $p < 0.01$) (Figure 4b). The transcripts for the *nfkbid* and *nfkbiz* ohnologs ranged between about 430 (*nfkbiz-a1.3+1.4* in the muscle) and 1.7×10^5 copies (*nfkbid-a1* in the gills) but showed few significant differences in transcript levels between the ohnologs (Figure 4c,d). With regard to tissue-specific expression patterns, the transcript levels of *nfkbia-a* and *nfkbie* were significantly higher in immune-relevant tissues, including head kidney, gill, and spleen, but (almost) no significant differential expression was observed for *nfkbia-c*, *nfkbid-a1*, *nfkbid-a2*, or *nfkbiz-a1.3+1.4* between tissues.

The tissue-wise expression profiles suggested that organs rich in immune cells generally have high levels of *nfkbi* transcripts. For this reason, we quantified the *nfkbi* transcripts in (i) a non-myeloid (mAb21N) fraction enriched with T- and B-lymphocytes, natural killer-like cells, and thrombocytes and (ii) a myeloid (mAb21P) fraction enriched with granulocytes, monocytes/macrophages, and dendritic cells from the head kidney of the rainbow trout (*O. mykiss*) (Figure 5).

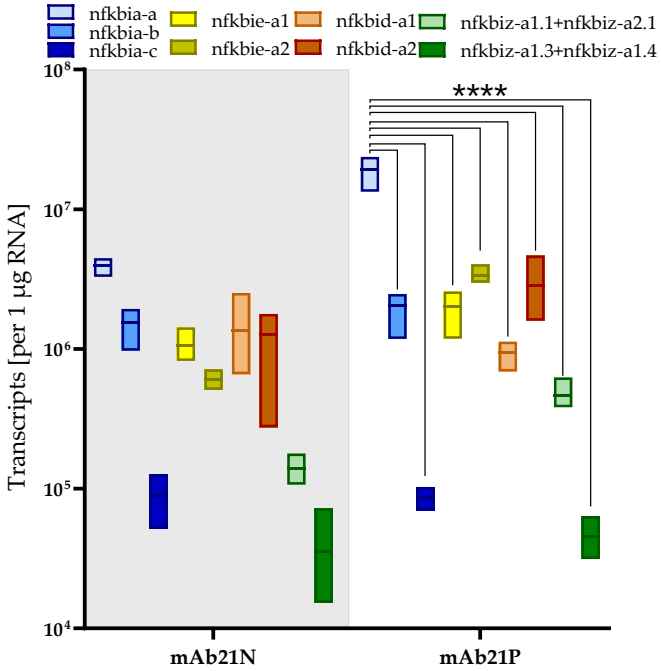

Figure 5. Levels of *nfkbi* transcripts per µg RNA in cell fractions from the rainbow trout (*O. mykiss*) (as listed on the abscissa). Bars represent the averaged copy numbers ($n = 3$) normalised against the reference genes *eef1a1* and *rps5*; error bars represent SEM. Asterisks represent significantly different transcript levels across different *nfkbi* genes (****, $p < 0.0001$).

Again, the level of *nfkbia-a* transcripts was the highest (19×10^6 and 4×10^6 copies in the mAb21P and mAb21N fractions, respectively) compared to the other *nfkbi* transcripts, but this difference was only statistically significant for the mAb21P fraction ($p < 0.0001$). The levels of *nfkbiz-a1.3+1.4* were the lowest (3.6×10^4 to 4.5×10^4 copies in the mAb21P and mAb21N fractions, respectively).

2.3. Iκbα and iκbε Localise to the Cytoplasm as well as the Nucleus of Salmonid Model CHSE-214 Cells

Their prominent expression in the immune tissues of rainbow trout suggested that *nfkbia-a2* and *nfkbie-a2* were appropriate iκb factors to seek the first insights into the regulation of nf-κb pathways in salmonid fish.

The ankyrin repeat is the signature motif of all IκB proteins (cf. Figure 3). We transiently overexpressed each of the three construct variants of iκbα or iκbε in CHSE-214 cells, including (i) the full sequence as well as truncated variants comprising (ii) the two N-terminal ankyrin repeats and (iii) the three C-terminal ankyrin repeats (Figure 3h). Confocal imaging indicated a differential localisation of the different iκbα or iκbε constructs (Figure 6). The concentration of the full-length iκbα was higher in the cytoplasm than in the nucleus (Figure 6a), while both iκbα-AR12 and iκbα-AR456 proteins were localised to a greater extent in the nucleus than in the cytoplasm (Figure 6b,c). The full-length iκbε factor and its derivative iκbε-AR12 and iκbε-AR456 proteins seemed to be evenly distributed in both the cytoplasm and nucleus (Figure 6d–f).

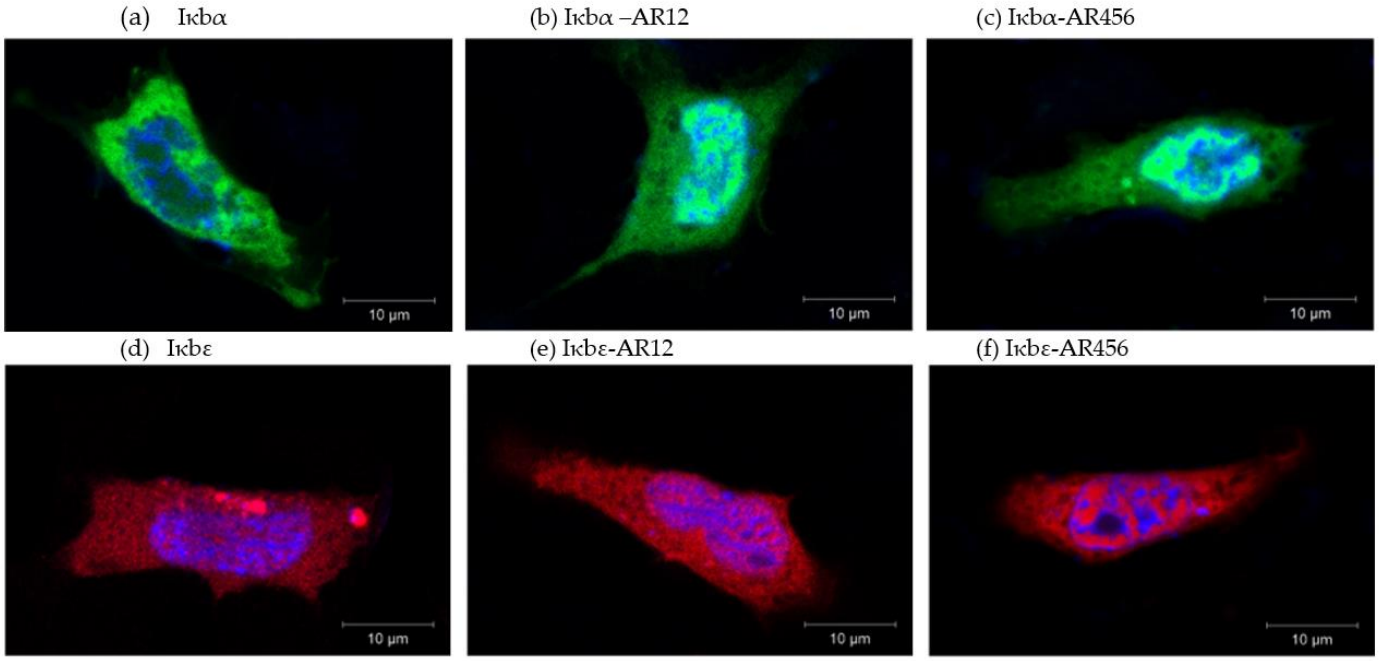

Figure 6. Overexpression of gfp-tagged iκbα (green fluorescence) or plum-tagged iκbε constructs (red fluorescence) in salmonid CHSE-214 cells. Confocal analysis of (**a**) iκbα, (**b**) ankyrin repeats 1 and 2 of iκbα, (**c**) ankyrin repeats 4, 5, and 6 of iκbα, (**d**) iκbε (red), (**e**) ankyrin repeats 1 and 2 of iκbε, and (**f**) ankyrin repeats 4, 5, and 6 of iκbε in CHSE-214. Nuclei were stained with Hoechst 33,342 dye (blue fluorescence); see Figure S1 for images without Hoechst staining. White scale bar represents 10 µm in all images.

2.4. Iκbα and iκbε Reduce the Basal and Stimulated nf-κb Activity

We used the six iκbα or iκbε expression constructs described in Sections 2.3 and 4.2 to assess their impact on the nf-κb activity in CHSE-214 cells. The overexpression of the full-length iκbα factor (1000 ng) robustly and significantly reduced the basal nf-κb activity down to 0.09-fold ($p = 0.0004$) (Figure 7a) compared to the non-transfected controls. Similarly, the overexpression of full-length iκbε (1000 ng) resulted in only 0.06-fold basal nf-κb activity (with $p < 0.0001$) compared to the controls (Figure 7b). In contrast to the full-length constructs, the two N-terminal ankyrin repeats or the three C-terminal ankyrin repeats of iκbα enhanced the basal nf-κb activity by 11.8-fold ($p = 0.08$) or 35.0-fold ($p < 0.00001$), respectively (Figure 7c). The pattern for the truncated iκbε constructs differed in the stronger nf-κb activation (5.2-fold; $p = 0.23$) caused by the N-terminal ankyrin repeats than by the C-terminal domains (2.3-fold; $p = 0.79$) (Figure 7d).

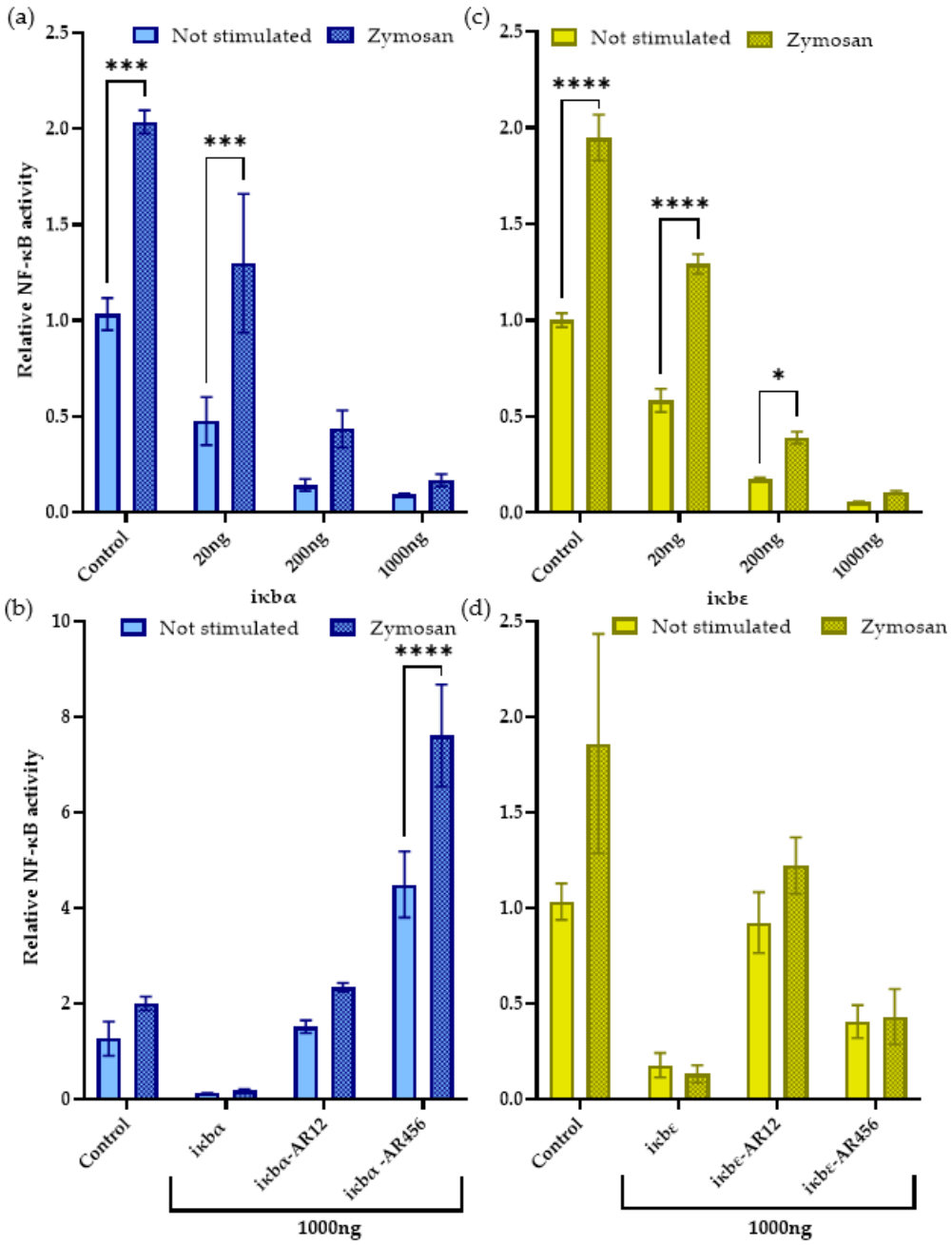

Figure 7. Overexpression of (**a**,**b**) gfp-tagged iκbα (green fluorescence) or (**c**,**d**) plum-tagged iκbε constructs (red fluorescence) in salmonid CHSE-214 cells. The luciferase activity of the ELAM-reporter vector was quantified in CHSE-214 cells co-expressing one of the six iκb constructs expressing (**a**,**b**) full-length iκbα and its truncated derivatives iκbα-AR12 and iκbα-AR456 and (**c**,**d**) full-length iκbε and its truncated derivatives iκbε-AR12 and iκbε-AR456. The concentrations of the iκb-expressing vector used for the transfection of the cells are indicated on the abscissa. Bars denote the mean values ± SEM. Statistical significance was assessed using two-way ANOVA (*, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$).

Stimulation of the non-transfected CHSE-214 cells with the fungal cell wall component zymosan doubled the nf-κb activity (2.0-fold; $p < 0.0001$) compared to the basal state (Figure 7a,b). Increasing the amounts of overexpressed iκbα factor from rainbow trout dose-dependently lowered this stimulated nf-κb activity down to 0.66-fold (20 ng expression vector; $p < 0.05$) and 0.05-fold (1000 ng expression vector; $p < 0.001$) compared to the non-transfected cells (Figure 7a). Again, the overexpression of iκbε had a very similar

effect on the stimulated nf-κb activity, as observed for iκbα ($p < 0.0001$) (Figure 7b). Cells overexpressing either the N-terminal or the C-terminal ankyrin repeats of iκbα or iκbε showed an already enhanced basal nf-κb activity, and stimulation induced a further increase in active nf-κb.

Admittedly, the expression constructs used for the above overexpression studies did not only encode distinct iκb factors but also a fluorescent protein (gfp or plum). To exclude the possibility that the fluorescent protein had an additional effect on the nf-κb activity, we verified that expression vectors encoding either iκbα or iκbε coupled to a fluorescent protein (gfp or plum) and an expression plasmid encoding either iκbα or iκbε without fluorescent protein had a similar effect on the nf-κb cellular activity (Figure 8).

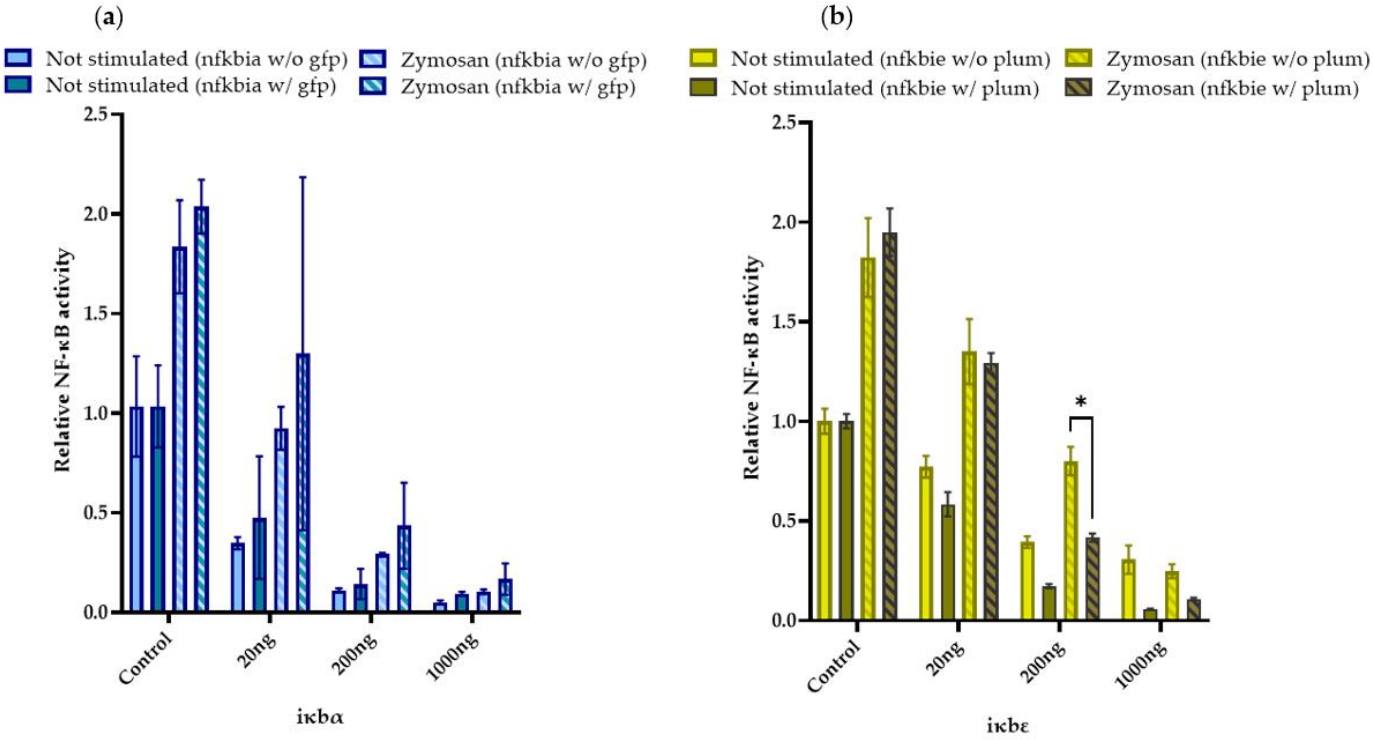

Figure 8. Overexpression of (**a**) green fluorescent gfp-tagged iκbα (w/gfp) and iκbα without gfp (w/o gfp) or (**b**) red fluorescent plum-tagged iκbε constructs (w/plum) and iκbε without plum (w/o plum) in salmonid CHSE-214 cells. The luciferase activity of the ELAM-reporter vector was quantified in CHSE-214 cells co-expressing the six iκb constructs. The concentrations of the iκb-expressing vector used for transfection of the cells are indicated on the abscissa. Bars denote the mean values ± SEM. Statistical significance was assessed using two-way ANOVA (*, $p < 0.05$).

Notably, zymosan stimulation induced a significantly different nf-κb activity in CHSE-214 cells transfected with 200 ng iκbε tagged with plum versus iκbε without plum, but this differential activity pattern was not consistent across the other concentrations. Therefore, we do not assume any significant influence of the fluorescent tag on the performance of the expressed iκb factor.

Having established that both iκbα and iκbε factors from rainbow trout significantly reduced the nf-κb activity in vitro, we used qPCR to test whether the overexpression of both factors would modulate the transcription of a panel of nf-κb-dependent immune genes in the same CHSE-214 model cells. The transcript levels of the seven selected immune genes were similar between untransfected cells and cells overexpressing iκbα or iκbε (Figure 9).

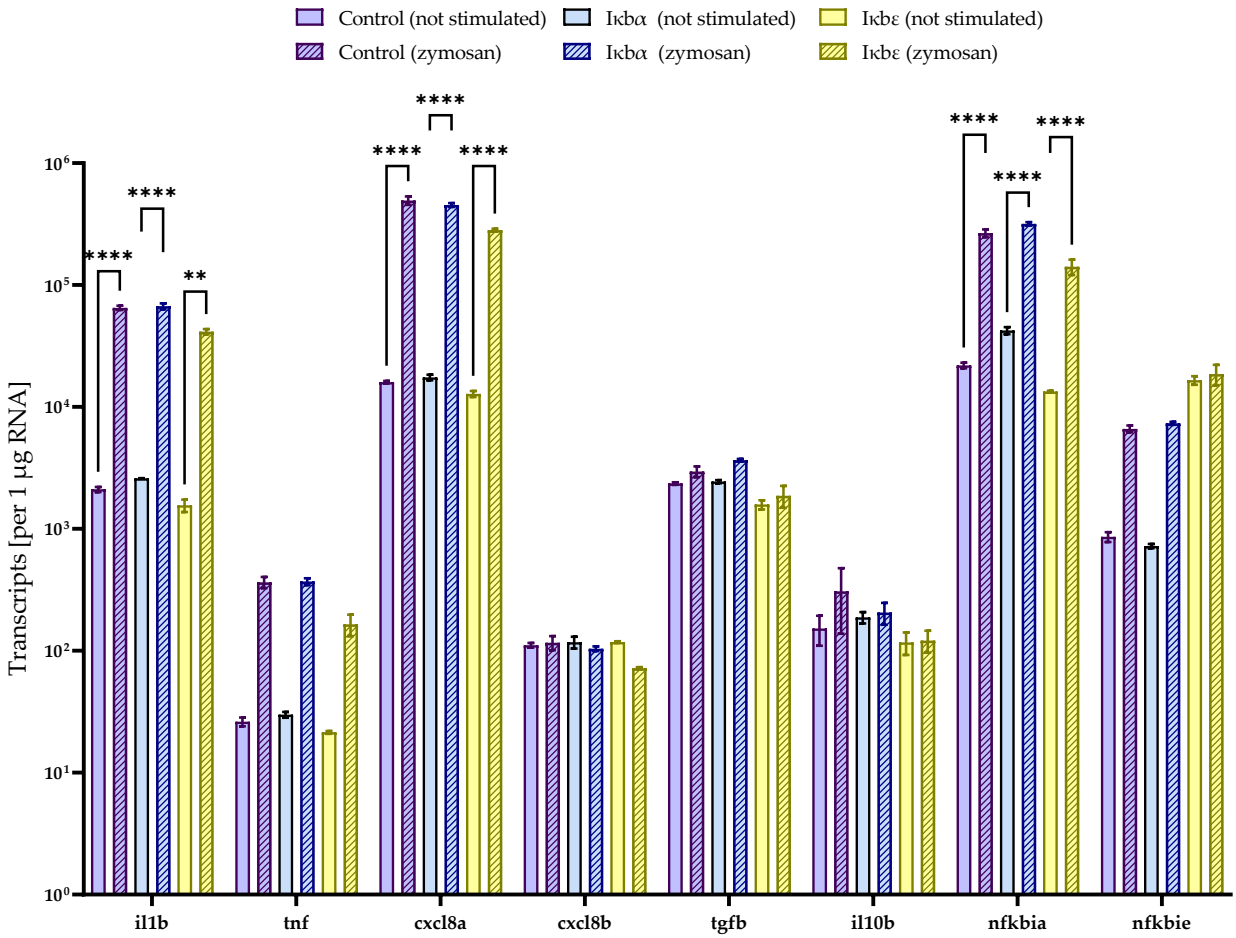

Figure 9. Expression profiling in CHSE-214 cells overexpressing iκbα and iκbε (representative of two experiments performed in triplicate). The bar chart illustrates the average number of specific transcripts (+SEM), as listed on the abscissa. Expression values were normalised against the geometric mean of the two reference genes. Transcript concentrations were quantified in unstimulated cells (unpatterned bars) and cells stimulated with zymosan for 4 h (striped), either transfected without (purple) or with iκbα- (blue) or iκbε-expression vectors (yellow). Statistical significance was assessed using two-way ANOVA (**, $p < 0.01$; ****, $p < 0.0001$).

Stimulation with zymosan for four hours significantly ($p < 0.01$) increased the transcript levels of characteristic inflammatory markers, such as il1b and cxcl8, but also of nfkbia (LOC112249975). Nevertheless, the differences in transcript levels of the induced immune genes in non-transfected cells versus cells expressing iκbα or iκbε were not statistically significant after stimulation with zymosan.

3. Discussion

Previous reports have suggested that rainbow trout possess one [53] or four [59] functional iκbα-encoding genes, but our research at the NCBI gene database revealed six *nfkbia* gene copies on different chromosomes of *O. mykiss*. Only one *NFKBIA* gene is present in mammals and two *nfkbia* paralogs have been characterised in several fish species, including zebrafish *D. rerio* [58], rock bream *Oplegnathus fasciatus* [55], orange-spotted grouper *Epinephelus coioides* [57], and blunt snout bream *Megalobrama amblycephala* [59]. It is rather unlikely that the additional teleost-specific whole-genome duplication in fish yielded three and not two *nfkbia* paralogs in salmonids, which then underwent an additional genome duplication [67] that eventually produced three pairs of *nfkbia* ohnologs. Several mechanisms could explain why certain genes are present in more copies than expected. For instance, the tandem duplication of genes arises from the unequal exchange between sister chromo-

somes [68]. Our structural and phylogenetic analyses suggest that one pair of the putative *nfkbia* ohnologs is the supposedly lost *nfkbib* gene. The sequence identity between the iκbα ohnologs a1/a2 or b1/b2 versus c1/c2 is comparably low, as reflected by our phylogenetic analysis that assigned the a1/a2 and b1/b2 pairs to the α-subfamily of IκB factors, while the c1/c2 pair was assigned to the β-subfamily of IκB factors. However, the genes flanking the human *NFKBIB* gene (including *SIRT2*, *RINL*, *SARS2*, and *CCR2*) [69] are either not present in rainbow trout or they are located on different chromosomes where they flank different genes. Conversely, the genes flanking *nfkbia-c* (presumably *nfkbib*) in rainbow trout (such as *gjd2*, *rab6a* and *zscan21*) are located on different chromosomes in the human genome, where they do not flank the same genes as they do in the trout genome. However, *nfkbib* genes are present in various teleost fishes, and *nfkbib* from the northern pike *Esox lucius*, for instance, is adjacent to the same genes as *nfkbia-c* (presumably *nfkbib*) in the rainbow trout genome (such as *rab6a* or *gjd1a*). The NCBI gene database (accessed on 14 December 2022) lists one *nfkbib* copy in 24 species, including 6 representatives of the Cypriniformes (carp fishes), 4 representatives of the Siluriformes (catfishes), 3 representatives each of the Characiformes (characins), and Clupeiformes (herring) and 2 representatives of the Osteoglossiformes (elephantfishes). In stark contrast, *nfkbia* and *nfkbie* are encoded in the genomes of more than 120 sequenced fish species, and *nfkbiz* is present in 115 species. The number of fish species possessing *nfkbid* is significantly lower at 63. We conclude from these indications that iκbβ has certainly been lost in many of the fish species sequenced thus far but not in salmonid fishes.

Three previous studies provide kaleidoscopic insights into the distinct characteristics and functions of teleostean iκbα paralogs: (a) iκbα-a is downregulated in the liver of the rock bream *O. fasciatus* a few hours after stimulation with flagellin, while its paralog iκbα-b is upregulated [55]; (b) the iκbα-a protein from the orange-spotted grouper *Epinephelus coioides* is distributed across the cytoplasm and nucleus, while its paralog iκbα-b mainly localises to the cytoplasm [57]; (c) the tnf-stimulated resynthesis of iκbα-b from the zebrafish *D. rerio* takes twice as long as the production of its paralog iκbα-a [58]. Our data on the iκbα paralogs and ohnologs of rainbow trout reveal that the expression of the iκbα-a-encoding gene is significantly higher in immune organs and in a head-kidney cell fraction enriched with granulocytes, monocytes/macrophages, and dendritic cells compared to its paralogs. Similar to what is observed in mammals, the iκb-encoding *nfkbi* genes from rainbow trout are constitutively expressed, albeit in a tissue-specific fashion [17,18,41]. These tissue-specific expression patterns obviously vary in different fish species, as demonstrated at least for *nfkbia*. The prominent *nfkbia* transcript level in the spleen of rainbow trout is in line with findings in the Japanese eel *Anguilla japonica* [63] and the mandarin fish *Siniperca chuatsi* [64], but it contrasts with the rather low splenic expression in the rock bream *O. fasciatus* [55], the blunt snout bream *M. amblycephala* [59] or the half-smooth tongue sole *Cynoglossus semilaevis* [60]. No comparable datasets are presently available for the expression profiles of the other *nfkbi* transcripts. In addition to the tissue-specific expression patterns, differential *nfkbia* transcript levels have been identified as indicative parameters for immune stimulation [70–72], exposure to toxic substances [73], or consumption of different diets [74,75] in diverse fish species, including rainbow trout [75,76]. The present study also confirms the significant upregulation of *nfkbia*, but not *nfkbie*, after in vitro fungal stimulation.

Iκbα and iκbε from rainbow trout localise to the cytoplasm and nuclei of unstimulated cells, as observed for IκBα orthologs from mammals [28] and bony fish [63]. Mammalian IκBε has been detected in the cytoplasm as well as in the nucleus [77], while the spatial distribution of iκbε in teleostean cells has not yet been analysed. In contrast to the full iκbα-protein from rainbow trout, the truncated iκbα versions were mostly located in the nucleus.

The overexpression of iκbα from rainbow trout blocked the basal and stimulated nf-κb activity in vitro, which is in line with many other reports on similar reporter-gene experiments in fish cells [56,57,63]. This again proves that the function of IκBα as an efficient

regulator of NF-κB signalling is well preserved across vertebrates. The effectiveness of iκbε from rainbow trout was similar to that of iκbα in terms of restricting nf-κb activity, but no comparative data on the biological activity of its orthologue in other teleost species are presently available.

Our expression constructs encoding either the first two or the last three ankyrin repeat motifs of iκbα and iκbε from rainbow trout increased the nf-κb activity, whereas the full-length iκbα and iκbε proteins did not. Moreover, we observed that the truncated iκbα variants had higher concentrations in the nucleus than in the cytoplasm, whereas the opposite was apparent for their full-length counterparts. The two N-terminal ankyrin repeat domains of the mammalian IκBα are known to contact the nuclear localisation signal of rela/nf-κB p65 [35,78,79]. Thus, the N-terminal domains of iκbα from rainbow trout quite plausibly have significant involvements in the oscillations of nf-κB. The three C-terminal ankyrin repeat domains in the mammalian IκB ortholog interact with the N-terminal domain of the Rel homology region or the PEST region of rela/nf-κB p65 and/or nfkb1/nf-κB p50 [35,78,79]. Accordingly, the ankyrin repeat domains seem to fulfil specific functions in fish, as they do in mammals, and their number and position may be crucial for their ultimate function.

The number of ankyrin-repeat motifs is probably not a criterion that discriminates iκbα from iκbβ. Iκbαa from orange-spotted grouper *E. coioides* contains five ankyrin repeat motifs, while iκbαb contains six ankyrin repeats [57]. In rainbow trout, the paralog pairs iκbα-a and iκbα-c/iκbβ possess six ankyrin-repeat motifs, while iκbα-b contains only five prototypical ankyrin-repeat motifs. Although the ankyrin-repeat domain is the most conserved feature of IκB proteins, it is found in many other proteins. Therefore, we can assume that more ankyrin repeat-containing proteins affect the activity of NF-κB than are currently known. In mammals, the ankyrin repeat-containing proteins INK4 and myotrophin, for instance, have been proven to modulate the efficacy of NF-κB functions [80,81], but their role in inflammatory processes in fish is not yet known.

In summary, the intensive structural comparisons presented here demonstrate that iκbβ does exist in salmonid fish, but its expression is significantly lower than that of the paraloguous iκbα proteins. Our comprehensive overexpression studies in trout cells provide insights into the regulation potential of a set of nf-κb inhibitors from rainbow trout, thereby providing the first functional results for iκbε in lower vertebrates. In ongoing studies, we are investigating the interplay between nf-κb inhibitors and nf-κb/rel factors from trout under defined environmental conditions.

4. Materials and Methods

4.1. Quantitative PCR (qPCR) Analysis

We recorded the expression of *nfkbia-a*, *nfkbia-b*, *nfkbi-c*, *nfkbie*, *nfkbid*, and *nfkbiz* in nine tissues (adipose tissue, gills, head kidney, heart, intestine, liver, muscle, spleen and trunk kidney) and sorted cells from rainbow trout. All analyses were conducted using animal materials left over from previous analyses [82]. We used the monoclonal antibody mAb21 to separate an mAb21-positive head-kidney cell fraction consisting of >95% myeloid cells from a mAb21-negative fraction consisting mostly of B- and T-lymphocytes, as well as thrombocytes. RNA was isolated from tissues and sorted cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and the ISOLATE II RNA Micro Kit (Bioline/Meridian Bioscience, Luckenwalde, Germany), respectively, including an in-column DNase treatment. We also profiled the expression of various Chinook salmon-specific immune genes (*il1b*, *tnf*, *cxcl8a*, *cxcl8b*, *tgfb*, *il10*, *nfkbia* and *nfkbie*) in transfected, stimulated CHSE-214 cells (derived from Chinook salmon [*O. tshawytscha*] embryos). After stimulation, the transfected CHSE-214 cells were washed twice with phosphate-buffered saline and then harvested by a 20-min incubation in lysis buffer (RNeasy Mini Kit; Qiagen, Hilden, Germany).

After isolation, RNA was reverse transcribed into cDNA using the SensiFAST cDNA Synthesis Kit (Bioline/Meridian Bioscience). Subsequently, the quantity of the *nfkbi* transcripts was selectively recorded by a panel of exon-skipping oligonucleotide primers

specific for rainbow trout (Pyrosequencing Assay Design software v.1.0.6; Biotage, Uppsala, Sweden; Table 2). These primers were either common for both *nfkbi* ohnologs (*nfkbia-a*, *nfkbia-b nfkbi-c*, and *bcl3-a*) or discriminated between *nfkbi* ohnologs (*nfkbie*, *nfkbid*, and *nfkbiz*). Of particular note, no discriminating primers could be designed for the individual *nfkbiz* gene variants a1 and a2 due to the high sequence identity (99–100%) (Table S1); instead, we derived common primers for each of the two similar *nfkbiz* transcript variants *nfkbiz-a1.1/a2.1* and *nfkbiz-a1.3/a1.4*. The primer pairs listed in Table 3 amplified fragments between 86 and 191 nucleotides in length. *Rps5* (ribosomal protein S5) and *eef1a1* (eukaryotic translation elongation factor) were used as reference genes to normalise the expression data. The qPCR analyses were conducted using the LightCycler-96 system (Roche, Basel, Switzerland) according to the following programme: initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 30 s, primer annealing at 60 °C for 15 s, elongation at 72 °C for 15 s, and the fluorescence measurement at 72 °C for 10 s. The amplicon quality was assessed by gel electrophoresis and melting-curve analysis. In addition, we checked the primer specificity by sequencing the amplicons (Azenta Life Sciences, Griesheim, Germany). The qPCR data were extracted using the LightCycler-96 analysis software v. 1.1.0.1320 (Roche).

4.2. Construction of Nfkbi-Expression Constructs

Three different vectors were used to express the complete ORFs of selected *nfkbi* genes (*nfkbia* and *nfkbie*) or distinct fragments of those genes (i.e., ankyrin repeat 1 and 2 or ankyrin repeat 4, 5, and 6) from rainbow trout. These vectors included the mammalian expression vector v280 [83] and two v280 derivatives attaching the red fluorescent protein mPlum or a green fluorescent protein (gfp) at the 3′ end of the inserted target fragments. These fragments were generated using Platinum Taq High-Fidelity DNA polymerase (Thermo Fisher Scientific, Bremen, Germany) and oligonucleotide primers linked with specific restriction sites (Table 4).

Table 4. Primers used in this study for the development of *nfkbi*-expression constructs.

Gene/Construct Name	Primer Sequence 5′→3′ (Sense, Antisense)	NCBI-Nucleotide Accession Code	Fragment Length [bp]
nfkbia	CCCAAGCTTGATATGGATGTTTATAGAGTTTCAAACG [a], GATGACATTACATTTGGTCAGAATGAATTCTCAAC [a]	NM_001124368 (position 50–991)	960
nfkbia-AR12	CCCAAGCTTATGGATGTTTATAGAGTTTCAAACG [a], GTGACCCGCGGATAGCAGACGAATTCGGG [a]	NM_001124368 (position 50–493)	465
nfkbia-AR456	CCCAAGCTTATGAGCGGACACAACTGCCTC [a], GATGACATTACATTTGGTCAGAATGAATTCGGG [a]	NM_001124368 (position 611–991)	402
nfkbie	CCCAAGCTTCTGATATGCAAAGCGCCGAAGATGCG [a], CCCGAATTCCTGATCAGAATGGCCCTCCAACCAC [a]	XM_021611602 (position 450–1511)	1080
nfkbie-AR12	CCCAAGCTTATGCAAAGCGCCGAAGATGCG [a], GGGGCCAGCCTGGAGCTGAGATCTGGG [a]	XM_021611602 (position 450–977)	546
nfkbie-AR456	CCCAAGCTTATGAGAGGTCTCACCTGTCTC [a], TCAGTGGTTGGAGGGCCATTCAGATCTGGG [a]	XM_021611602 (position 1104–1511)	429

[a] Underlining marks the attached sequences (composed of the restriction site, optionally a start codon and three additional nucleotides at the 5′-end).

The Biometra TAdvanced cycler (Analytik Jena, Jena, Germany) was used to amplify the gene fragments according to the following programme: initial denaturation at 94 °C for 2 min, followed by 40 cycles of denaturation at 94 °C for 30 s, primer annealing at 60 °C for 30 s, elongation at 72 °C for 2 min, and a final extension step at 68 °C for 5 min. Amplicons were inserted into the above expression vectors by double digestion with *Hind*III and *Eco*RI (*nfkbia*) or *Bgl*II (*nfkbie*). All expression vectors were sequenced and checked for correct assembly before use.

4.3. Cell Transfection, Luciferase Assay and Confocal Microscopy

The salmonid cell line CHSE-214 was cultured as described previously [83]. The CHSE-214 cells were transfected in six-well plates with a total of 2050 ng endotoxin-free

prepared DNA (ZymoPure II Plasmid Maxi Prep Kit, ZymoResearch, Freiburg, Germany) using X-tremeGENE HP DNA Transfection Reagent (Roche, Mannheim, Germany). The co-transfection assays contained 50 ng of the NF-κB-responsive promoter (endothelial-leukocyte adhesion molecule)-reporter (luciferase) construct ELAM-1-luc, defined concentrations (20 ng–1000 ng) of *nfkbi* expression vectors, and varying amounts of empty-vector DNA to ensure that the total DNA amount per assay remained constant.

For the stimulation experiments, co-transfected cells were split into 24-well plates: Three wells per row remained unstimulated, while the other three wells were challenged with 1 mg/mL zymosan from *Saccharomyces cerevisiae* (tlrl-zyn; Invivogen, Toulouse, France) for 4 h or 24 h. After incubation, the cell lysates were collected, and the luciferase activity of each assay was measured using the Dual-Luciferase Reporter Assay System (Promega, Mannheim, Germany) at the Lumat LB9501 luminometer (Berthold, Bad Wildbad, Germany). The resulting relative light units were normalised by the protein concentrations of the CHSE-214 cell extracts. Each transfection experiment was measured in triplicate and conducted at least twice.

The IκBα and IκBε factors were localised by transfecting CHSE-214 cells with different vectors expressing *nfkbia* and *nfkbie* tagged with green fluorescent protein (gfp) or plum, respectively. Hoechst 33342 dye (250 µg/mL; Sigma-Aldrich/Merck, Hamburg, Germany) was used to stain the nuclei 30 min before fixation of the CHSE-214 cells with 4% paraformaldehyde (Merck KGaA, Darmstadt, Germany). The cells were then examined with confocal microscopy (LSM 780; Carl Zeiss Microscopy, Oberkochen, Germany), using a 63× oil-immersion differential interference contrast objective.

4.4. Data Analysis

The qPCR data was normalised against the reference genes and based on gene-specific standard curves, and the individual copy numbers were calculated ($R^2 > 0.99$; 10^7–10^3 copies per 5 µL). The GraphPad Prism software (v9.1.0) was used for the statistical analysis of the normalised qPCR data. Significant differences between the different tissues/cell fractions were assessed using two-way analysis of variance (ANOVA) followed by a Holm-Šídák's post-hoc test to correct for multiple comparisons. A parametric *t*-test conducted using GraphPad Prism software v.9.5.1 was run to evaluate the statistical significance of the reporter-gene measurements. *p*-values less than 0.05 were considered statistically significant.

Orthologous *NFKBI* gene sequences were retrieved from the NCBI gene database. The protein sequence identity was determined by using https://npsa-prabi.ibcp.fr/NPSA/npsa_clustalw.html (accessed on 16 January 2023).

The ClustalW alignment tool [84] was used to align the NFKBI amino acid sequences. The phylogeny of orthologous IκB proteins was assessed using ETE3 on the GenomeNet (https://www.genome.jp/tools/ete/, accessed on 3 January 2023) [85].

A phylogenetic dendrogram was reconstructed with the neighbour-joining method based on log-corrected distances and optimised manually. Node robustness was evaluated on a bootstrap analysis based on 1000 iterations. SMART (Simple Modular Architecture Research Tool) [86] was applied to identify motifs and domains of the IκB proteins. The three-dimensional iκb protein structures were predicted using I-TASSER (Iterative Threading ASSEmbly Refinement) [87] in complement with UCSF ChimeraX v.1.1 [88].

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms241210229/s1.

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Institutional Review Board Statement: All BioFia experiments were approved by the animal welfare officer of the 'Fraunhofer IMTE Büsum' and the local authority of Schleswig-Holstein, according to the German animal welfare law (approval ID: NTP—ID 00043858-1-0). Note: the present study exclusively utilised samples from previous experiments [51,82] following the 3R principles in science.

Data Availability Statement: The qPCR and reporter-gene data generated during the current study are not publicly available but are available on request. The nucleotide and amino acid sequences of the analysed IκB factors are available in the NCBI database; the respective accession numbers are provided in the manuscript.

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Abbreviations

Aa, amino acid(s); bcl3, B-cell chronic lymphatic leukemia protein 3; CDS, coding sequence; CHSE, Chinook salmon embryo; cxcl8, C-X-C motif chemokine ligand 8; eef1a1, eukaryotic translation elongation factor; ELAM, endothelial cell-leukocyte adhesion molecule; gfp, green fluorescent protein; IKK, IκB kinase; IκB, inhibitor of NF-κB; il1b, interleukin-1 beta; il10, interleukin-10; mAb21N, non-myeloid fraction enriched with T- and B-lymphocytes, natural killer-like cells and thrombocytes; mAb21P, myeloid fraction enriched with granulocytes, monocytes/macrophages and dendritic cells; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NLS, nuclear translocation signal; nt, nucleotide(s); PAMP, pathogen-associated molecular pattern; PEST, proline (P), glutamate (E), serine (S), threonine (T); RHD, Rel homology domain; rps5, ribosomal protein S5; tgfb, transforming growth factor-β; tnf, tumour necrosis factor; WGD, whole-genome duplication.

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Declarations

Statement of contributions

I hereby declare that my share of the publications summarized in this thesis is as follows:

Publication I

Microalgae as functional feed for Atlantic salmon: effects on growth, health, immunity, muscle fatty acid and pigment deposition.

Mueller, J., Pauly, M., Molkentin, J., Ostermeyer, U., **van Muilekom, D. R.**, Rebl, A., ... & Schulz, C. (2023). *Frontiers in Marine Science*.

- Fish sampling
- RNA isolation
- Data analysis
- Visualization
- Reviewing and editing draft

Publication II

Can dietary *Chlorella vulgaris* supplementation improve health of Atlantic salmon? Insights from combined analysis of microbiota, host immunity and the response to oxidative stress

Jonas Mueller†, **Doret R. van Muilekom†**, Jannick Ehlers, Marvin Suhr, Stéphanie C. Hornburg, Corinna Bang, Marie Wilkes, Thekla Schultheiß, Edmund Maser, Alexander Rebl, Tom Goldammer, Henrike Seibel, Carsten Schulz. **Submitted in Scientific Reports on 4th of March 2024.**

- Fish sampling
- Data analysis
- Statistical analysis
- Visualization
- Writing and preparation of original draft

Publication III

Salinity change evokes stress and immune responses in Atlantic salmon with microalgae showing limited potential for dietary mitigation.

van Muilekom, D. R.†, Mueller, J.†, Lindemeyer, J., Schultheiß, T., Maser, E., Seibel, H., ... & Goldammer, T. (2024). Frontiers in Physiology, 15, 1338858.

- Fish sampling
- RNA isolation
- Data analysis
- Statistical analysis
- Visualization
- Writing and preparation of original draft

Publication IV

Lost and Found: The Family of NF-κB Inhibitors Is Larger than Assumed in Salmonid Fish.

van Muilekom, D. R., Collet, B., Rebl, H., Zlatina, K., Sarais, F., Goldammer, T., & Rebl, A. (2023). *International Journal of Molecular Sciences*, 24(12), 10229.

- Conceptualization
- Primer design
- RNA isolation
- qPCR analysis
- Vector design
- Cloning
- Cell culture
- Cell experiments
- Reporter-gene analyses
- Data analysis
- Statistical analysis
- Visualization
- Writing and preparation of original draft

Publications and conferences

Peer-reviewed manuscripts

** part of the doctoral thesis; † These authors have contributed equally to this work and share first authorship.*

1. *** van Muilekom, D. R.**, Collet, B., Rebl, H., Zlatina, K., Sarais, F., Goldammer, T., & Rebl, A. (2023). Lost and Found: The Family of NF-κB Inhibitors Is Larger than Assumed in Salmonid Fish. *International Journal of Molecular Sciences*, 24(12), 10229.
2. * Mueller, J., Pauly, M., Molkentin, J., Ostermeyer, U., **van Muilekom, D. R.**, Rebl, A., ... & Schulz, C. (2023). Microalgae as functional feed for Atlantic salmon: effects on growth, health, immunity, muscle fatty acid and pigment deposition. *Frontiers in Marine Science*.
3. *** van Muilekom, D. R**.†, Mueller, J.†, Lindemeyer, J., Schultheiß, T., Maser, E., Seibel, H., ... & Goldammer, T. (2024). Salinity change evokes stress and immune responses in Atlantic salmon with microalgae showing limited potential for dietary mitigation. *Frontiers in Physiology*, 15, 1338858.
4. *Mueller, J.†, **van Muilekom, D.R**.†, Ehlers, J., Suhr, M., Hornburg, S.C., Bang, C., Wilkes, M., Schultheiß, T., Maser, E., Rebl, A., Goldammer, T., Seibel, H. & Schulz, C. Can dietary *Chlorella vulgaris* supplementation improve health of Atlantic salmon? Insights from combined analysis of microbiota, host immunity and the response to oxidative stress. **Submitted to Scientific Reports on 4th of March 2024.**
5. Chaumont, L., Jouneau, L., Huetz, F., **van Muilekom, D.R.**, Peruzzi, M., Raffy, C., Le Hir, J., Minke, J., Boudinot, P & Collet, B. Unexpected regulatory functions of cyprinid Viperin on inflammation and metabolism. **Submitted to *BMC Genomics* in April 2024.**

Conference abstract

1. **van Muilekom, D.**, Collet, B., Rebl, H., Zlatina, K., Sarais, F., Goldammer, T., & Rebl, A. (2023). Regulators of NF-κB activation: characterisation of the large NF-κB inhibitors family in salmonid fish. *Developmental & Comparative Immunology*, *148*, 104996.

Internal presentations

1. **Van Muilekom, D.R.**, Rebl, A. & Goldammer, T. (2021) Characterization of the influence of microalgae diets and transfer into marine farming systems on the transcriptome of *Salmo salar*. **Day of Doctoral Student (Beginners)**, Dummerstorf, Germany (**Presentation**).
2. **Van Muilekom, D.R.**, Mueller, J., Schlachter, M., Brunner, R.M., Seibel, H., Starke, S., Rebl, A. & Goldammer, T. (2021) Characterization of the influence of microalgae diets, transfer into marine farming systems and slaughtering process on the transcriptome of *Salmo salar*. **Meeting Institute of Genome Biology**, Dummerstorf, Germany (**Presentation**).
3. **Van Muilekom, D.R.**, Rebl, A. & Goldammer, T. (2022) The effect of salinity change and its dietary mitigation potential using microalgae on the immune and stress response of Atlantic salmon *Salmo salar*. **Day of Doctoral Student (Advanced)**, Dummerstorf, Germany (**Presentation**).

4. **Van Muilekom, D.R.** & Mueller, J., Schlachter, M., Lindemeyer, J., Schultheiss, T., Maser, E., Brunner, R.M., Seibel, H., Schulz, C., Rebl, A. & Goldammer, T. (2023) Effect of microalgae in feed on the health status of Atlantic salmon under stress. **Friday Seminar Institute 3.0,** Dummerstorf, Germany (**Presentation**).
5. **Van Muilekom, D.R.** & Mueller, J., Schlachter, M., Lindemeyer, J., Schultheiss, T., Maser, E., Brunner, R.M., Seibel, H., Schulz, C., Rebl, A. & Goldammer, T. (2023) Effect of microalgae as functional feed on the immune status of stressed Atlantic salmon. **PhD students seminar**, Dummerstorf, Germany (**Presentation**).

Contributions to national conferences

1. **Van Muilekom, D.** & Mueller, J., Schlachter, M., Brunner, R.M., Seibel, H., Starke, S., Rebl, A. & Goldammer, T. (2022). Untersuchungen zum Einfluss von Mikroalgen auf die Expression von Immun- und Stressgenen des Atlantischen Lachses Salmo salar bei unterschiedlichen Salinitäten. **Deutscher Fischerei Tag**, Berlin, Germany (**Poster & Pitch**)
2. **Van Muilekom, D.**R. (2022) Transcriptomic profiling of the stress and immune response in Atlantic salmon Salmo salar to salinity change and microalgae diets. **BAMS symposium 2022**, Stralsund, Germany (**Presentation**)
3. **Van Muilekom, D.R.** & Mueller, J., Schlachter, M., Lindemeyer, J., Schultheiss, T., Maser, E., Brunner, R.M., Seibel, H., Schulz, C., Rebl, A. & Goldammer, T. (2023) Effect of microalgae in feed on the health status of Atlantic salmon under stress. **14. Büsumer Fischtag 2023**, Büsum, Germany (**Presentation**).
4. **Van Muilekom, D.R.** & Mueller, J., Schlachter, M., Lindemeyer, J., Schultheiss, T., Maser, E., Brunner, R.M., Seibel, H., Schulz, C., Rebl, A. & Goldammer, T. (2023) It's getting salty – Microalgae diet effects on salmon. **Symposium Blaue Bioökonomie 2023**, Oldenburg, Germany (**Presentation**).

Contributions to international conferences

1. **Van Muilekom, D.** & Mueller, J., Schlachter, M., Brunner, R.M., Seibel, H., Starke, S., Rebl, A. & Goldammer, T. (2021). The transcriptomic response of Atlantic salmon *Salmo salar* to microalgae diets and environmental stress. **Aquaculture Europe 2021**, Madeira, Portugal (**Poster**)
2. **Van Muilekom, D.** & Mueller, J., Schlachter, M., Brunner, R.M., Seibel, H., Starke, S., Rebl, A. & Goldammer, T. (2021). The transcriptomic response of Atlantic salmon *Salmo salar* to microalgae diets and environmental stress. **Fish Immunology Workshop 2021**, Wageningen, the Netherlands (**Poster & Presentation**)
3. **Van Muilekom, D.R.** & Mueller, J., Schlachter, M., Brunner, R.M., Seibel, H., Rebl, A. & Goldammer, T. (2022) The transcriptomic response of Atlantic salmon Salmo salar to microalgae diets and environmental stress. **Aquaculture Europe 2022**, Rimini, Italy (**Presentation**).
4. **Van Muilekom, D.R.**, Collet, B., Rebl, H., Zlatina, K., Sarais, F., Goldammer, T. & Rebl, A. (2023) Regulators of NF-κB activation: characterisation of the large NF-κB

inhibitors family in salmonid fish. **ISDCI 15th Congress**, Wageningen, the Netherlands (**Presentation**).

5. **Van Muilekom, D.R.**, Collet, B., Rebl, H., Zlatina, K., Sarais, F., Goldammer, T. & Rebl, A. (2023) Fighting threats with many others: Charachterisation of the large NF-κB inhibitors family in salmonid fish. **Aquaculture Europe 2023**, Vienna, Austria (**Presentation**).

Declaration of the doctoral candidate according to § 4 (1) of the doctoral degree regulations of MNF of the University of Rostock

I hereby certify that:

- I have written this thesis independently and without outside help, that I have not used any aids or sources other than those indicated by me, and that I have marked the content and literal passages taken from the works used as such.
- The opportunity for this PhD project was not communicated to me commercially. In particular I have not engaged any organisation that for money seeks supervisors for the drawing up of dissertations or that performs entirely or partially on my behalf the duties incumbent upon me regarding the examinations.

Doret Rosie van Muilekom,

Rostock, 10-04-2024

Doktorandin - Erklärung gemäß § 4 Absatz 1 der Promotionsordnung der MNF der Universität Rostock

Hiermit versichere ich:

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