MASTER THESIS

Course code: BIO5011 Name: ACHUMIE OKWUKWEKA U. Candidate no: 1

Effect of dietary algal polysaccharides on immune-related gene expression in zebrafish (*Danio rerio*) mucosal tissues.

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ABSTRACT

Fish meal replacement by soybean meal is considered one of the approaches to improve sustainability of aquaculture. However, the presence of antinutritional factors in soybean meal limits its use in aquafeeds as they can have severe intestinal and extra intestinal effects in fish. Skin and gills are important mucosal organs which serve to regulate the welfare of the fish. In this study, the effect of soybean meal alone (potential intestinal inflammation inducing diet) or algae-derived alginate/β-glucan supplemented soybean meal-based diets on immune-related genes in the skin and gill of zebrafish was explored. Five experimental diets (diet groups CT control, SBM - 50**%** soybean meal, BG - SBM +2.5% algal β-glucan, AL - SBM + 0.5% low molecular alginate oligosaccharide (AOS), AH –SBM +0.5% high molecular weight AOS) were formulated and fed to adult male zebrafish (AB strain) for 30 days. At the end of the 30 days feeding trial, the expression of targeted immune-related: [*interleukin-1b* (*il1b*), *interleukin-8 (il8*), *tumor necrosis factor-alpha (tnfα*), *mucin 5.1* (*muc5.1*)], antioxidant-related: [*catalase* (*cat*), *superoxide dismutase* (*sod*)], and antimicrobial: [*lysozyme* (*lyz*), *hepcidin (hep*)] genes in the skin and gill of the zebrafish were examined. We observed that skin tissue showed higher response to dietary changes in terms of gene expression with six (*il1b, il8, tnfα, muc5.1, sod, cat*) out of the 8 genes showing significant alterations. On the other hand, whereas expression of only 3 genes (*sod, cat, tnfα*) was altered in the gills. Changes in diets altered the expression of *muc5.1, il1b and il8* only in the skin but not in the gills. Furthermore, the result obtained showed that SBM (inflammation-causing) diet induced the expression of *il8* in the skin and *cat* in the gills of zebrafish. We also observed that AL diet group showed significantly higher of *sod* and *tnfα* in the skin compared to AH diet group. Dietary β-glucan also stimulated the expression of *il8* and *muc5.1* in the skin of zebrafish. Overall, insights from this study could be used to promote the use of AOS and β-glucans as dietary additives that can modulate mucosal immune response in fish.

1.0 INTRODUCTION

1.1 Relevance of aquaculture in global food production

Aquaculture is currently one of the fastest growing industries in the world. Farmed fish have contributed immensely to human nutrition as a source of proteins and essential micronutrients that play important roles in human health. In the last decade, aquaculture has supplied one-third of the seafood consumed worldwide (Reverter et al., 2014). Considering the importance of aquaculture industry in global food production, considerable research focus is being laid on sustainability issues of the industry, particularly the fish feeds. The use of fish meal by the aquafeed industry is economically and ecologically unsustainable (Gause & Trushenski, 2011). Awareness about unsustainability has prompted the search for alternative plant-based proteins that could be used as fish meal substitutes in aquafeeds. Among the plant-based ingredients, soybean meal is one of the most promising options due to its high protein content and balanced amino acid profile. However, soybean meal contains certain anti-nutritional factors that evoke inflammatory responses in the intestine of some farmed fishes which can hamper the hamper growth performance and welfare during culture conditions. To prevent such undesirable effects of dietary soybean meals, several anti-inflammatory feed additives can be used as mitigation strategies. Alginate oligosaccharides (AOS) and β-glucans are well known bioactive compounds which can be used as dietary supplements to inhibit the soybean associated inflammatory response in the different tissues. β-glucans, the natural polysaccharide composed of glucose monomers linked by β (1,3) glycosidic bonds, are derived from different sources like yeasts, seaweeds, mushrooms, algae, and bacteria. *Euglena gracilaris* is a rich source of β-glucans which consists of β-1,3-linked glucose subunits, and molecular weight between 100 and 500 kDa. These novel sources of β-glucans have also been explored as anti-inflammatory agents (Hou et al., 2020; Jayachandran et al., 2018). Alginate is a naturally occurring compound found mainly in brown algae. Alginate oligosaccharides (AOS) are produced from this brown algae-derived compound. AOS are linear polymers of 2-25 monosaccharides composed of β-D-mannuronic acid (M) and α-L-guluronic acid (G) monomers linked by 1-4 glycosidic linkages and exhibit anti-inflammatory properties (Lu et al., 2022). Considering the anti-inflammatory properties of both AOS and β-glucans, several studies have evaluated their potential as dietary supplements for fish (Bledsoe et al., 2022; Li et al., 2022; Machuca et al., 2022; Thompson et al., 2016). Fish represent early vertebrates and are regarded as an important model in comparative immunology research because of their position as an important link to early vertebrate evolution. Zebrafish (*Danio reiro*) is a freshwater fish belonging to the minnow

family (Cyprinidae) of the order Cypriniforms, native of South Asia (Meyers, 2018). Zebrafish is considered a model organism in aquaculture research and has been used in various studies to extend knowledge regarding gene function and nutritional outcomes related to development, health, and disease in fishes (Brugman, 2016; Meyers, 2018). Zebrafish has also been established as an ideal and validated screening model for the discovery of novel anti-inflammatory compounds that can be used as therapeutic additives (Yu et al., 2021). Adult zebrafish have a complete (innate and adaptive) immune system, a valuable feature as a model in innate immunity and inflammation studies. Skin and gills of fish are portals of exposure for various xenobiotics and external factors. Therefore, these tissues are equipped with a repertoire of immune cells which prevent unwanted exposure to microbes or antigenic factors. Evaluation of the effect of soluble bioactive compounds on these immune tissues is critical to promote their use as anti-inflammatory agents. Analysis of gene expression is an easy and reliable approach in understanding the mode of action of novel bioactive compounds. Therefore, the present study was undertaken to understand the anti-inflammatory potential of dietary AOS and β-glucans on the skin and gill tissue of zebrafish.

1.2 Aim and Objectives

The aim of the present study is to evaluate the effect of dietary AOS and β-glucans on the immune related gene expression in zebrafish mucosal organs skin, and gill with emphasis on their, anti-inflammatory and antioxidant effect by examining the expression of the targeted immune genes.

1.3 Overview of fish immune system

The fish mucosal system is in constant touch with its surroundings and can easily be exposed to pathogens, pollutants, and other stressors. Unlike invertebrates, teleost fish have evolved both innate and adaptive immunity to protect themselves against pathogens residing in the aquatic environment. Like in mammals, B and T-cells constitute the first adaptive mechanism in all bony vertebrates (Yu et al., 2020). Fish can protect themselves with the help of a complex.

innate defense mechanism, and its components can have constitutive (already present) expression or be responsive (inductive) in nature (Ellis, 2001). Under farm conditions, the infection pressure would be much greater due to the commonly adopted intensive culture practices (Kiron, 2012). For instance, physical abrasions on the primary defense barriers such as mucus and epidermis can grant easy access for pathogens to the fish tissues. At this stage, the systemic innate response can take over, thus the operation of the immune system is both at local and systemic level. (Kiron, 2012). Despite certain differences, fish depends on both

cellular and humoral immune response as in the higher vertebrates and have organs dedicated to immune defense. (Kiron, 2012). Apart from lymphatic nodules and bone marrow which are functionally replaced by the head kidney, most of the generative and secondary lymphoid organs that are seen in mammals are also found in fish (Press & Evensen, 1999). The adaptive immune system of teleost fish can be found associated with each of their mucosal body surfaces and occurs at different mucosal sites. The principal mucosa-associated lymphoid tissues (MALT) of teleost are the gut-associated lymphoid tissue (GALT), skin-associated lymphoid tissue (SALT), the gill-associated lymphoid tissue (GIALT) and the recently discovered nasopharynx-associated lymphoid tissue (NALT) (Salinas, 2015). In the present study we focused our attention on skin and gill.

1.3.1 Skin

Fish skin is a multi-purpose tissue that has vital functions including physical protection, sensory activity, behavioral purpose, or hormone metabolism (Rakers et al., 2010). The skin of teleost, like every vertebrate, comprises of two basic layers, the outer epidermis, and the inner dermis. Unlike mammals, fish skin is not keratinized, but covered by epithelial cells with abundant mucus producing cells which are in direct contact with water. The skin, therefore, is considered as the primary barrier that provides the physical and chemical protection in association with its mucus which consists of glycoproteins, proteoglycan, and proteins forming a layer that exist as an interface between the fish and its environment. The aquatic environment is rich in pathogenic organisms; hence the skin of aquatic vertebrates is extremely important as the first line of defense against the invasion of environmental pathogens (Magnadottir, 2010). It also has a vast assortment of antimicrobial peptides (AMPs) like hepcidin which offers a unique opportunity for innate antimicrobial defense system research.

1.3.2 Gills

The gills are a multifunctional organ, serving as respiratory organ for evaginated gas exchange. Fish have four gill arches on each side of buccal cavity. The gills are also involved in osmoregulatory functions, pH balancing regulation, ammonia excretion, hormone regulation, detoxification functions and immune defense through the mucosal associated lymphoid tissue (GIALT- gill associated lymphoid tissues) that harbor macrophages, neutrophils, lymphocytes and mast cells/eosinophilic granulocytes, T-cells.

1.4 Soybeans meal dietary effects

Soybean meal (SBM) is widely used as a cost-effective alternative for fish meal in aquafeeds (Storebakken, 2000) due to its favorable protein content, amino acid profile and additional advantage of absence of the contamination problems reported in some animal protein sources., However, SBM is low in methionine (an essential amino acid that plays a crucial role in metabolism and health of many species including humans) and contains many antinutritional factor such as protease inhibitors, lectins, phytic acid, saponins, phytoestrogens, antivitamins enzymes and allergens (Francis et al., 2001). Antinutritional factors are biological components that can reduce nutrient uptake in animals which could disrupt gastrointestinal functions, metabolic performance and cause intestinal inflammation. Although inflammation is a natural protective response by the tissue, a prolonged and chronic inflammation could be detrimental, causing pathogenesis of diseases (Hou et al., 2020). Experiments have shown that soybean meal-base diet can induce intestinal inflammation in zebrafish (Rehman et al., 2022). Hence the need to further research on food additives that could reduce these negative dietary effects associated with SBM.

1.5 β-glucan dietary effects

β-glucan comprises a group of β-D-glucose polysaccharides (glycans) linked by 1-3 β-glycosidic bonds and naturally occurring in the cell walls of cereals, bacteria, and fungi (yeast) with significantly differing physicochemical properties dependent on source (Li et al., 2021). Their immunomodulating activities have been attributed to chemical composition, structural conformation, and molecular weight among other factors (Machuca et al., 2022). Contrary to SBM which could evoke diet-induced inflammation (Rehman et al., 2022), β-glucan feed additive exhibits anti-inflammatory properties. The anti-inflammatory effect is mediated through the regulation of various inflammatory cytokines such as interleukins (*ILs*), tumor necrosis factor alpha (*tnfα)*, interferon gamma (*inf)-y* as well as non-cytokine mediator, prostaglandin (Du et al., 2015). β-glucan exhibits antioxidant activities, antibacterial, wound healing and stress tolerance effects in fish (Guzmán-Villanueva et al., 2014). In this study, these known bioactive effects of β-glucan diet are examined alongside alginate oligosaccharides in zebrafish skin and gill.

1.5.1 Anti-inflammatory effects of β-glucan

Several studies have revealed that dietary B-glucan exhibits wide spectrum of biological activities which includes anti-inflammatory ability (Akramienė et al., 2007; Michael et al., 2011; Ye et al., 2011). In vivo experiment by (Suchecka et al., 2015) revealed the anti-inflammatory activities of β-glucan extract from oat.

1.5.2 Antioxidant effects of β-glucan

Oxidative stress is regarded as one of the main causal factors for different diseases (Kofuji et al., 2012). The antioxidant effect of β-glucan was evidential in a 30 days experiment trial by (Barera et al., 2016) which confirm its capacity to lower oxidative stress. Another experiment revealed that the antioxidative property of β-glucan was responsible for its antiplatelet activity which confirms its benefit as a supplementation in the prevention of excess blood platelet activation-related diseases (Saluk-Juszczak et al., 2010).

1.5.3 Anti-microbial effects of β-glucan

Dietary inclusion of β-glucan has been found to enhance different types of immune responses, resistance to bacterial and viral infections and to environmental stress in many fish species(Vetvicka et al., 2013) (Vetvicka et al., 2019). In vivo experiment has revealed that β-glucan can directly activate leukocytes and further stimulate their phagocytic, cytotoxic and antimicrobial activities (Pelizon et al., 2005).

1.6 Dietary properties of alginate oligosaccharide (AOS)

Alginates are fiber derivatives obtained mainly from brown algae used and is as prebiotics. In diet, they serve as food for health promoting microorganism in the host. Alginates are linear acidic polysaccharide found widely in cell walls of brown algae (Sari-Chmayssem et al., 2016). They are made up of variable amounts of (1,4)-linked β-D-mannuronate and α-L-guluronate residues. Structurally, the monomers are distributed as continuous 6 blocks of mannuronate residues (M-blocks), guluronate residues (G-blocks), or alternating residues (MG-blocks) as depicted in the Fig 1 (Rehm & Valla, 1997). The biochemical and biophysical properties of alginates are dependent on their individual molecular weight M:G ratio sequence and the G-block lengths (Brownlee et al., 2005).

1.6.1 Anti-inflammatory effects of alginate oligosaccharide

Alginate can be digested chemically or enzymatically to produce alginate oligosaccharides (AOS) which have lower molecular weight and lower viscosity, with better solubility and bioavailability (Trincone, 2015). AOS can mitigate the inflammatory reaction induced by *tnf-α* by reducing the pro-inflammatory cytokine (*il-6* and *tnf-α*) concentrations in porcine enterocytes(Wan et al., 2020). (Bi et al., 2022) reported the inflammation suppressing ability of AOS through attenuation of nitric oxide and prostaglandin E2 production and inactivation of the nuclear-factor (NF)-κB and mitogen-activated-protein-kinase (MAPK) signalling pathways.

1.6.2 Antioxidant effects of alginate oligosaccharide

Antioxidants are molecules that inhibit the oxidation (Sugihara et al., 2021) of other molecules (Kelishomi et al., 2016). These molecules provide protection against damage caused by free radicals which play crucial roles in the development of many chronic diseases including inflammation. In an experiment conducted by (Falkeborg et al., 2014), AOs were able to completely (100%) inhibit lipid oxidation emulsions, superior to ascorbic acid (89%). In an in vivo study with grass carp, it was observed that feeding AOS for a duration of six-weeks was able to stimulate the expression of antioxidant genes like *sod, cat, gpx* (Yang et al., 2021).

1.6.3 Antimicrobial effects of alginate oligosaccharides

Antimicrobial substances destroy or prevent the growth of microorganisms. Experiment has shown that alginates consisting of 96% of α-L-guluronic acid and 4% of the β-D-mannuronic acid isomer possesses antibacterial and anti-biofilm properties (Xing et al., 2020). The antibacterial activity of AOS was reported (Asadpoor et al., 2021) by its ability to significantly reduce the growth of pathogenic bacterial stain *GBS V* and *S. aureus*.

1.7.0. Brief description of the functions of the targeted genes

1.7.1 Immune genes, interleukin-1b (il1β), interleukin 8 (il8), tumor necrosis factor α (tnfα) and mucine gene (muc5.1)

Interleukins are a group of cytokines, a general term used for secreted proteins that are key modulators of inflammation. Cytokines are produced by different cells of the mucosal tissues in response to invading pathogens or antigenic factors to stimulate, recruit and proliferate immune cells (Kany et al., 2019). The pro-inflammatory cytokines are secreted by the T_{H1} cells, CD4+ cells, macrophages, and dendrite cells. They play essential roles in the activation and differentiation of immune cells as well as proliferation, maturation, migration, and adhesion of immune cells.

The tumor necrosis factor a (*tnfα)*, like other T_{H1} pro-inflammatory cytokines has an important role during the inflammatory response both locally and systemically. *tnfα* mainly produced by activated macrophages, T-lymphocytes, and natural killer cells triggers a series of inflammatory molecules, including other cytokines and chemokines (Jang et al., 2021). Besides, it increases the expression of vascular endothelial cells as well as enhances the leucocyte adhesion against viral infection by enhancing the infiltration of lymphocytes to the site of infection (Kobayashi et al., 2016).

il1b is a pro-inflammatory cytokine that has been implicated in pain, inflammation and autoimmune conditions(Ren & Torres, 2009). It is a member of β-trefoil family of the cytokines produced as an inactive precursor molecule that is processed by interleukin converting enzyme (ICE) to produce a biologically active peptide (Secombes et al., 2001). It is produced by monocytes, astrocytes and brain endothelial cells and believed to be involved in inflammatory reactions (Heidary et al., 2014). *il1β* differentially modulates the expression of gene which are suppressors of the immune response (Zou & Secombes, 2016). *il1β* production is regulated by intracellular complex inflammasome (Ren & Torres, 2009). Evidence has shown that fish produces *il1β* during immune response (Secombes et al., 1999)

il18 is a member of interleukin-8 supergene family and a pro-inflammatory chemoattractant cytokine produced by a variety of tissue and blood cells (Bickel, 1993). Unlike many other cytokines, it has a distinct target specificity for the neutrophil, with only a weak effect on other blood cells (Harada et al., 1994).

Mucins are high molecular weight glycoprotein secreted mainly by goblet cells (Marcos-López et al., 2018). Fish skin and gill is covered by mucus which is composed of mucin proteins and a complex mixture of enzymes, ions, and lipids. They play a crucial role in the innate immunity of the teleost. Mucins are essential for not only maintaining the viscous and elastic properties of mucus but also for enabling its barrier function (Thornton & Sheehan, 2004). They primarily create defensive barriers that hamper access of the pathogens to the host mucosal surface. Mucin genes have also been discovered in several fish species including zebrafish (Jevtov, 2014) (Lang et al., 2004). The genes *muc5.1* and *muc5.2* are expressed in skin, gill and the pharynx/esophagus of zebrafish, while *muc2.1* is the major mucin in the gut and shares its tissue localization with human *muc2*.

1.7.2 Anti-microbial genes, lysozyme (lyz) and hepcidin (hep)

Lysozyme is a ~14kDa protein present in different living organisms with a wide variability in origin, quantity, structure, chemical and enzymatic properties (Ferraboschi et al., 2021). It plays a crucial role in innate immunity, providing protection against bacteria, viruses, and fungi. The bacteriolytic activity of lysozyme in the fish skin mucus and other tissues contribute to its host defense mechanism (Ángeles, 2012). The three main isoforms of lysozyme detected in skin mucus are the c-type (chicken or conventional type), the g-type (goose type) and the i-type (invertebrate type) (Nigam et al., 2012). They function as antimicrobial agents by cleaving the peptidoglycan component of the bacteria cell wall, which lead to cell death.

Hepcidin is the hormone responsible for the regulation of iron recycling and balancing. Hepcidin synthesis increases in response to iron and inflammation and decreases to erythropoiesis. In other words, it controls how much iron is available for essential body function like making hemoglobin and red blood cells. Many harmful germs like bacteria use iron to thrive. Increase in hepcidin and decrease in iron prevents these germs from accessing the body of the organism (Ganz, 2006).

1.7.3 Antioxidant genes, catalase (cat), and superoxide dismutase (sod)

Oxidation is an inevitable reaction in every living organism. *sod* and *cat* represents the primary enzymatic defense against reactive oxygen species (Bartosz, 2005). Both are metalloproteins found in virtually all types of aerobic cells and efficiently employ the dismutation reaction to dispose the two most common reactive oxygen species formed (the superoxide radical anion and hydrogen peroxide). Experiments have shown that catalase, a key enzyme in the oxidation defense grid of organisms, scavenges free radicals to curtail their harmful effect on the host, supporting proper immune function (Gayashani et al., 2021). Therefore, the expression of the antioxidant genes, catalase and superoxide dismutase in the skin and gill of zebrafish were evaluated in this study.

1.8 Reference genes in zebrafish actb1, eef1a1l1, rpl13a

Selection of the most suitable reference genes is a vital approach in quantifying gene expression by real-time PCR. Reference genes used in this study for the purpose of normalization and comparison in the qPCR analysis are majorly housekeeping genes. The key factor for their selection is that they are characterized by a permanent and unchanging expression in each of the samples tested, despite the impact of the experimental factors (Kozera & Rapacz, 2013). *actb1*, *rpl3a* and *eef1a1l1* are zebrafish reference genes with a known range of CT values (average threshold cycle value below 20). Their expression ratio was found to be identical in all samples regardless of cell type, this agrees with GeNorm software package principles of ideal housekeeping gene (Tang et al., 2007).

1.8.1 β-actin (actb1)

β-actin is one of the six different actin isoforms with molecular weight approximately 42kDa which have been identified in humans, it interacts with RNA-binding protein Sam68 and activate eNOS (endothelia nitric oxide an enzyme that synthesizes nitric oxide) (Gunning et al., 2015). It is a cytoskeletal protein involved in cell structure and motility and has been frequently considered as constitutive housekeeping gene for RT-PCR and used to normalize changes in specific gene expression (Mori et al., 2008). Study has described *actb1* as one of the validated

reference genes for developmental time course study of zebrafish (Tang et al., 2007), report shows it has high transcript abundance (average Ct value below 20). The expression of *actb1* was validated during this study to ensure its expression does not change under experimental conditions and the average Ct value was below 20 which is in consonance with previous report (Tang et al., 2007). *actb1* as a reference gene shows high expression stability with low expression stability value.

1.8.2 eukaryotic translation elongation factor 1 alpha 1, like 1(eef1a1l1)

The eukaryotic translation elongation factor1 alpha 1 gene is a highly conserved ubiquitous protein involved in protein translation believed to have desirable properties for phylogenetic inference (Roger et al., 1999). It is an isoform of *eEF-1* complex alpha subunit, an elongation factor protein (involve in protein synthesis), a GTPase and an actin building protein. (Becker et al., 2013). In addition to its role in translation, it has been shown to play a vital role in the nuclear export of protein (Khacho et al., 2008). Moreover, it participates in several processes required for cell growth and proliferation including cytoskeleton organization, mitotic apparatus formation and signal transduction (Hamrita et al., 2011). It is regarded as one of the most suitable reference genes for tissue analysis (Tang et al., 2007), report shows it also has high transcript value (average Ct value below 20). The expression of eef*1a1l1*was validated during this study to ensure its expression does not change under experimental conditions and the average Ct value was below 20 which is in consonance with previous report (Tang et al., 2007). It also showed high expression stability with low expression stability value.

1.8.3 ribosomal protein L13a (rpl13a)

The *rpl13a* gene is a component of the large ribosomal subunit, a large ribonucleoprotein complex responsible for the synthesis of protein in the cell (Dresios et al., 2006). Report shows it also has high transcript value (average Ct value below 20) and a suitable reference gene for oxidative stress study in various tissues (Mohindra et al., 2014). The expression of *rpl13a* was validated during this study to ensure its expression does not change under experimental conditions and the average Ct value was below 20 which is in consonance with previous report (Tang et al., 2007).

1.9 Advantages of qPCR technique

Real time PCR is an alternative to some well-established laboratory techniques with several features which makes it the choice for several types of study. Compared with the other techniques presently available, it allows the detection of a given nucleic acid target in a rapid,

specific, and very sensitive way. In addition, it affords the absolute quantification of the initial target. To date, the reliability of real-time PCR has never been questioned. Real-time PCR provides a high sensitivity for the detection of DNA or RNA due to a combination of the amplification for double stranded. Thus, real-time PCR allows the amplification to be followed in real-time during the exponential phase of the run, and thus allows the amount of starting material to be determined precisely. (Gachon et al., 2004). Three phases are involved in QPCR, preincubation, 3 step amplification and melting phase. The product can be detected in each amplification cycle in QPCR. Data is collected during the exponential phases of the reaction. QPCR has very high resolution. Uses fluorescent dyes to detect the product (SYBR Green). QPCR is less time consuming. The role of QPCR is to quantify a particular fragment of a sample. QPCR is a combination of PCR and spectrofluorometry.

1.10 Hypothesis of the study

The aim of the present study is to evaluate the effect of dietary AOS and β-glucans on the gene expression profile of genes in zebrafish mucosal organs, skin, and gill.

The hypothesis is that alginate oligosaccharide (AOS) and β-glucan have potentially beneficial dietary effects on the mucosal organs- skin and gills in fish. These beneficial effects include alterations in the expression of antioxidant, anti-inflammatory and antimicrobial genes. Therefore, the expression of the selected genes in the five treatment groups were evaluated using qPCR approach.

2.0 MATERIALS AND METHODS

2.1 Experimental design

The experiment consists of five different dietary treatment groups. The control diet was based on a standard zebrafish formulation based on 40% fish protein. In the SBM diet, soybean meal was added at an inclusion of 50% replacing fish meal and wheat meal. The BG group (SBM + Algal β- glucan) had an inclusion of β- glucan at 2.5% inclusion rate. AL (SBM + Alginate 1, alginate with lower molecular weight) and the AH group (SBM + Alginate 2, alginate with higher molecular weight) contained AOS of varying molecular weights at an inclusion of 0.5% each. The feeding trial was conducted in five biological replicates for each treatment, 12 fish per replicate tank.

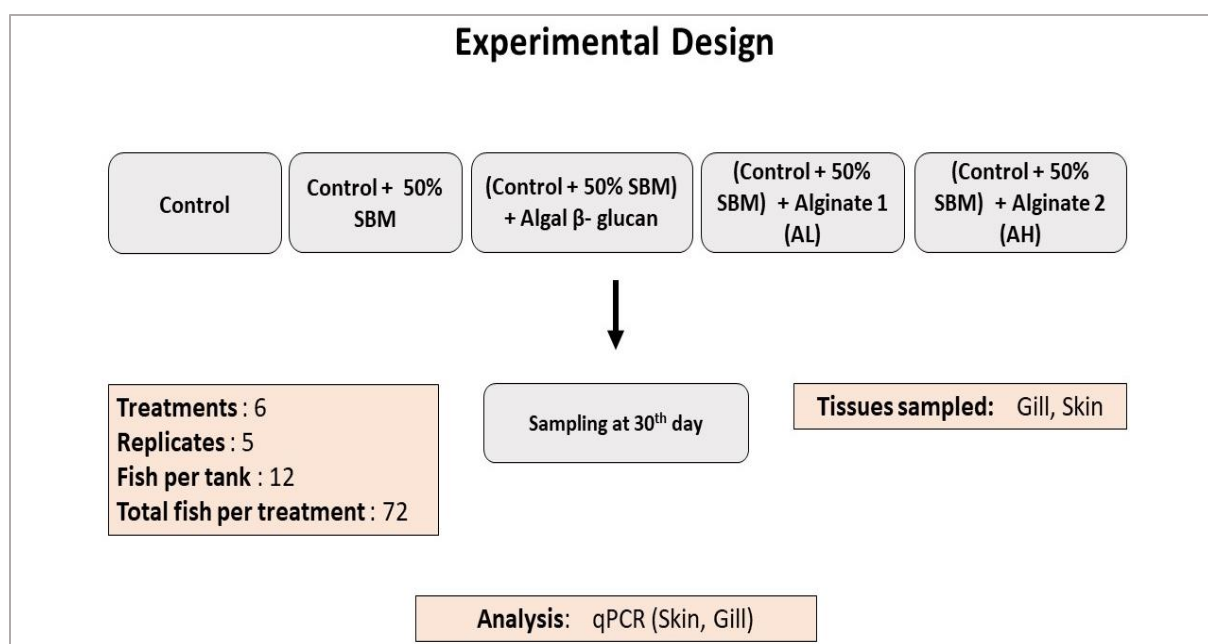


Figure 1 Schematic representation of the experimental design of the study.

Table 1 Table 1 Ingredient composition of the diets used in the experiment.

Ingredients %	CT	SBM	BG	AL	AH
Fish meal	35				
Fish protein hydrolysate	2.5	2.5	2.5	2.5	2.5
Fish gelatin	2.5	2.5	2.5	2.5	2.5
Soy protein isolate	8.7	11.1	11.1	11.1	11.1
Wheat gluten	8	8	8	8	8
Corn gluten meal	8	8	8	8	8
Soybean meal 44		50	50	50	50
Wheat meal	24.2	3.4	0.9	2.9	2.9
Vitamin mineral premix	1	1	1	1	1
Monoammonium phosphate		2	2	2	2
Soy lecithin	3	3	3	3	3
Fish oil	3	3	3	3	3
Palm oil	4.1	5.5	5.5	5.5	5.5
Beta-glucans			2.5		
Low molecular weight AOS				0.5	
High molecular weight AOS					0.5
Total	**100**	**100**	**100**	**100**	**100**

Low molecular weight AOS: <3kDa alginate composed 31% of the total AOS.

High molecular weight AOS: <3kDa alginate composed 3% of the total AOS.

2.2 Zebrafish husbandry

Healthy Adult zebrafish were used for the experiment. The Zebrafish were bred in the Nord University zebrafish laboratory, Bodø, Norway following the standard protocol (Westerfield, 2000). The experiment was conducted in a ZebTEC Stand-Alone Toxicology Rack, Tecniplast, Varese, Italy) which is a part of freshwater flow through system with the treatments randomly.

arranged in the rack, maintaining the water temperature in the tank at 28 ± 0.5°C, the water flow rate was maintained at 1 L/h, and dissolved oxygen was 7-8 ppm (Oxygen saturation >

85%). A 14L:10D photoperiod was maintained throughout the experiment period. The fish were fed 3 times daily for 30 days, starting from 7th February 2022 to 7th March 2022.

2.3 Sampling

Prior to weighing and fork length measurement, the fish were anaesthetized in a lethal dose of 200mg/L MS 222 Tricaine methane sulfonate (Argent Chemical Laboratories) buffered with an equal amount of sodium bicarbonate. Individual fish were weighed, and respective lengths were recorded before dissection. The condition factor of fish was calculated using the formula:

$$K = 100 \times \frac{W}{L^3}$$

Where, K = Fulton's condition factor

W = whole body wet weight in grams

L = length in cm

Skin and gill tissues were carefully dissected and snap frozen in dry ice -78.5°C and later stored and preserved in -80°C pending usage. Care was taken to obtain the skin tissue from the same region of the fish as shown in figure below:

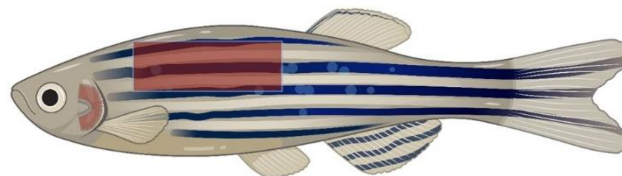


Figure 2 Details of fish tissues used for RNA extraction.

Four gill arches from the left gill of the zebrafish were dissected. A lateral incision on the skin anterior to the dorsal fin was made to obtain the skin tissue.

2.4 Total RNA isolation using Trizol

Total RNA from the frozen samples (skin and gills) were extracted using the Trizol, RNA MiniPrep kit (Zymoresearch, CA, USA) following the manufacturer's instructions. Briefly, the workstation was safeguarded with RNAase away. Frozen samples from -80°C were homogenized in bead with cold 800 ml Trizol. 200ml of chloroform was added to isolate the RNA, centrifuged for 15 minutes 4°C at 12000 rpm**.** To precipitate the mRNA, 100 per cent alcohol was added to the isolated RNA, this was further centrifuged at 1 minute at 4°C and 12000rpm**,** followed by two times prewash process (by adding 400ml of the prewash buffer to

the insoluble mRNA) and a wash process with 700ml of the RNA wash buffer, centrifuged for 1 minute 4°C 12000. 25/30ul of NFW was added to dissolve the insoluble mRNA. The products were safeguarded by placing them on ice. The purity, concentration and quality of the RNA were checked using the NanoDropTM 1,000 (Thermo Fisher Scientific) and Tape Station 2,200 (Agilent Technologies). The 260/280 and 260/230 ratio were also noted. RNA samples (RIN value>7) were used for cDNA synthesis.

2.5 cDNA synthesis protocol

The cDNA (complimentary DNA) was synthesized from the mRNA template by reverse transcription using reverse transcriptase enzyme.

Several steps are involved following the protocol as described below.

First, from the concentraion (from the nanodrop convert to 1000 µl or less depending on the figure) of the RNA samples µl + add 2µl of the gDNA + NFW = 14µl.

e.g RNA sample = µl (variable volumes)

gDNA = 2µl

NFW = µl (variable volumes)

Total = 14µl.

Incubate at 42°C for 2minutes, then place on dry ice.

Second prepare the reverse transcription master mix.

RT Buffer = 4µl

RT enzyme (reverse transcriptase) = 1µl

RT Primers Mix= 1µl

Incubate at 42 ºC for 15 minus also 95 ºC for 3 minutes to inactivate the reverse transcriptase. Place the product on solid ice/ store in -20 ºC for a long-term use.

2.6 Primers

Short strands of DNA or RNA molecules, 18-22 nucleotides long, serve as starting point of DNA synthesis (complimentary strand of the targeted gene) in other words, it is used to initiate DNA replication. Specificity and complementarity are the primary factors in the designing of primers (Mallona et al., 2010). Other factors that should be considered during the designing of primers include > (1). Melting Temperature ™ the optimal melting temperature of both the

forward and reverse primer should be 60-64^{0}C. (2). Annealing temperature. The annealing temperature of the experiment should be 5^{0}C below the melting temperature of each primer. (3). GC content, the GC content of the primer should be 35-65% (Mallona et al., 2010). Primers are hybridized by single stranded DNA and can be labeled on purpose. Primers for the selected genes were designed using the Primer-BLAST tool in NCBI. The primers were also checked for secondary structures such as hairpin, repeats, self, and cross dimer by NetPrimer (Premier Biosoft, Palo Alto, USA). The primers for the target genes are listed Table 2**.**

Table 2 . Details of the primers used for the qPCR.

Gene name	**Symbol**	**Primer Sequence**	**Amplicon (bp)**	**Efficiency (%)**	**Accession**
interleukin-1b	*il-1b*	GGACAGGAAAGACACCGAGC-F TTGGATGGAAGCACAGCAG-R	90	100	NM_212844.2
interleukin-8	*Il-8*	AGTGTGTGTTATTGTTTTCCTGGC-F TGCGTCGGCTTTCTGTTTCA-R	90	100	XM_001342570.7
lysozyme	*lyz*	GATTTGAGGGATTCTCCATTGG-F CCGTAGTCCTTCCCCGTATCA-R	200	102	NM_139180
actin, beta 1	*actb1*	TCAAACGAACGACCAACCTAAAC-F GCCATACAGAGCAGAAGCCA-R	71	100	AF057040.1
elongation factor 1 alpha	*eef1a1l1*	GCTTCTCTACCTACCCTCCTCT-F CCACCGATTTTCTTCTCAACGC-R	100	100	L23807.1
ribosomal protein L13a	*rpl13a*	CCACACAAAACCAAGAGGGG-F ATCGTCCAAGCAGGGCAAAT-R	157	100	NM_212784.1

hepcidin	*hep*	GAGCCGAGCAGAAGACAAGT-F	140	109	AY130989.1
		TGGAAGACACAGACACAGGT-R			
mucin 5.1	*muc5.1*	GTCCCTCTTTCCGCCTTGTC-F	95	103	XM_021470624.1
		TGTATTCTGTGCCGTTGCCT-R			
superoxide dismutase	*sod*	CAACACAAACGGCTGCATCA-F	110	100	NM_131294.1
		TTTGCAACACCACTGGCATC-R			
tumour necrosis factor-α	*tnf* α	TCACGCTCCATAAGACCCAG-F	123	108	NM_212859.2
		GATGTGCAAAGACACCTGGC-R			
catalase	*cat*	TCTCCTGATGTGGCCCGATA-F	169	88	NM_130912
		GGTTTTGCACCATGCGTTTC-R			

2.7 Gene expression analysis by quantitative real-time PCR (qPCR technique/RT-qPCR)

The analysis of the mRNA expression of genes was conducted using SYBR Green in LIGHT Cycler96® Real-Time PCR System (Roch Holdings AG, Basel Switzerland).

To calculate the efficiency of each primer, the following equation was used:

$$Efficiency(\%) = (10^{\frac{-1}{slope}} - 1) \times 100$$

Where *slope* is slope of the Ct value curve obtained when qPCR reactions are run in six dilutions of the cDNA (diluted 10 times at each dilution).

Normalized expression of the selected genes was determined based on the geometric mean of the three reference genes used (*actb1, rpl13a* and *eef1a1l1*).

The gene expression in the treatment groups, relative to control was obtained by using the following formula:

$$Relative\ expression = 1 + X^{-\Delta\Delta Ct}$$

where, $X = \frac{Primer\ Efficiency\ (\%)}{100}$

$\Delta\Delta Ct = \Delta Ct\ (treated\ sample) - \Delta Ct\ (untreated\ sample)$

$\Delta Ct = Ct\ (gene\ of\ interest) - Ct\ (housekeeping\ gene)$

The R package *ggplot2* was used for the visualization of the data. Data were also checked for assumption of normality (Shapiro Wilk) and homogeneity of variance (Bartlett's test). Based on the result of the assumption check, the statistical difference was determined by analysis of variance (ANOVA) or Kruskal-Wallis test for three groups.

3.0 RESULTS

3.1. Condition factor of fish from different treatment groups

We estimated Fulton's condition factor from the fish of different experimental groups to understand if the different diets have any effect on the condition of the fish. We observed that the fish from SBM and AL group had slight lower condition factor than the rest of the treatment groups, but this difference was not statistically significant (Figure 3). Overall, we did not observe any effect of the different diets on the condition of the fish.

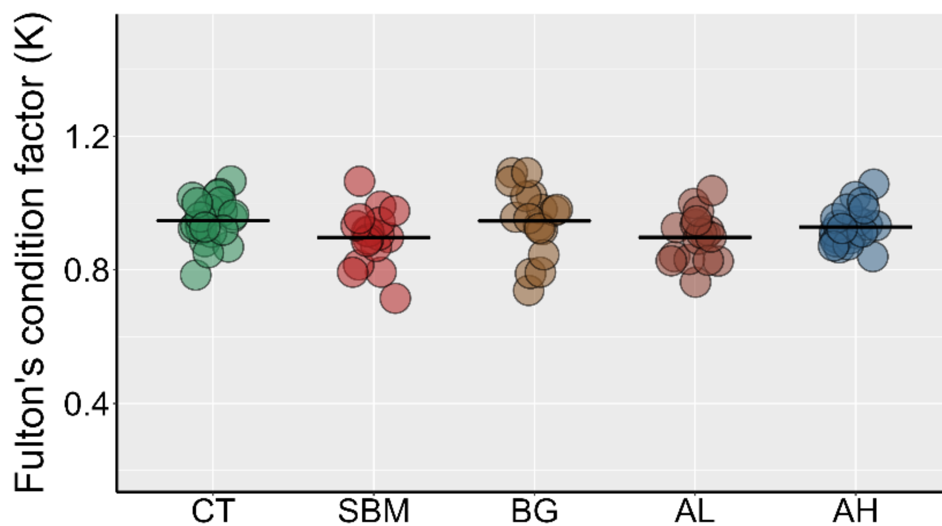
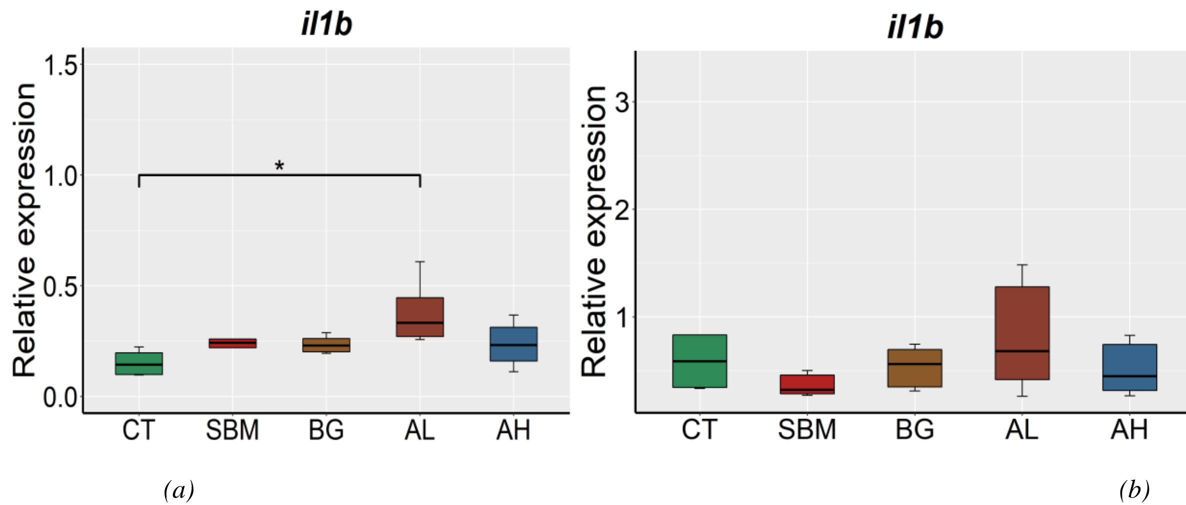


Figure 3 Fulton's condition factor (K) of the fish from different treatment groups after the feeding experiment of 30 days. CT: control group; SBM: soybean meal fed group; BG: fish fed with soybean meal and 2.5% beta glucan; AL: fish fed with soybean meal-based diet with 0.5% low molecular weight alginate oligosaccharide; AH: fish fed with soybean meal-based diet with 0.5% high molecular weight alginate oligosaccharide.

3.2 il1β expression in zebrafish skin and gill

From the result obtained as shown in figure 4a, in the zebrafish skin, the AL diet group had significantly higher level of expression of *il1β* (p value <0.05) compared to CT group. The diet groups SBM, BG and AH did not show any significant difference in the expression of *il1b* compared to each other or compared to CT group. In the gill tissue also, there was no observed significant difference in the expression of il1β among the treatment groups. (figure 4b).

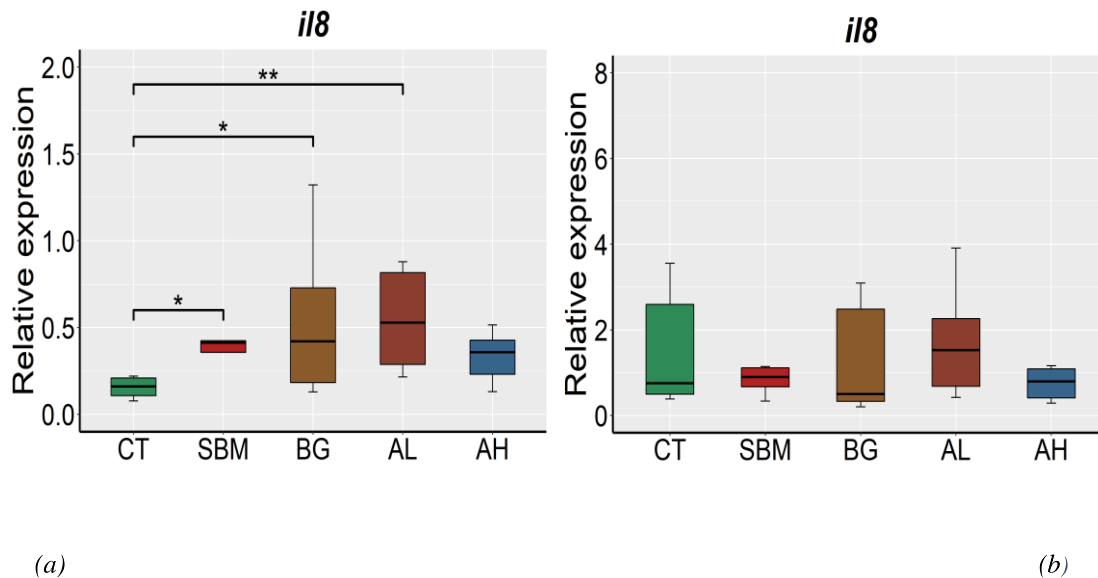


(a) *(b)*

Figure 4 Expression of il1b in zebrafish skin and gill (a) and gill (b) fish from different treatment groups after the feeding experiment of 3o days. *CT: control group; SBM: soybean meal fed group; BG: fish fed with soybean meal and 2.5% beta glucan; AL: fish fed with soybean meal-based diet with 0.5% low molecular weight alginate oligosaccharide; AH: fish fed with soybean meal-based diet with 0.5% high molecular weight alginate oligosaccharide. Asterisk * indicates p value < 0.05. Each treatment group consisted of five biological replicates.*

3.3 il8 expression in zebrafish skin and gill

The result of *il8* expression in the skin as shown in fig 5a. We observed a significant upregulation of *il8* expression in the SBM, BG and AH groups compared to CT. There was no statistically significant difference in the expression of *il8* in the AH group compared to CT. On the other hand, the gill tissue did not display any significant difference in the expression of *il8* in the different diet groups. Figure 5b.

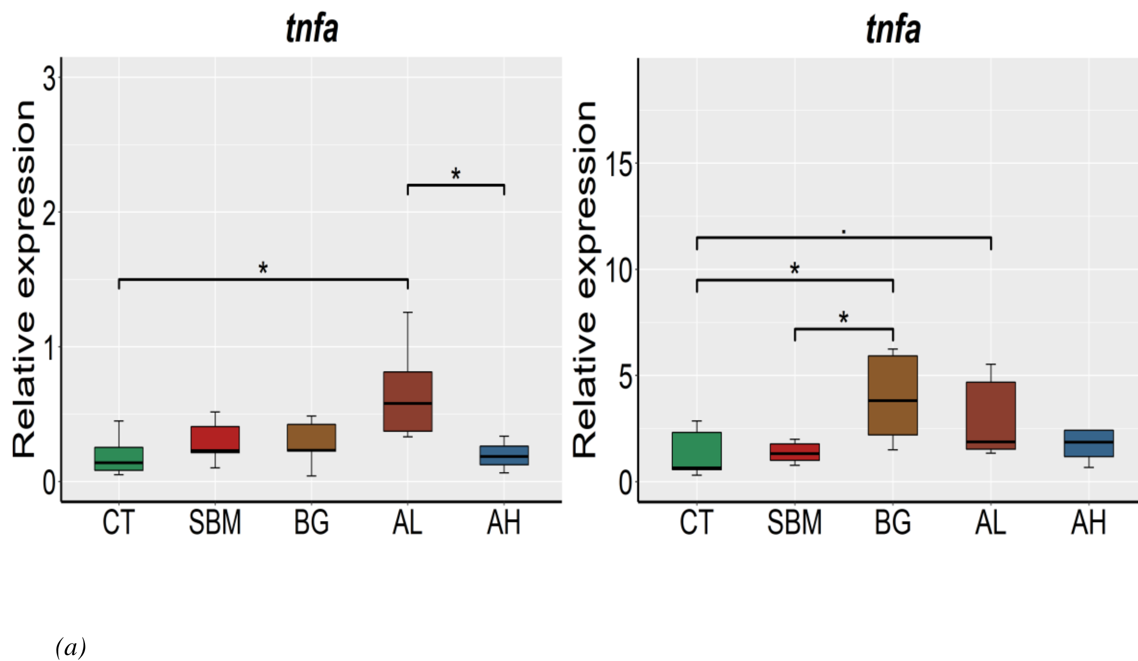


(a) *(b)*

Figure 4 Expression of il8 in zebrafish skin (a) and gill (b) fish from different treatment groups after the feeding trial of 30 days. *CT: control group; SBM: soybean meal fed group; BG: fish fed with soybean meal and 2.5% beta glucan; AL: fish fed with soybean meal-based diet with 0.5% low molecular weight alginate oligosaccharide; AH: fish fed with soybean meal-based diet with 0.5% high molecular weight alginate oligosaccharide. Asterisk * indicates p value < 0.05. Each treatment group consisted of five biological replicates.*

3.4 tnfα expression in zebrafish skin and gill

AL treatment group showed significantly higher expression of *tnfα* compared to CT and AH groups in the skin, whereas SBM and BG diet groups did not show any significant difference in the expression (figure 6a). However, in the gill tissue, the BG groups showed significantly higher expression of *tnfα* compared to Ct and SBM diet groups. There was no significant difference in the expression of *tnfα* in the gills of AH and AL diet groups compared to other treatment groups. See figure 6b.

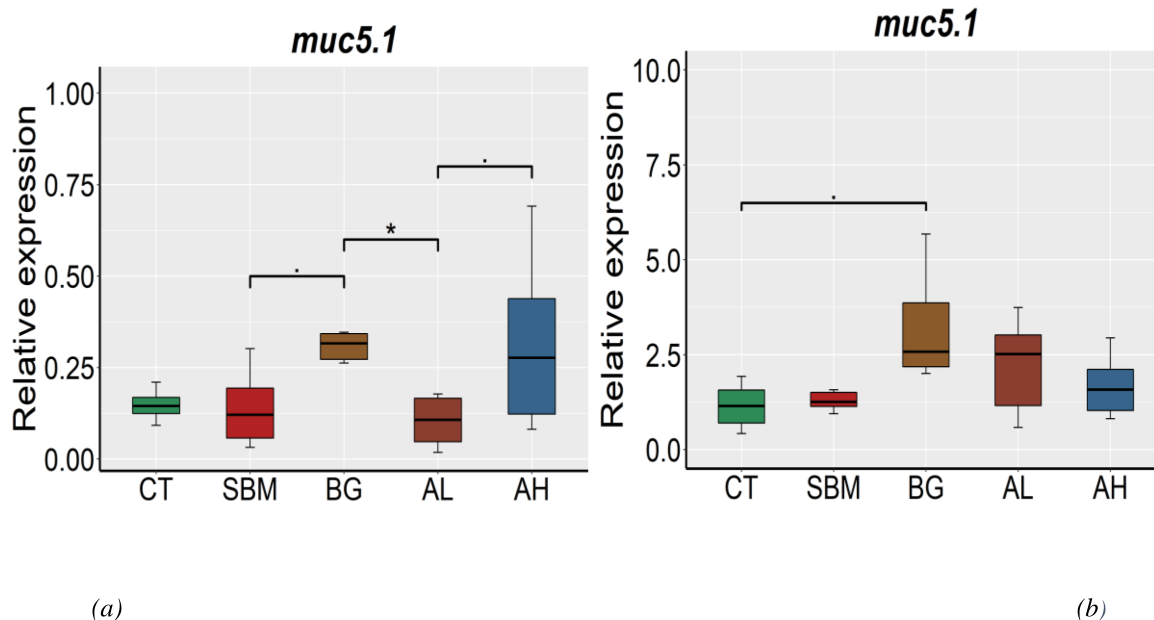


(a) *(b)*

Figure 5 Expression of tnfa in zebrafish skin (a) and gills (b) fish from different treatment groups after the feeding experiment of 30 days*. CT: control group; SBM: soybean meal fed group; BG: fish fed with soybean meal and 2.5% beta glucan; AL: fish fed with soybean meal-based diet with 0.5% low molecular weight alginate oligosaccharide; AH: fish fed with soybean meal-based diet with 0.5% high molecular weight alginate oligosaccharide. Asterisk * indicates p value < 0.05. Each treatment group consisted of five biological replicates.*

3.5 muc5.1 (Mucin gene) expression in zebrafish skin and gill

The result obtained for *muc5.1* was quite different from other genes that were examined in the skin as shown in figure 7a. The diet group AL showed a significant relative downregulation of the expression of *muc5.1* compared to BG. All expression differences in all other diet groups were not statistically different. However, the gill tissue did not show any significant difference in the expression of muc5.1 between treatment groups. See figure 7b.



(a) *(b)*

***Figure 6 Expression of muc5.1 in zebrafish skin (a) and gills (b) fish from different treatment groups after the feeding experiment of 30 days**. CT: control group; SBM: soybean meal fed group; BG: fish fed with soybean meal and 2.5% beta glucan; AL: fish fed with soybean meal-based diet with 0.5% low molecular weight alginate oligosaccharide; AH: fish fed with soybean meal-based diet with 0.5% high molecular weight alginate oligosaccharide. Asterisk * indicates p value < 0.05. Each treatment group consisted of five biological replicates.*

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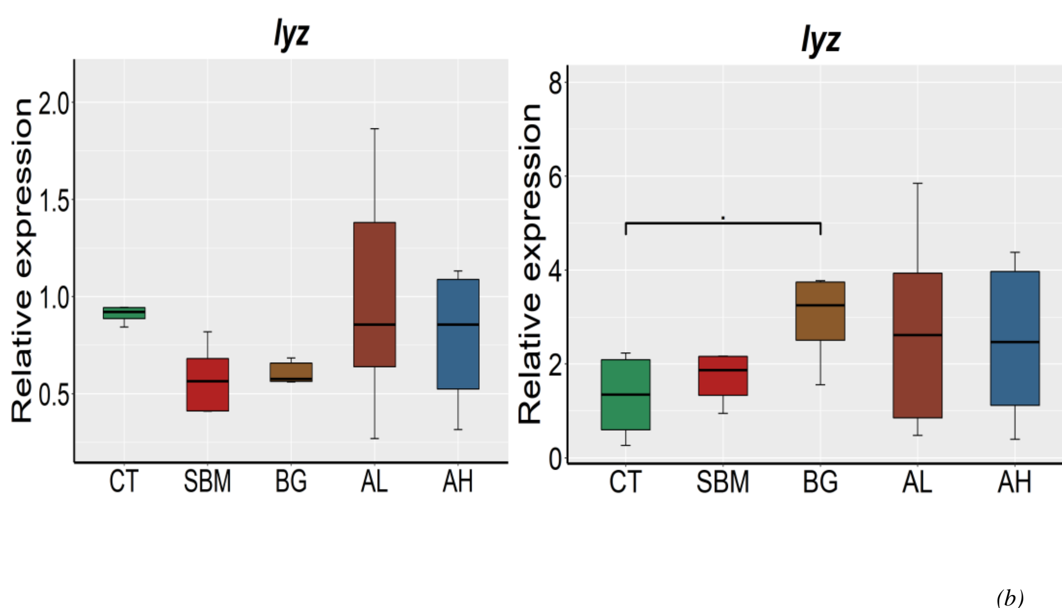
3.6 lyz expression in zebrafish skin and gill.

The result obtained for lysozyme relative expression showed no significant difference for the treatment groups compared to the control in the zebrafish skin and gills as shown in figure 8a and 8b.



(a) *(b)*

***Figure 7 . Expression of lyz in zebrafish skin (a) and gills (b) fish from different treatment groups after the feeding experiment of 30 days**. CT: control group; SBM: soybean meal fed group; BG: fish fed with soybean meal*

*and 2.5% beta glucan; AL: fish fed with soybean meal-based diet with 0.5% low molecular weight alginate oligosaccharide; AH: fish fed with soybean meal-based diet with 0.5% high molecular weight alginate oligosaccharide. Asterisk * indicates p value < 0.05. Each treatment group consisted of five biological replicates.*

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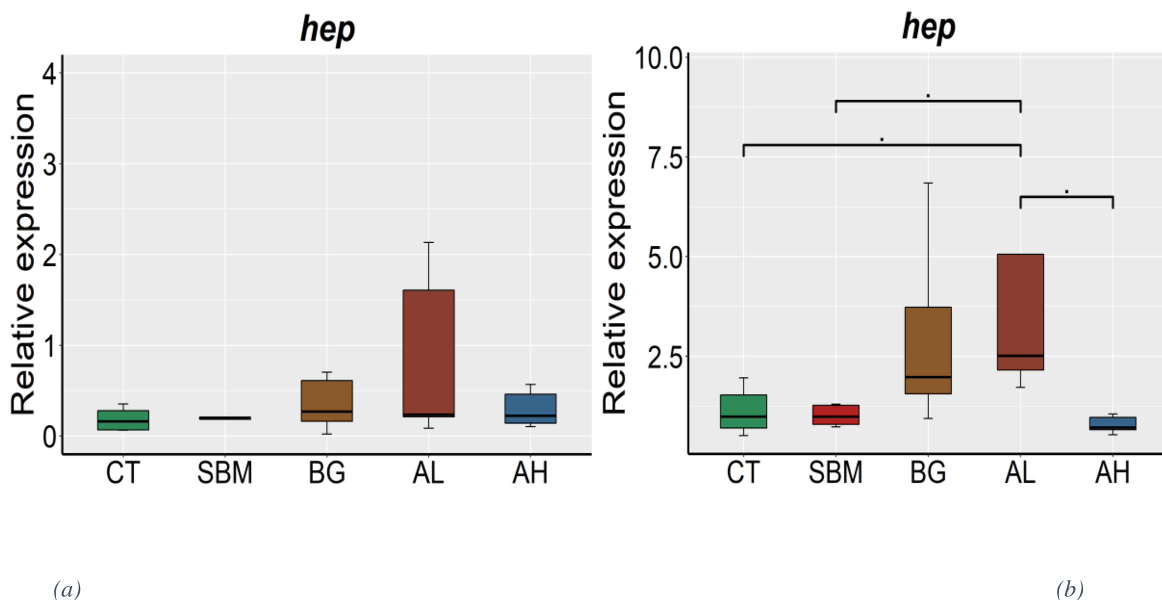
3.7 hep expression in zebrafish skin and gill

The relative expression of *hep* gene also showed no significant difference in the skin and gill tissues from different treatment groups. See figure 9a and 9b.

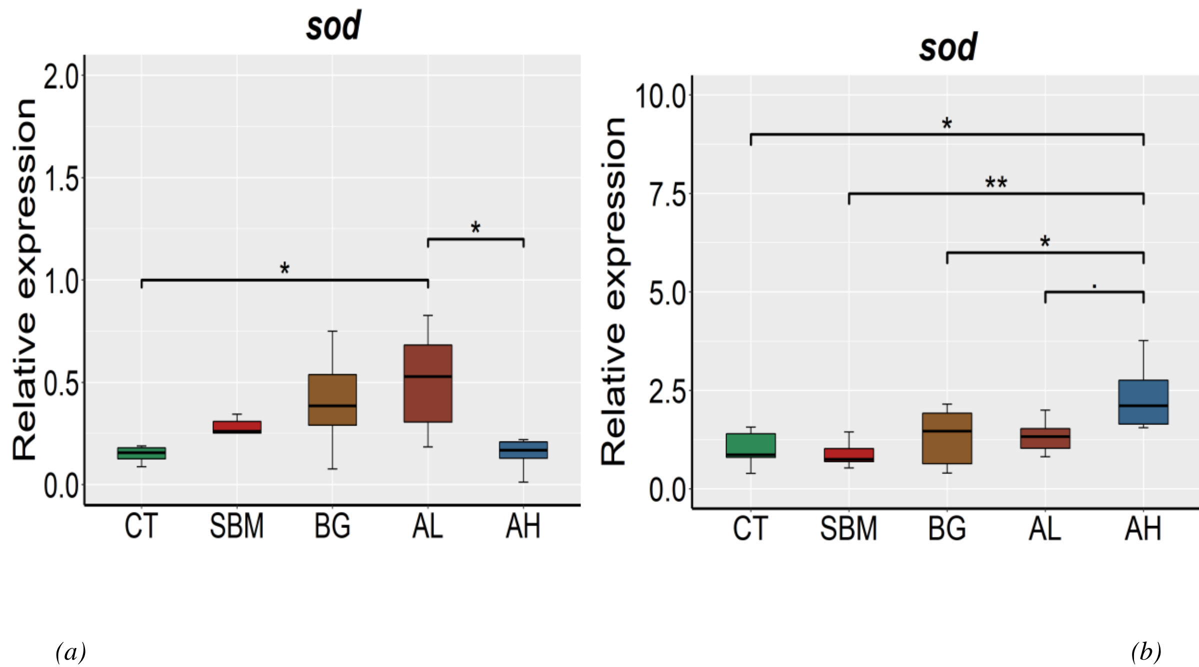


(a) *(b)*

Figure 8 Expression of hep in zebrafish skin (a) and gills (b) fish from different treatment groups after the feeding experiment of 30 days. *CT: control group; SBM: soybean meal fed group; BG: fish fed with soybean meal and 2.5% beta glucan; AL: fish fed with soybean meal-based diet with 0.5% low molecular weight alginate oligosaccharide; AH: fish fed with soybean meal-based diet with 0.5% high molecular weight alginate oligosaccharide. Asterisk * indicates p value < 0.05. Each treatment group consisted of five biological replicates.*

3.8 sod expression in zebrafish skin and gill

In the AL group, the relative expression of *sod* in the skin tissue was significantly higher than CT and AH groups (Figure 10a). The SBM and BG diet groups did not show any significant change in the expression compared to other groups. However, in the gill tissue, the AH group showed significantly upregulated expression, compared to CT, SBM and BG diet groups. See figure 10b.

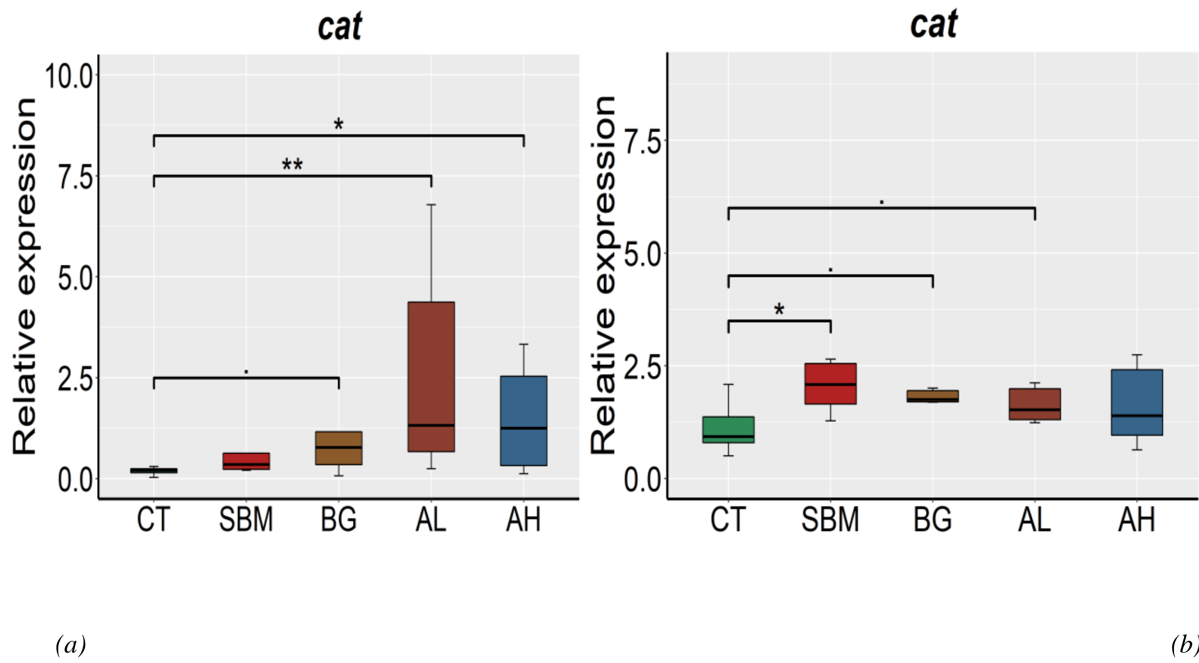


(a) *(b)*

Figure 9 Expression of sod in zebrafish skin (a) and gills (b) fish from different treatment groups after the feeding experiment of 30 days*. CT: control group; SBM: soybean meal fed group; BG: fish fed with soybean meal and 2.5% beta glucan; AL: fish fed with soybean meal-based diet with 0.5% low molecular weight alginate oligosaccharide; AH: fish fed with soybean meal-based diet with 0.5% high molecular weight alginate oligosaccharide. Asterisk * indicates p value < 0.05. Each treatment group consisted of five biological replicates.*

3.9 cat expression in zebrafish skin and gill

In the skin tissue, expression of *cat* gene was significantly upregulated in both AL and AH diet groups compared to CT group. The SBM and BG diet groups did not show any significant change in expression compared to other diet groups (figure 11a). In the gill, however, there was a significant upregulation in the expression of *cat* in the SBM treatment group compared to the CT group. The diet groups BG, AL and AH did not show any significant difference in the expression compared to SBM or CT. see figure 11b.



(a) *(b)*

***Figure 10 Expression of cat in zebrafish skin (a) and gills (b) fish from different treatment groups after the feeding experiment of 30 days**. CT: control group; SBM: soybean meal fed group; BG: fish fed with soybean meal and 2.5% beta glucan; AL: fish fed with soybean meal-based diet with 0.5% low molecular weight alginate oligosaccharide; AH: fish fed with soybean meal-based diet with 0.5% high molecular weight alginate oligosaccharide. Asterisk * indicates p value < 0.05. Each treatment group consisted of five biological replicates.*

4.0 DISCUSSION

Soybean meal is considered as one of the best alternative feed ingredients which can replace fish meal in aquafeeds. However, the use of soybean meal as a fish meal replacer is hampered by presence of antinutritional factors (Francis et al., 2001). The removal of antinutritional factors from soybean meal is expensive and can reduce cost-effectiveness of the aquafeeds. In addition, the use of soybean meal does not only cause intestinal inflammation but can also have severe extra-intestinal effects in fish (Rehman et al., 2022). Some studies have reported the impact of dietary soybean meals on the immune system and behavior of fish. The skin and gills are two important mucosal organs whose health determines the over-all welfare of fish (Rehman et al., 2022. Therefore, we evaluated the expression of several immune, antioxidant and antimicrobial genes in these tissues after feeding the fish with soybean meal. Supplementing soybean meal-based diets with certain bioactive compounds that can partly alleviate its detrimental effects (Guzmán-Villanueva et al., 2014). Therefore, we explored the effect of supplementing soybean meal diet with AOS and beta-glucan on the gene expression profile of the skin and gills of the zebrafish. Our study revealed that dietary soybean meals can upregulate the expression of genes in the gills of the zebrafish. We further observed that dietary bioactive compounds can affect the expression of several genes in the skin and gill tissues.

4.1 Skin and gill showed variable response to diets

Teleost adaptive immunity is related to each of their mucosal surfaces which includes the skin and the gill. Their skin is a multi-purpose tissue that serves numerous vital functions including physical protection, sensory activity, behavioral purpose, or hormone metabolism (Rakers et al., 2010). The gill on the other hand is a multifunctional organ serving as respiratory organ, osmoregulation function, pH balancing regulation, amino acid excretion and involved in immune defense through the gill associated lymphoid tissue. Both tissues as mucosal surfaces serve as a primary barrier to the pathogenic microbes that may invade the host. The health of these mucosal surfaces is vital as they are in constant touch with the environment. Both β-glucans and alginate oligosaccharides are water soluble polysaccharides. Although, they mainly affect the gut functions, the possibility of these bioactive compounds having an effect on skin and gills cannot be ignored. In general, we found that zebrafish skin showed higher response to dietary changes in terms of gene expression profile. Six (*il1b, il8, tnfa, muc5.1, sod, cat*) out of the 8 genes evaluated were altered in the skin tissue whereas expression of only 3 genes (*sod*, *cat*, *tnfa*) was altered in the gills. Changes in diets altered the expression of *muc5.1, il1b and*

il8 only in the skin but not in the gills. Similar variability in the expression of immune genes has been observed in the skin and gills of Atlantic salmon when fed with mannan-oligosaccharide alone or in combination with coconut oil (Bledsoe et al., 2022). This study also shows that diet can significantly impact the microbiota composition of the skin but not the gills. Since both β-glucans and AOS are soluble in nature and so have prebiotic properties, it may be speculated that they may alter the microbiota profile of the skin and thus generate a more pronounced response in the zebrafish host.

4.2 Dietary soybean meal altered the expression of il8 and cat

Although we evaluated several immune, antioxidant and antimicrobial genes in the skin and gill tissues, soybean meal feeding altered the expression of only two genes−*il8* in the skin and *cat* in the gills of zebrafish. The gene *il8* codes for a proinflammatory cytokine expressed in skin and gills and is involved in chemoattraction of neutrophils during an inflammatory response (Oehlers et al., 2010)Neutrophils are one of the earliest cells to arrive at the site of inflammation where they produce ROS and release enzymes such as proteinases, and cationic peptide from granules. The cat expression was also increased in the gills in response to soybean meal feeding. Previous study has shown an increase in the *il8* and *cat* expression in response to dietary soybean meal in zebrafish (Hedrera et al., 2013) (Pervin et al., 2020). This indicates that dietary soybean meals may generate inflammatory response and oxidative stress responses beyond the intestine.

4.3 Source specific effect of alginate oligosaccharides

The AL group displayed significantly higher expression of *il1b, il8, tnfa* and *sod* in the skin compared to different treatment groups, whereas in the Ah group only *sod* expression of gill was increased. Furthermore, the expression of *tnf-α* and sod was significantly higher in the AL group compared to AH. This result indicates that AL diet was more effective in altering the gene expression profile compared to AH. AL diet contained a higher percentage of low molecular weight AOS. AOS have better biological activities when they have lower molecular weight (MW) and better water solubility *(Lu et al., 2022).* Dietary low molecular weight sodium alginate extracted from brown algae enhanced liver antioxidant activities including superoxidase dismutase, catalase, and glutathione-S-transferase in Asian sea bass (*Lates calcarifer*) (Ashouri et al.,2020) Previous study has revealed that improving the solubility of AOS can increase its bioactivity. For instance, Hu et al reported the sulfated derivative of AOS which is a more soluble form of AOS, exhibited better anti-tumor activity than AOS (Hu, et al.,

2004). Although we have not estimated the solubility of low molecular weight AOS in the present study, it may be speculated that the better solubility of low molecular weight AOS in the AL diet may have enabled access of AOS to the mucosal tissues.

4.4 β-glucan increased the expression of immune genes

β-glucan is a well-known immunomodulator which has been used for many decades in aquafeeds. β-glucans impact beneficial effects by strengthening the mucosal barrier and improving immune response in the tissues. In our study β-glucan altered the expression of *il8* and *muc5.1* in the skin of zebrafish. Previous studies have also found an alteration in the skin expression of these genes. A study with a skin wound model of common carp revealed that bath exposure of β-glucans can alter the expression of *il8* and *muc5b* in the skin (Przybylska-Diaz, et al., 2013). Another study in common carp revealed that dietary beta glucan can also stimulate the expression of *muc5b* by 2.7 fold in the skin (van der Marel et al., 2012). Our study corroborates with these results and indicates the protective role of dietary β-glucan in enhancing the mucosal barrier strength of fish.

5.0 CONCLUSION

In this study, the effect of soybean meal alone or alginate/β-glucan supplemented soybean meal-based diets on immune-related genes in the skin and gill of zebrafish was explored. We found that zebrafish skin showed a comparatively more pronounced response to dietary changes in terms of gene expression profile, compared to the gill. The results obtained imply dietary soybean meals altered the expression of *il8* and *cat* genes in the skin and gills respectively. We also found that feeding zebrafish with AOS with low molecular weight alginate generates a differential gene expression response compared to dietary AOS with higher molecular weight alginate. The low molecular weight AOS was more effective in altering the gene expression profile compared to high molecular AOS. Dietary β-glucan supplementation was also able to stimulate the expression of mucin and immune genes. Overall, insights from this study could be used to promote the use of AOS and β-glucans as dietary additives that can modulate mucosal immune response in fish.

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