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Diet-induced inflammation in zebrafish and its alleviation by functional oligo- and polysaccharides

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Preface

This thesis is submitted in fulfilment of the requirements for the degree of Philosophiae Doctor (PhD) at the Faculty of Biosciences and Aquaculture (FBA), Nord University, Bodø, Norway. The studies included in this dissertation represent original research that was carried out over a period of 4 years from 01.11.2018 to 05.06.2023. Saima Rehman was financially supported by Netaji Subhas-ICAR International Fellowships (NS-ICAR IFs) from the Indian Council of Agricultural Research, India

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Saima Rehman
Bodø, 5th June 2023

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Saima

Contents

List of abbreviations

AOS	-	Alginate oligosaccharides
ATP	-	Adenosine triphosphate
2HB	-	2-hydroxybutyric acid
AMP	-	Antimicrobial peptides
CD	-	Cluster of differentiation
CR3	-	Complement receptor 3
DC	-	Dendritic cell
DP	-	Degree of polymerisation
DPF	-	Days post fertilization
DSS	-	Dextran sodium sulphate
ENS	-	Enteric nervous system
ER	-	Endoplasmic reticulum
FAE	-	Follicle associated epithelium
GALT	-	Gut-associated lymphoid tissue
IBD	-	Inflammatory bowel disease
IEC	-	Intestinal epithelial cell
IEL	-	Intraepithelial lymphocytes
IKK	-	IκB kinase
IL	-	Interleukin
M cells	-	Microfold cells
MAC	-	Membrane attack complex
MHC	-	Major histocompatibility complex
MLN	-	Mesenteric lymph nodes

MMPs	-	Matrix metalloproteases
MW	-	Molecular weight
NF-κB	-	Nuclear factor kappa-light-chain-enhancer of activated B cells
PP	-	Peyer's patches
PRRs	-	Pattern recognition receptors
RANK	-	Receptor activator of NF-κB ligand
RNA-Seq	-	RNA-sequencing
ROS	-	Reactive oxygen species
SCFAs	-	Short chain fatty acids
SED	-	Subepithelial dome
TJ	-	Tight junction
TLR	-	Toll-like receptors
TNBS	-	2,4,6-Trinitrobenzene sulfonic acid
Treg	-	Regulatory T-cells
VB5	-	Vitamin B5

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List of Papers

Paper I

Rehman, S., Gora, A. H., Siriyappagouder, P., Brugman, S., Fernandes, J. M., Dias, J., & Kiron, V. (2021). Zebrafish intestinal transcriptome highlights subdued inflammatory responses to dietary soya bean and efficacy of yeast β-glucan. Journal of Fish Diseases, 44(10), 1619-1637.

Paper II

Rehman, S., Gora, A. H., Varshney, S., Dias, J., Olsvik, P. A., Fernandes, J.M., Brugman, S., & Kiron, V. (2022). Developmental defects and behavioural changes in a diet-induced inflammation model of zebrafish. Frontiers in Immunology, 13, 1018768.

Paper III

Rehman, S., Gora, A. H., Abdelhafiz, Y. A., Dias, J., Pierre, R., Meynen, K., Fernandes, J.M., Sørenson, M Brugman, S., & Kiron, V. (2023) Potential of algae-derived alginate oligosaccharides and β-glucan to counter inflammation in adult zebrafish intestine. Frontiers in Immunology, 14, 1183701.

SUMMARY

The intestinal mucosa acts as a selective barrier by preventing the entry of pathogens and food toxins while maintaining tolerance to commensal microbiota. Hence, a compromised mucosal barrier increases the permeability of the intestine, allowing undesirable luminal antigens to cross the barrier and activate an inflammatory response in the tissue. Chronic inflammation-associated damage can eventually result in the disturbance of the overall intestinal physiology. Though previous studies have reported soybean meal-induced inflammation in the intestine of zebrafish, there is a paucity of information regarding the underlying molecular changes. Furthermore, the extraintestinal effects of soybean meal-associated inflammation have not been studied in detail. For instance, the behavioural changes associated with feeding soybean meal are underexplored in animal models. Also, the shift in plasma metabolomic landscape of zebrafish by dietary soybean meal has not been investigated to understand the metabolites associated with inflammation. Such information will establish the reliability of zebrafish as a nutritional model for assessing therapeutic agents against inflammatory diseases.

In this PhD project, employing transcriptomic approaches and through histological evaluation I elucidated the changes marking soybean meal-induced inflammation in the intestine of zebrafish. In this first study, I also examined the effects of yeast β-glucan supplementation. Dietary soybean meal increased the expression of several genes that are associated with small GTPase-mediated signal transduction and ATP dependent peptidase activity. The downregulation of genes linked to inhibition of GTPase-mediated signal transduction, guanyl nucleotide binding, and intracellular signal transduction, reduced lamina propria width and increased villi height of the yeast β-glucan fed group point to their protective responses in the intestine.

The second study focused on soybean meal-induced defects in behaviour and organ development during the early stages of zebrafish, using transcriptome analysis, morphological observations, and oxygen consumption measurements. Furthermore,

an algal β-glucan supplement was evaluated for its ability to mitigate specific behavioural and developmental aberrations induced by dietary soybean meal in zebrafish. The dietary soybean meal was noted to reduce the locomotor activity, induce developmental defects, and increase the oxygen demand in zebrafish larvae. In addition, the transcriptomic analysis pointed to the suppression of genes linked to visual perception, organ development, phototransduction pathway and activation of genes linked to the steroid biosynthesis pathway and retinoic acid metabolism. However, the algal β-glucan counteracted the behavioural and phenotypic changes induced by dietary soybean meal.

In the third paper, I studied the intestinal transcriptome and plasma metabolome, along with gene markers and histological analysis to evaluate the anti-inflammatory potential of alginate oligosaccharides (AOS) and an algal β-glucan in zebrafish fed dietary soybean meal. The two functional feed additives could reduce the expression of certain inflammatory genes associated with soybean meal intake. Transcriptomic analysis indicated that dietary AOS with a higher percentage of the low molecular weight fraction suppressed the expression of genes related to complement activation, inflammatory and humoral response while algal β-glucan suppressed several genes linked to endopeptidase activity and proteolysis. The plasma metabolomic profile further revealed the increase of a short chain fatty acid in the AOS group and pantothenic acid in the algal β-glucan group, all these responses likely indicate the effectiveness of the additives to alleviate the inflammatory signs in the intestine. Histological evaluation also revealed the increased goblet cell number and villi length in the intestine of the group fed AOS, indicating the ability of the product to possibly enhance nutrient absorption and aid in mucosal defence.

Overall, this thesis provides insights into the transcriptomic, metabolomic and behavioural changes that mark soybean-induced intestinal inflammation in zebrafish. The project further elucidated the molecular changes and histological alterations accompanying the administration of oligo and polysaccharides to counter intestinal inflammation in the zebrafish model.

SAMMENDRAG

Tarmslimhinnen fungerer som en selektiv barriere for å forhindre inntreden av patogener og mattoksiner samtidig som toleransen for kommensal mikrobiota opprettholdes. En kompromittert slimhinnebarriere øker permeabiliteten til tarmen, slik at uønskede luminale antigener kan krysse barrieren og aktivere en inflammatorisk respons i vevet. Kronisk betennelsesassosiert skade kan til slutt resultere i en forstyrret tarmfysiologi. Selv om tidligere studier har påvist at soyabønner induserer endringer i tarmen til sebrafisk, mangler det informasjon om de underliggende molekylære endringene. Videre har de ekstraintestinale effektene av soyabønnemel-assosiert betennelse ikke blitt studert i detalj. For eksempel er atferdsendringer forbundet med fôring av soyabønnemel underutforsket i dyremodeller. Hvordan soyabønnemel i dietten påvirker metabolitter i plasma og induserer betennelse har heller ikke blitt undersøkt. Slik kunnskap vil bidra til å etablere sebrafisk som en ernæringsmodell for vurdering av terapeutiske midler mot inflammatoriske sykdommer.

I dette doktorgradsprosjektet bruke jeg transkriptomikk og histologi til å belyse hvordan soyabønnemel induserer betennelse i tarmen hos sebrafisk. I den første studien undersøkte jeg også effekten av gjær-β-glukantilskudd. Soyabønnemel økte uttrykket av flere gener som er assosiert med liten GTPase-mediert signaltransduksjon og ATP-avhengig peptidaseaktivitet. Nedregulering av gener knyttet til inhibering av GTPase-mediert signaltransduksjon, guanylnukleotidbinding og intracellulær signaltransduksjon, redusert lamina propria-bredde og økt tarmvilli-høyde av gjær-β-glukan tyder på at tilsetning av gjær-β-glukan i fôret beskytter tarmen.

Den andre studien fokuserte på hvordan soyabønnemel påvirker atferd og organutvikling i tidlige livsstadier i sebrafisk. Dette ble undersøkt ved å studere transkriptomikk, morfologiske endringer og oksygenforbruk. Videre ble det undersøkt hvordan tilsetning av alge-β-glukan i fôret til sebrafisk motvirker atferds- og utviklingsavvik indusert av soyabønnemel. Tilsetning av soyabønnemel i fôret medførte redusert bevegelsesaktivitet, induserte utviklingsdefekter og øke oksygenbehovet i

sebrafisklarver. I tillegge pekte transkriptomikk-analysen på inhibering av gener knyttet til visuell persepsjon, organutvikling, fototransduksjonsvei og aktivering av gener knyttet til steroidbiosynteseveien og retinsyremetabolisme. Tilsetning av alge-β-glukan i fôret motvirket de negative effektene av soyabønnemel på atferdsmessige og fenotypiske parametre.

I den tredje studien brukte jeg analyser av det intestinale transkriptomet og plasmametabolomet, sammen med genmarkører og histologisk analyser, for å evaluere det antiinflammatoriske potensialet til alginatoligosakkarider (AOS) og alge-β-glukan i sebrafisk fôret med en soyabønnediett. De to funksjonelle fôrtilsetningene kan redusere uttrykket av inflammatoriske gener knyttet til inntak av soyabønnemel. Transkriptomikk-analyse indikerte at dietten som inneholder en høyere prosentandel av lavmolekylvektsfraksjonen av AOS inhiberte uttrykket av gener relatert til komplementaktivering, inflammatorisk og humoral respons mens alge-β-glukan inhiberte flere gener knyttet til endopeptidaseaktivitet og proteolyse. Plasmametabolitt-profilen avslørte videre at en kortkjedet fettsyre i AOS-gruppen og pantotensyre økte i alge-β-glukangruppen. Disse responsene indikerer at de studerte tilsetningsstoffene effektivt kan lindre soyabønnediett-indusert inflammasjon i tarmen. Histologiske analyser viste et økt antall av begerceller og økt lengde av tarmvilli i tarmen til den AOS-fôrede gruppen, noe som indikerer at de to funksjonelle fôrtilsetningene kan forbedre næringsabsorpsjon og hjelpe til med slimhinneforsvar.

Samlet gir oppgaven innsikt i de transkriptomiske, metabolomiske og atferdsmessige endringene som kjennetegner soyabønne-indusert tarmbetennelse hos sebrafisk. Prosjektet belyste videre hvordan tilsetning av oligo-/polysakkarider påvirker molekylære og histologiske prosesser som bidrar til å motvirke tarmbetennelse hos sebrafisk.

1. INTRODUCTION

1.1. Intestinal immune components of mammals

The intestine of vertebrates is a multifunctional organ that performs diverse physiological functions such as food digestion, nutrient absorption, and immune defense (Ahluwalia et al., 2017). The epithelial layer of the intestine is constantly exposed to food and microbe-derived antigens (Allaire et al., 2018) and acts as a selective barrier to prevent the entry of pathogens and food toxins while maintaining tolerance to commensal microbiota and harmless food-derived ligands. Different immune components of the intestine are essential to achieve the complex task of maintaining homeostasis and sustaining the overall tissue integrity. The mucosal immune sites can be functionally classified as i) inductive sites (e.g., gut-associated lymphoid tissues, GALT) where naïve immune cells first go through priming and differentiation or ii) effector sites (e.g., lamina propria and epithelium) where activated immune cells localize and are maintained to support barrier integrity (**Figure 1**). The intestinal epithelial cells (IECs) include enterocytes, goblet cells, Paneth cells, and microfold (M) cells (Allaire et al., 2018). Among these cells, goblet cells are specialized cells that are primarily responsible for secreting mucus, which serves as a first layer of the physical barrier. Mucus contains glycoproteins that are known as mucins. Mucins are broadly divided into secreted gel-forming or transmembrane mucins based on their structural and functional properties (Grondin et al., 2020). The gel-forming mucins, namely mucin 2 (MUC2), MUC5AC, MUC5B and MUC6, provide viscoelastic property to the mucus layer. MUC2 and MUC5AC are the major gel-forming mucins that are responsible for barrier formation near the luminal region (Hasnain et al., 2011). Transmembrane mucins, such as MUC1, MUC3, MUC4, MUC13, and MUC17, which form a carbohydrate-rich layer called glycocalyx, are present on the apical surfaces of epithelial cells and act as an outer barrier (Pelaseyed et al., 2014). The enterocyte is a columnar intestinal epithelial cell type that is responsible for nutrient and water absorption. Large molecules are transported via the transcellular route of the

absorptive enterocytes that are equipped with different membrane transporter proteins. Certain molecules for example short chain fatty acids (SCFAs) such as butyrate are transported via passive diffusion or with the help of transporter proteins. Furthermore, enterocytes can also secrete antimicrobial peptides (AMPs; such as β-defensins, cathelicidin, lysozyme) and the cohesion between the enterocytes facilitated by different cell junctions and their interactions with other intestinal cells help in maintaining the barrier integrity. The tight junctions (TJs) and adherens junctions are present near the apical surface of the enterocytes (**Figure 1**). Desmosomes and gap junctions are located on the baso-lateral membranes and hemidesmosomes connect the epithelial cells to the basal membrane. The important function of the TJ proteins is to regulate the paracellular transport and the passage of water and ions (Wells et al., 2011). On the other hand, the interepithelial flow of ions and small molecules is controlled by the intercellular channels of gap junctions (Mowat, 2003). The tight junctions consist of transmembrane proteins such as claudin, occludin, tricellulin, and junctional adhesion molecule-A and cytosolic proteins such as zonula occludens and cingulin, which are impermeable to large organic molecules (e.g., amino acids and glucose). Adherens junction allows cell-cell adhesion and maintain intracellular actin-cytoskeleton organization. Desmosomes attach to the intermediate filament cytoskeleton and create a strong adhesive bond to give mechanical strength to tissues. Paneth cells that are at the base of the crypt also reinforce barrier integrity by producing several AMPs including α-defensins and lysozyme (Moretti and Blander, 2014). The intraepithelial lymphocytes (IELs) that reside between the adjacent epithelial cells also take part in maintaining the barrier integrity (Olivares-Villagómez and Van Kaer, 2018). IELs are polarized into antigen-experienced phenotypes after their encounter with foreign antigens present in the lumen.

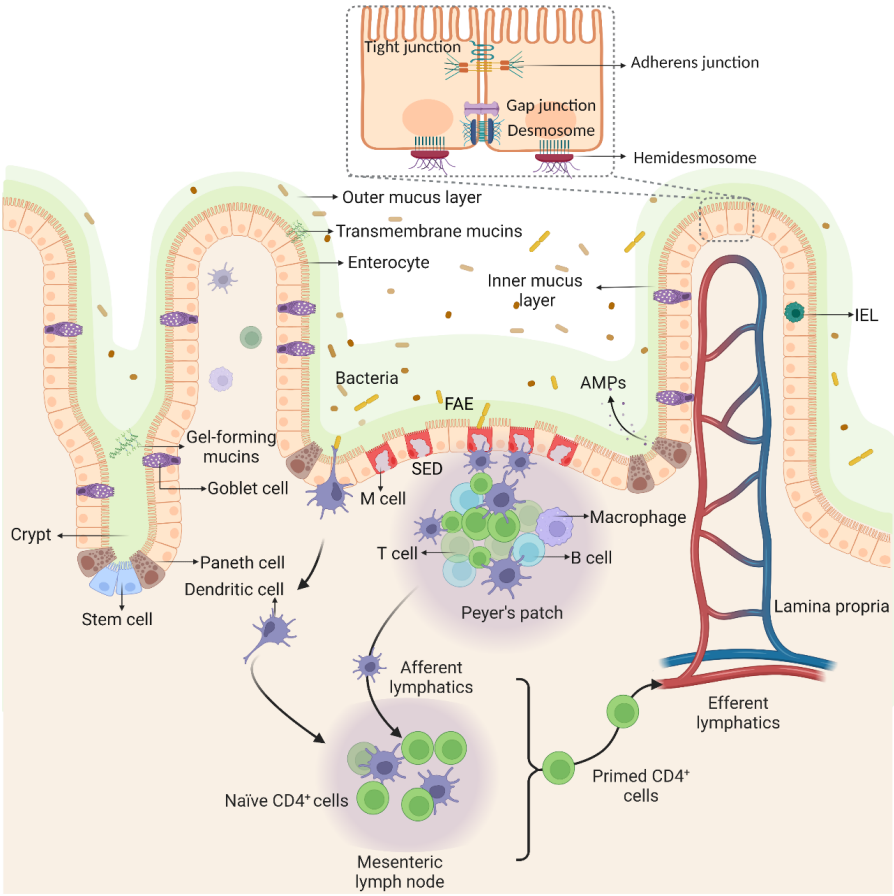


Figure 1. **Overview of the intestinal immune components of mammals.** The intestine has the largest mucosal surface that has a single layer of intestinal epithelial cells, which originate from stem cells that reside at the base of crypts. The epithelial cells with its cell junctions (such as tight junction, adherens junction and gap junction) maintain the barrier integrity. Goblet cells help in the barrier functions by secreting mucus that prevents microbial invasion and entry of unwanted antigens. Mucin proteins present in the mucus are broadly divided into secreted gel forming or transmembrane mucins. The Paneth cells ensure the physical barrier by secreting AMPs. The intraepithelial lymphocytes (IELs) that are present between the adjacent epithelial cells are antigen-experienced phenotypes. Antigen presenting cells (dendritic cells, macrophages) sample luminal antigens and present the processed antigens to naïve T and B cells. Peyers's patches include follicle associated epithelium (FAE) and subepithelial dome (SED). *Created with BioRender.com*

Specific sites that have critical immune components play crucial roles in immune defence. GALT is significant among them and has components both in the epithelial layer and the connective tissue directly under the intestinal epithelium known as the lamina propria (LP). GALT constitutes key antigen sampling sites and its components include the Peyer's patches (PP) and mesenteric lymph nodes (MLN)(Mowat, 2003). PP contains large B-cell and T-cell (naïve) clusters, follicle-associated epithelium (FAE) and subepithelial dome (SED) (Ahluwalia et al., 2017). The FAE covering the PP contains specialized antigen-sampling cells called microfold (M) cells, a specialized IEC subtype that can transcytose luminal antigens to the dendritic cells (DCs) to elicit immune responses. The second component of GALT, the MLN, contains naïve lymphocytes and antigen presenting cells (APCs), and is the site where the antigens are recognized by naïve T and B cells in the context of proper antigen-presentation to initiate immune responses (Mowat, 2003). The lamina propria contains both the innate (macrophages, DCs, neutrophils, eosinophils) and adaptive immune cells (T and B cells).

To mount an immune response to a particular antigen, APCs such as macrophages, DCs and B cells use their pattern recognition receptors (PRRs) to identify pathogen-associated molecular patterns (PAMPs) (Wells et al., 2011). After the PRR-mediated recognition of antigens, APCs internalize their target for eventual phagocytosis or signal transduction (Moretti and Blander, 2014). The phagocytosed antigens are processed and presented as peptides to naïve T cells via the major histocompatibility complex (MHC) class I or II molecules. While $CD8^+$ T cells recognize intracellular peptides presented by MHC class I molecules, $CD4^+$ T cells interact with extracellular antigenic peptides presented by the MHC class II molecules (Wells et al., 2011). Activation and differentiation of naïve T lymphocytes requires two signals, the first one is T-cell receptor (TCR) binding to peptide/MHC complexes and the second signal is initiated upon engagement with different co-stimulatory molecules. For example, cluster of differentiation 28 (CD28) present on the T-cells bind with the ligand B-lymphocyte activation antigen (B7-1) on the APC cells and such interactions are necessary for T cell activation (Gaudino and Kumar, 2019). Upon activation, $CD4^+$T-cells

differentiate into $CD4^+$ effector T-cells and $CD4^+$ memory T-cells. The $CD4^+$ effector T-cells differentiate into T-helper 1 (Th1), Th2, Th17 cells and regulatory (Treg) cells. The differentiation of $CD4^+$ T cells into different subsets is affected by several factors, including the cytokine milieu, strength of the TCR signal, affinity of the TCR for the antigen and the nature of the different co-stimulatory molecules (activation or inhibition cascades that enhance or diminish TCR signalling) (Magee et al., 2012). These $CD4^+$ T-cell subsets are functionally distinct, and they produce specific cytokines. Th1 cells primarily secrete interferon-γ (IFN-γ) which is associated with cellular immunity against intracellular microbes. Th2 cells secrete cytokines such as interleukin-4 (IL-4), IL-5, IL-9, IL-10, IL-13 to promote humoral immunity (for B cell activation and differentiation) and to protect the host from extracellular parasites. Th17 cells secrete cytokines IL-17A, IL-17F, IL-21 and IL-22 that are essential for protection from extracellular bacteria and fungi (Gaudino and Kumar, 2019). The Treg is an important population of T cells that secrete cytokines such as IL-10, IL-35 and transforming growth factor beta (TGF-β) and support their polarization to anti-inflammatory phenotypes (Okeke and Uzonna, 2019). Activation of $CD8^+$ T lymphocytes requires an antigen-specific signal initiated through the binding of TCR to peptides derived from intracellular pathogens (e.g., virus, signal 1). In addition, optimal $CD8^+$ T lymphocyte differentiation may require costimulatory molecules such as CD134 and CD137, expressed on activated $CD8^+$ T cells to differentiate into surviving T effector cells (signal 2) (Karginov et al., 2022, Duttagupta et al., 2009). Most effector $CD8^+$ T cells die after the virus is cleared off from the system, but many of them can remain in circulation and tissues as resting memory cells (Duttagupta et al., 2009, Okeke and Uzonna, 2019).

In addition to T lymphocytes, B cells which provide humoral immunity can be activated by binding of the B cell receptor (BCR) to antigens and these cells differentiate into plasma cells that secrete immunoglobulins. Based on the type of the encountered antigen, the naïve B cell activation occurs in two ways- T cell independent and T cell dependent activation (Kato et al., 2020). If a particular antigen (e.g., viruses) displays many highly repetitive surface antigens (epitopes), it can engage with multiple BCRs on

the B cell, resulting in T-independent activation. Weak binding affinity antigens can drive T-dependent activation wherein naïve B cells depend on signals from helper T cells ($CD4^+$). Furthermore, activated B cell differentiates into short-lived plasma cells (plasmablasts) and long-lasting plasma cells and the relative differentiation into these distinct states depends on the integration of signals received by the BCR and T helper cells (Cyster and Allen, 2019). Both T (CD4+ and CD8+) and B memory cells resides in the LP and a re-exposure to specific antigens leads to a rapid response against a particular antigen. Thus, the brief overview presented here highlights that the different intestinal immune components are crucial to maintaining homeostasis.

1.2. Intestinal components that have a bearing on brain function

The intestine is also densely innervated by a complex web of neurons (enteric neurons) that govern important physiological functions (Jacobson et al., 2021). Different nerves are localized close to immune cells to form neuro-immune cell units. These units can independently initiate different responses and can also communicate with each other to form a neuro-immune axis. Enteric neuronal stem cells are present in the adult mouse and human intestine, however, these cells do not develop new neurons under steady-state conditions (Belkind-Gerson et al., 2015). Since intestine immune cells express neurotransmitters and neuropeptide receptors and neurons express cytokine receptors, cross talk along the neuro-immune axis is essential for inflammatory responses. It was reported that intestinal inflammation can induce the development of new enteric neurons via serotonin dependent signalling mechanism and enteric neuronal hyperplasia can exacerbate inflammation (Margolis et al., 2011). Furthermore, a specific macrophage population, which is an anti-inflammatory phenotype, resides in the muscularis regions of the intestine near the enteric ganglia (Gabanyi et al., 2016). These muscularis macrophages exhibit a tissue-protective phenotype characterized by the expression of genes such as *arginase 1* (*ARG1), IL10, CD163* and resemble M2 macrophages (Matheis et al., 2020). It was also reported that muscularis macrophages that are similar to M2 phenotype displayed a tissue-protective role via β2 adrenergic receptor (AR) signalling and upregulation of arginase

1 (Matheis et al., 2020). These macrophages can utilize arginine to produce ornithine and urea, limiting arginine availability for nitric oxide synthetase (required by pro-inflammatory M1 phenotype), thus promoting anti-inflammatory M2 phenotype of macrophages important for cellular proliferation and tissue repair (Rath et al., 2014). In addition to neuro-immune axis, cross talk between central and enteric nervous system, aided by microbiota regulates the immune response in the intestine (Maiuolo et al., 2021). In this regard, SCFAs, the metabolites produced by the microbiota through the anaerobic fermentation of indigestible polysaccharides play an important role (Parada Venegas et al., 2019). These SCFAs (acetate, propionate, and butyrate) enter the enterocytes by passive diffusion or with the aid of different transporters (mainly via H^+-dependent monocarboxylate transporters or sodium-dependent monocarboxylate transporters). Particularly butyrate can influence mucosal immunity by modulating the differentiation of the Treg cells and production of interleukins (e.g., IL-10). Furthermore, SCFAs can enhance intestinal barrier by stimulating mucus production, alteration of tight junction proteins and production of AMPs (Peng et al., 2009, Parada Venegas et al., 2019). They can also cross the blood-brain barrier via monocarboxylate transporters located on endothelial cells of the blood vessels (Silva et al., 2020) and enter the brain, where they affect anti-inflammatory signals of the microglia and regulate the production of neurotransmitters like dopamine and serotonin (Silva et al., 2020). Together, the interaction of SCFAs with these gut-brain pathways can affect behaviour. Thus, intestinal inflammation is regulated by microbial metabolites, and neuro-immune axis, among others. These understudied aspects can affect behavioural characteristics which should be explored in detail in connection with inflammation.

1.3. Intestinal immune homeostasis and inflammation

Evoking appropriate immune responses at the epithelial barrier is key in sustaining intestinal homeostasis. For example, IECs communicate with both microbiota and immune cells to establish a tolerogenic response (Peterson and Artis, 2014). IECs express a range of PRRs to sense microbes. However, PRR signalling towards the

commensal microbiota have been adapted to avoid inflammatory responses in the gut. Among the different PRRs of IECs, members of Toll-like receptors (TLRs) are localized on the cell membrane and/or endosomal membrane to recognize extracellular and endocytosed microbe associated molecular patterns (MAMPs). Polarized activation of TLRs at either the apical or basolateral membranes of IECs can help to distinguish between commensal and pathogen-signals. For example, TLR9 activation through the apical membrane of IECs by resident *Escherichia coli* DNA inhibited IL-8 production and supressed transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signalling (Lee et al., 2006). Furthermore, a key strategy for maintaining homeostasis is to reduce the contact between luminal antigens and the IECs (Peterson and Artis, 2014). For instance, by goblet cells that secrete mucin glycoproteins and by other epithelial cells such as enterocytes and Paneth cells that secrete AMPs help to eliminate bacteria that penetrate the mucus layer. There are two layers of mucus; while the outer mucus layer is home to microbes, the inner layer is impenetrable to them (Hansson, 2019).

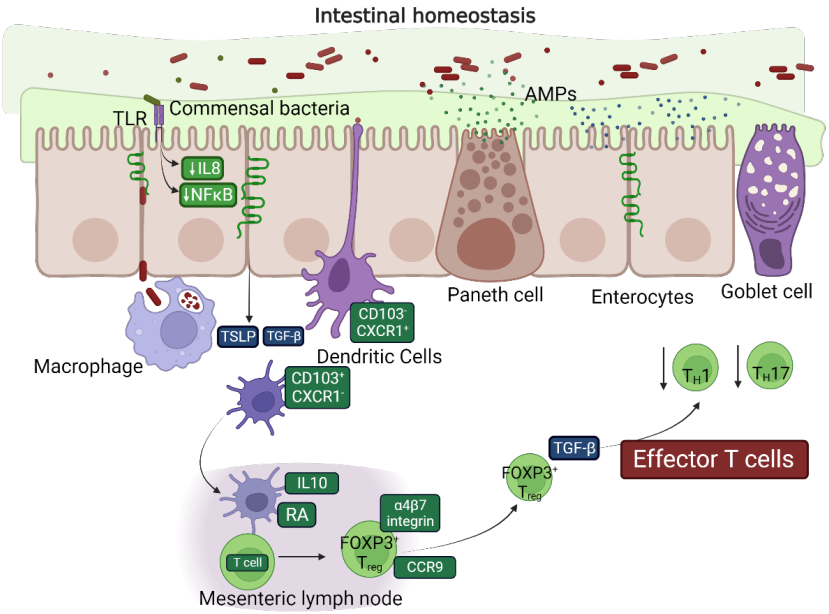
If the pathogenic bacteria succeed in crossing the barrier, APCs can prevent their entry into intestinal tissue. Macrophages rapidly phagocytose microbes and kill the ingested organisms that penetrate beyond the epithelial cells (Hooper and Macpherson, 2010). Furthermore, IECs secrete soluble factors such as thymic stromal lymphopoietin (TSLP) and TGFβ; these humoral components along with other factors promote the development of DCs and macrophages with tolerogenic phenotypes (Li and Guo, 2009, Rimoldi et al., 2005). Two major subsets of DCs ($CD103^+$ $CX3CR1^-$ and $CD103^-$ $CX3CR1^+$) are present in the LP to perform different immune functions (Ruane and Lavelle, 2011). The $CD103^-$ $CX3CR1^+$ DCs lack migratory properties in their steady state and are found near IECs. These DCs use their dendrites to clear the pathogens and opportunistic commensal bacteria that traverse the mucus barrier (Schulz et al., 2009). $CD103^+$ $CX3CR1^-$ DCs act as migratory antigen-presenting cells and upon activation transport antigens to MLN for presentation to adaptive immune cells. Conditioned by their previous interactions with IECs at the intestinal barrier (by TSLP, TGFβ and other

factors), these migratory $CD103^+$ DCs promote immune tolerance via the differentiation of forkhead box P3 ($FOXP3^+$) Treg cells (from naïve $CD4^+$ cells) and production of retinoic acid in the MLN (Ruane and Lavelle, 2011, Coombes et al., 2007). This interaction further induces the expression of α4β7 integrins and C-C chemokine receptor 9 (CCR9) on $FOXP3^+$Treg cells for allowing them to migrate to the lamina propria (Ruane and Lavelle, 2011). There are two main subtypes of Treg cells: $FOXP3^+$ Treg cells and $FOXP3^-$ IL-10^+ Treg cells. Treg cells secrete TGFβ or IL-10 that have negative regulatory effects on effector T cells (Th1 and Th17) (Sakaguchi et al., 2008). This balance between the functions of Treg cells and the $CD4^+$ effector T cells in the intestinal mucosa is crucial for intestinal homeostasis.

An imbalance in the immune functions can result in dysregulation of the intestinal homeostasis (**Figure 2**). Defects in the barrier components including junction proteins, mucins etc. can increase intestinal permeability leading to increased infiltration of luminal antigens and abnormalities in the paracellular and transcellular transport of ions (Allaire et al., 2018). Furthermore, binding of the MAMPs to PRRs expressed on different IECs and immune cells triggers downstream signalling cascades, leading to the activation of transcription factor NF-κB (Wells et al., 2011). The mechanism for NF-κB activation is the degradation of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα). The phosphorylation of IκBα by the multi-subunit IκB kinase (IKK) complex results in rapid and transient translocation of NF-κB dimers (particularly the p50/RelA and p50/c-Rel dimers) into the nucleus, where it induces the expression of proinflammatory cytokines such as IL-1β, and chemokines like IL-8. The proinflammatory cytokine IL-1β is secreted by immune cells. The cytokine, IL-8 is a neutrophil chemoattractant that induces the migration of neutrophils from peripheral blood into an inflamed site (Hammond et al., 1995) Neutrophils can kill pathogens by producing reactive oxygen species (ROS) and releasing lytic enzymes such as proteinases, and cationic peptides from their granules. On the other hand, chronic activation of neutrophils damages the epithelium and destroys the barrier integrity (Herrero-Cervera et al., 2022). Neutrophils can also produce matrix

metalloproteases (MMPs), which degrade extracellular matrix proteins that regulate tissue remodelling (Yabluchanskiy et al., 2013).

The complement system in the intestine is another key component of the innate immune system that supports the antibodies and phagocytes to clear the damaged cells and microbes (Sina et al., 2018). The complement system is activated via 3 main pathways: classical, lectin, and alternative pathways. The classical and lectin pathway is initiated by pathogens or damaged cells while alternative pathway is activated by hydrolysis of complement component C3. The classical pathway is activated when C1q of the C1 complex binds to antigen-antibody complex or non-immunoglobulin activators like components of bacteria and viruses. The lectin pathway is activated when PRRs such as mannose-binding lectins binds to specific carbohydrate structures (PAMPs) on damaged cell surfaces and pathogens. The alternative pathway is activated after the spontaneous hydrolysis of C3 to reactive molecules such as C3(H_2O) and C3b, both of which bind to positively charged surfaces of pathogens. Cleavage of C3 convertase that is formed via one of the abovementioned pathways can generate C3a, C3b, C5a, C5b. Of these, C3b leads to opsonization of pathogens and subsequent phagocytosis by neutrophils/macrophages. While C3a, C4a and C5a act as chemoattractants, i.e., attracting neutrophils to the site of inflammation, C5b, C6, C7, C8 and C9 will form membrane attack complex to form pores on pathogens. Appropriate activation of the intestinal complement system helps in the resolution of chronic intestinal inflammation, while over-activation or dysregulation may worsen it. Furthermore, the expression of complement regulators such as CD59 on the apical side of the intestinal epithelial cells regulate the complement activation and formation of membrane attack complex (MAC) to prevent destruction of intestinal epithelial cell (Sina et al., 2018). Inflammation models should be used to obtain a complete understanding of the changes in the intestinal immune components especially in relation to dietary factors that stimulate as well as counter inflammation.


Intestinal homeostasis
TLR
Commensal bacteria
AMPs
↓IL8
↓NFκB
Paneth cell
Enterocytes
Goblet cell
TSLP
TGF-β
CD103⁻ CXCR1⁺
Dendritic Cells
Macrophage
CD103⁺ CXCR1⁻
TH1
TH17
TGF-β
Effector T cells
FOXP3⁺ Treg
IL10
RA
α4β7 integrin
FOXP3⁺ Treg
T cell
CCR9
Mesenteric lymph node

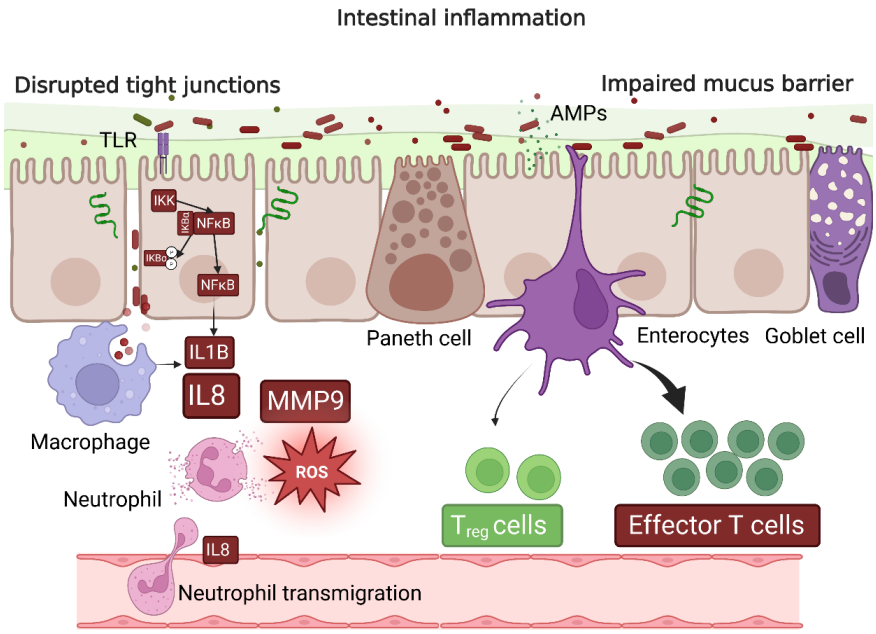

Intestinal inflammation
Disrupted tight junctions
Impaired mucus barrier
TLR
AMPs
IKK
IκBα
NFκB
IKBα
NFκB
Paneth cell
Enterocytes
Goblet cell
IL1B
IL8
MMP9
Macrophage
ROS
Neutrophil
Treg cells
Effector T cells
IL8
Neutrophil transmigration

Figure 2. Schematic representation of a healthy and inflamed condition in the intestine of mammals. The intestinal mucosa is constituted by, among others, mucus and antimicrobial peptides (AMPs) that helps to eliminate bacteria that breach the mucus layer. Macrophages phagocytose and kill the microorganisms that infiltrate beyond the epithelial cells. Mucosal $CD103^+$ dendritic cells promote immune tolerance through the differentiation of forkhead box P3 ($FOXP3^+$) regulatory T cell (Treg) which depends on the TGFβ and retinoic acid (RA). The RA programs $CD103^+$DCs to migrate to the mesenteric lymph nodes and induce the expression of CC-chemokine receptor 9 (CCR9) and α4β7 integrins on T cells. Treg can secrete TGF-β which have negative regulatory effects on effector T cells. Toll-like receptors (TLRs) recognizes antigens, leading to phosphorylation of IkBα and the translocation of NF-κB dimers into the nucleus. The release of proinflammatory cytokines shifts the T cell balance towards effector T cells (Th1, Th17). *Created*

1.4. Zebrafish as a model of intestinal inflammation

Zebrafish (*Danio rerio*) is an important model organism that is widely used for studying intestinal inflammation as well as its behavioural responses to various factors (Brugman, 2016). The transparent larval stage, small size, high fecundity, inexpensive maintenance, shorter time between generations, ease of genetic manipulation, and fully annotated genome are among the attractive characteristics that make the fish an ideal experimental model. Furthermore, approximately 70% of human genes have a corresponding orthologue in the fish (Howe et al., 2013), and zebrafish and humans have conserved transcriptional factors that control intestinal development and physiology (Lickwar et al., 2017). Moreover, the late development of adaptive immunity (3-4 weeks) enables us to examine the relationship between innate immune components and intestinal inflammation (Brugman, 2016). Zebrafish has three morphologically distinct gut segments: the anterior, mid, and the posterior parts (Wallace et al., 2005, Wallace and Pack, 2003). The typical acidification during the digestive process does not occur in this fish as it lacks a stomach. Instead, the intestinal bulb, a dilated portion of the intestine functions as a food storage and mixing site (Lickwar et al., 2017, Wallace et al., 2005). In the anterior and mid intestine, the folds/villi are longer and their size decreases antero-posteriorly. Digestive enzymes and solute transporters are also present in these two segments, suggesting their role in nutrient absorption. However, the posterior region is the controller of ion and water

absorption and this segment plays a role in mucosal immunity (Wallace et al., 2005). Zebrafish has many genes coding for gel-forming mucins; four *muc2*, six *muc5*, and one *muc19*. In addition, this teleost fish has genes that code for the transmembrane mucins; *muc13* and *muc17* have been identified in zebrafish (Lang et al., 2006, Lang et al., 2016). Zebrafish intestine expresses multiple defensin-like genes that resemble beta-defensin family members of mammals (Brugman, 2016); the expression of defensin beta-like gene was higher in the mid-intestine when examined along the antero-posterior regions (Oehlers et al., 2011a). The order of formation of different junctions in the developing zebrafish intestine (like adherens junction prior to tight junction and desmosome) is similar to that in mammalian (Wallace et al., 2005). However, zebrafish gut does not have intestinal crypts which are the source of stem cells in mammals; instead, in the fish, cells at the base of the folds divide and migrate to the tip where they become apoptotic (Wang et al., 2010). Furthermore, several cell types of the intestine (enterocytes, goblet cells) and a functional brush border are similar to mammalian intestine, but Paneth cells and M cells have not been clearly identified in zebrafish (Wallace et al., 2005, Wang et al., 2010). Zebrafish neutrophils can phagocytose, degranulate and produce ROS. In addition, eosinophils can also degranulate in response to antigenic exposure, another immune reaction that is similar to that in mammals (Flores et al., 2020). IELs and APCs like macrophages, DCs, and B cells have also been described in zebrafish. Furthermore, the immune cells in zebrafish express several homologues of mammalian PRRs (López Nadal et al., 2020). The TLR adaptor molecule MyD88 and downstream intracellular signalling are conserved in zebrafish (Hu et al., 2021). Several cytokines and chemokines identified in zebrafish are homologous to mammalian cytokines. However in zebrafish, several genes are duplicated which might affect the functionality of these cytokine subtypes (Oltova et al., 2018). In addition, several genes that code for the different components of the complement system have been identified in zebrafish and they exhibit functional similarities to their mammalian counterparts (Houseright et al., 2020)

In addition, three classes of immunoglobulins, IgM, IgD, and IgZ have been reported in zebrafish, with IgZ being the functional equivalent of mammalian mucosal IgA (Lewis et al., 2014). Zebrafish intestine also has DCs that express conserved co-stimulatory molecules (CD80/CD86) with phagocytic ability and capacity to appropriately stimulate and assist in differentiating T-cells (Shao et al., 2022). Moreover, $CD4^+$ cells isolated from zebrafish expressed T cell subsets and a population of regulatory T cells (with transcriptional factor $FOXP3^+$) resides in the zebrafish gut mucosa, as found in the intestine of mammals (Dee et al., 2016). However, T cell subset markers and their signature cytokines have not been fully characterized in zebrafish. Because the gut draining lymph nodes (like MLNs, Peyer's patches) have not been identified in zebrafish it has been suggested that APCs and adaptive immune cells interact in the spleen, the secondary lymphoid organ (Brugman, 2016, Lewis et al., 2014). Other initiators of the adaptive immune system, like MHC class I and II molecules, are also found to act in the intestine of the fish (Lewis et al., 2014).

The intestinal architecture of the fish reveals the mucosa, muscularis externa, and serosa layers (Wang et al., 2010), but not muscularis mucosa and submucosa that are found in mammals (**Figure 3**). In zebrafish, blood capillaries and muscle fibres are located below the lamina propria. Surrounding the mucosa of the fish intestine is the muscularis externa composed of circular and longitudinal smooth muscle fibres. Unlike the mammalian enteric nervous system (ENS) which is composed of myenteric and submucosal plexus with their own interconnected ganglia, zebrafish ENS develops into a single myenteric plexus with non-ganglionated network of neurons (Kuil et al., 2021).

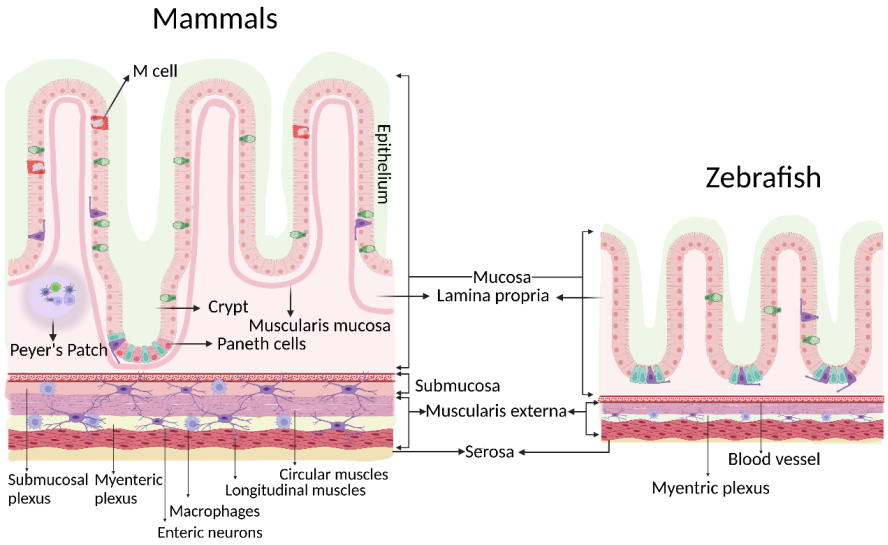


Figure 3. **Comparison of intestinal cell types and structures in mammals and zebrafish.** Zebrafish intestine has irregular folds as opposed to normal villi in mammals. There are no crypts, Paneth cell, M cells and organised lymphoid tissues like Peyer's patches. The different intestinal layers include mucosa, muscularis externa with circular and longitudinal muscle layers and serosa layer. However, zebrafish intestine lacks muscularis mucosa and submucosa. Mammalian and zebrafish intestines have stem cells, enterocytes, enteroendocrine cells, and goblet cells. *Created with BioRender.com*

Notwithstanding the morphological differences between human and zebrafish intestine, inflammation models have mimicked several aspects of intestinal disorder in humans (**Figure 4**). For example, a chemically-induced inflammation model has been developed in adult zebrafish by intra-rectal administration of oxazolone (Brugman et al., 2009). This model is characterized by a distorted intestinal fold architecture, depletion of goblet cells, increased infiltration of eosinophils and neutrophils and upregulation of pro-inflammatory cytokines (*il1b*, *tnfa*). Another model developed by immersing larval zebrafish in 2,4,6-trinitrobenzenesulfonic acid (TNBS) has the following features: increased expression of *mmp9*, altered lipid metabolism (Oehlers et al., 2011b) and modified microbiota composition (He et al., 2013). The dextran sodium sulphate (DSS)-induced inflammation model in zebrafish larvae had reduced proliferation of goblet cells and increased acidic mucins in the intestinal bulb with no

change in the goblet cell number (Oehlers et al., 2012). In the presence of microbiota, TNBS exposure induced inflammation through TLR signalling, but in germ free zebrafish such a signalling and inflammation were absent (He et al., 2014). The non-steroidal anti-inflammatory drug, glafenine, is used to develop intestinal inflammation model in zebrafish larvae, and the salient inflammation-linked feature included increased apoptosis and shedding of intestinal epithelial cells and increased endoplasmic reticulum (ER) stress (Goldsmith et al., 2013, Espenschied et al., 2019). There are also other zebrafish models; a neurotoxic pesticide (rotenone) exposure induced intestinal inflammation, affected brain function and antioxidant status and also caused inflammation in the brain (Cansız et al., 2021). In addition to chemically triggered inflammation models, diet-based models have been developed to study the gut and brain axis in zebrafish. For example, a high fat diet caused an undesirable shift in the microbiota, generated intestinal inflammation (Progatzky et al., 2014, Arias-Jayo et al., 2018) and impaired cognitive functions of zebrafish (Meguro et al., 2019, Uyttebroek et al., 2022). Intestinal inflammation models that rely on dietary triggers are better suited to explain the development and progression of such diseases in humans because specific diets can stimulate the development and progression of such diseases.

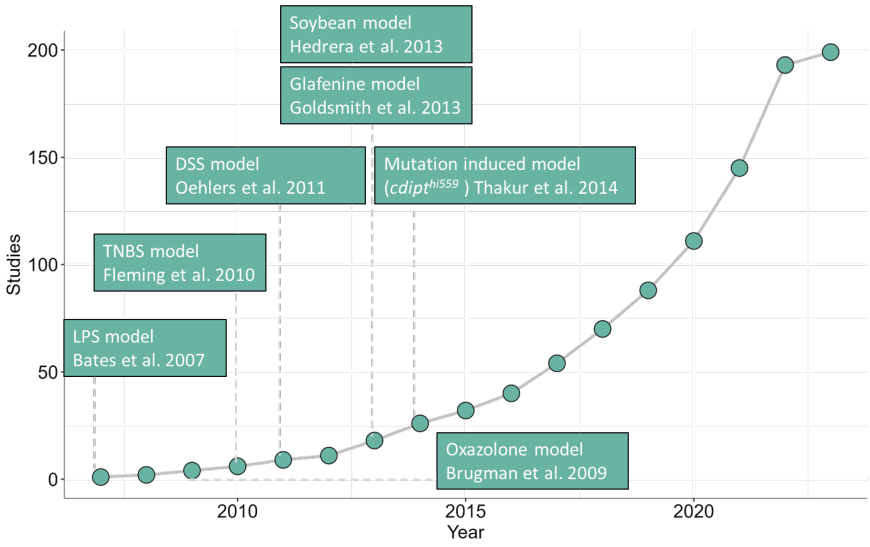


Figure 4. Surge in articles on zebrafish models of intestinal inflammation. This increase suggests that the model is a relevant tool for biomedical science. The Y-axis represents the number of studies and X-axis indicates the year. Shown in boxes are prominent articles that have presented the different methods for developing zebrafish model of intestinal inflammation. Data collected from PubMed using keywords "zebrafish intestinal inflammation" and the articles are from the year 2000 onwards".

1.5. Soybean meal as an inducer of intestinal inflammation in zebrafish

Soybean meal (SBM) is a feed ingredient that is known to cause inflammation in the intestine of zebrafish (Hedrera et al., 2013). This condition is characterized by increased migration of neutrophils, macrophages, higher proportion of T-lymphocytes with Th17 profile (Coronado et al., 2019) and elevation of pro-inflammatory cytokines like *il1b, il8, mmp9, tnfa, il17a/f3, nfkb, cox2*. Furthermore, disruption of the epithelial barrier (altered barrier related genes like *muc2.2*, *β-defensin*, *claudin, occludin*) and changes in the composition of the intestinal microbiota have been reported (Wu et al., 2020) (Solis et al., 2020). The different life stages (larvae, juveniles, and adults) of zebrafish have been used to develop inflammation models (Xie et al., 2021a, Li et al., 2022c).

Additionally, the extraintestinal effects like delayed bone formation associated with soybean-induced inflammatory response have been reported previously (Carnovali et al., 2021, Carnovali et al., 2022). The inflammation inducing ability of this dietary component is mainly due to anti-nutritional factors including soy saponin that can bind to cholesterol in the intestinal epithelial membrane and subsequently form pores to increase the membrane permeability (Böttger and Melzig, 2013). Soya saponins are amphiphilic molecules, with triterpenoid aglycone (or sapogenin) moieties attached to carbohydrate residues via glycosidic bonds (Xu et al., 2021). A strong interaction of saponin with cholesterol depends on both the hydrophobic (aglycone) and the hydrophilic components (sugar moieties). Saponins can also penetrate into the lipid layers and disturb the interaction of cholesterol with other membrane lipids (phospho- and sphingolipids), thereby disturbing the overall barrier integrity (Wojciechowski et al., 2016). It is reported that such interaction with membrane components increases the cell permeability (Francis et al., 2002) and adversely affect nutrient transport (Johnson et al., 1986). In addition, soya saponin can reduce the digestion of proteins and lipids (Chikwati et al., 2012), reduce growth and feed intake (Chen et al., 2011), leading to weight loss and gastroenteritis in animals (Gu et al., 2018). Furthermore, dietary saponin increased relative abundance of *Vibrio* and *Shewanella* in zebrafish gut (López Nadal et al., 2023). Considering the negative impacts of SBM on the intestine, this ingredient can be selected to develop a diet-induced intestinal inflammation model of zebrafish (Coronado et al., 2019). On the other hand, several additives have been identified to counteract the intestinal disorder caused by SBM; the list includes lactoferrin (Ulloa et al., 2016), microalgae (Bravo-Tello et al., 2017), aloe vera (Fehrmann-Cartes et al., 2019), phytase-producing strain of *Bacillus subtilis* (Santos et al., 2019), cholinesterase inhibitor galantamine (Wu et al., 2020), dipeptide supplementation (Molinari et al., 2021), sinomenine hydrochloride (Xie et al., 2021a) and functional feed additives.

1.6. Efficacy of functional feed additives in countering intestinal inflammation

Polysaccharides are important structural and storage components of plants and other organisms (Liu et al., 2015). These complex compounds are held together by glycosidic bonds and are classified based on the number of sugar units. For example, a monosaccharide contains one sugar unit compared to two, 3-9 and ten or more in the case of disaccharides, oligosaccharides, and polysaccharides, respectively (Navarro et al., 2019). Many polysaccharides are immune modulatory in nature and can bind to PRRs present on various immune (macrophages, dendritic cells, neutrophils, mast cells, lymphocytes) or non-immune cells (fibroblasts). Receptors like TLR4, C-type lectins (dectin-1 and mannose), scavenger receptors (SR), complement receptor 3 (CR3), CD14, and CD44 can recognize polysaccharides. They can stimulate macrophages when they interact with TLR4, CD14 and dectin-1 simultaneously (Sindhu et al., 2021). Their immunomodulatory effects can also be through their interaction with the gut microbiota. For instance, gut microbiota ferment non-digestible polysaccharides (e.g. those with a β-glycosidic bonds that resist digestion in the intestine due to lack of β-1,3 glucanase in the digestive system) to produce SCFAs that activate $FOXP3^+$ T cells (Kim, 2021). In addition, these bioactive compounds promote the growth of specific bacterial groups (Hayes and Tiwari, 2015) that evoke appropriate receptor-mediated signals to release cytokines that stimulate certain innate lymphoid cells or regulate B cell responses to produce appropriate immunoglobulins (Kim, 2021). These immunomodulatory effects can have several beneficial effects; they reduce intestinal inflammation and support gut barrier functions (Davani-Davari et al., 2019). These properties of bioactive oligo- and polysaccharides make them ideal anti-inflammatory feed additives. β-glucans and alginate oligosaccharides are such additives which have, among others, prebiotic, immunomodulatory and antioxidant properties.

β-glucan is a polysaccharide that serves as storage and structural components of plants, fungi, algae and bacteria (Han et al., 2020). They contain glucose polymers that are generally linked by β-(1,3) glycosidic bond, with variable levels of branching, depending

on the origin. This structure of β-glucans can be recognized by different PRRs like dectin-1, TLR, CR3, scavenge receptors, and lactosylceramide present on the surface of macrophages, dendritic cells, neutrophils, and lymphocytes. In mammals, β-glucans are predominantly recognized by lectin receptor dectin-1 that is a type II transmembrane receptor containing a single extracellular C-type lectin-like carbohydrate recognition domain and an immunoreceptor tyrosine-based activation motif in the cytoplasmic tail (Goodridge et al., 2009). Dectin-1 particularly recognizes β-(1-3)(1-6) and β-(1-3) glucans from fungi, plants, and bacteria, but it is not reactive toward β-(1-4) glucans. As regards β-glucan receptors in fishes, common carp C-type lectin domain family 4 member C (*clec4c*)*, salmon C-type lectins* (*sclra, sclrb, sclrc*) *and cr3* in several fishes have been identified (Petit et al., 2019, Kiron et al., 2016). Another β-glucan receptor CR3 is an integrin dimer consisting of CD11b (α_M) and CD18 (β2) and is highly expressed on neutrophils, monocytes and to a lesser extent on macrophages (Goodridge et al., 2009). CR3 also acts as an opsonic receptor for the complement component iC3b (inactive form of C3b)(Bose et al., 2013). The opsonization of pathogens by complement can lead to direct killing of the pathogen by formation of MAC or along with C3 fragment the opsonized pathogen will be recognized by CR3 present on immune cells (Bose et al., 2013). The CD11b component of CR3 has two binding sites, one for β-glucan located within the C terminus (lectin domain), while the other for iC3b located within the N-terminus (40). The dual binding of CD11b by complement iC3b and β-glucan activates CR3 and CD18 transmits the signal of CD11b to the downstream signalling cascade. Furthermore, it has been reported that the lectin site of CR3 can bind to other carbohydrates (Goodridge et al., 2009).

Several studies have indicated that β-glucans can induce pro-inflammatory phenotypes of immune cells characterized by increased expression of pro-inflammatory cytokines (Pedro et al., 2021). However, the intended bioactivity of β-glucans i.e., to inhibit pro-inflammatory factors could be revealed when immune cells are stimulated with an inflammatory agent. It was reported that pre-treatment with β-glucan can inhibit LPS-stimulated IL-1β production in macrophages (Camilli et al., 2020). Furthermore, dectin-

1-mediated signalling is key for the anti-inflammatory effect of β-glucans (Karumuthil-Melethil et al., 2014, Camilli et al., 2020). In an *in vitro* gut-inflammation model (in which Caco-2 cells were grown along with RAW264.7 cells in a co-culture system and then stimulated with LPS to mimic a gut-inflammation condition), the mRNA level of IL-8 was significantly lower in cells co-stimulated with LPS and β-glucans, compared to cells stimulated with only LPS (Mizuno et al., 2009). Similar inflammation subduing effects of β-glucans were also reported in studies which employed *in vivo* models of inflammation. For instance, dietary yeast β-glucan reduced the infiltration of macrophages and neutrophils and decreased the production of proinflammatory cytokines-TNF-α, IL-6, and IL-8, nitric oxide synthase (NOS), cyclooxygenase-2 (COX-2) and prostaglandin E synthase2 (PTGES2) in the intestine of a DSS-induced inflammation model of mice (Han et al., 2017). Overall, these studies indicate that β-glucans are effective in mitigating the inflammatory response induced by various pro-inflammatory agents. The immunomodulatory potential of β-glucans is influenced by molecular characteristics such as backbone, side chain branching and molecular weight. The branching pattern determines the bioactivity of β-glucans (Han et al., 2020). A study reported that the side-chains of the glucan can increase the binding affinity to dectin-1 (Adams et al., 2008). Furthermore, the same study reported that non-β-linked glucans and glucans with a mixed β-(1,3) and β-(1,4) linkages were not recognized by dectin-1. The molecular weight (MW) can also affect the immunomodulatory potential of β-glucans (Han et al., 2020); higher MW types have more immunomodulatory activity possibly because larger polymers can crosslink spatially separated receptors and alter immune cell function (Sletmoen and Stokke, 2008). However, there are conflicting reports on immunomodulating capacities of low and high MW β-glucans. For example, a low MW yeast β-glucan was better as an antioxidant and immunostimulant compared to the high MW type (Lei et al., 2015). This indicates that structural variations can affect immunomodulation by β-glucans.

Among the different β-glucans, MacroGard® derived from the cell wall of baker's yeast *Saccharomyces cerevisiae* (about 55–65% of β-glucan) and paramylon (about 80% of

dry weight) derived from the unicellular alga *Euglena gracilis* are well-studied in terms of their immunomodulatory properties (Machuca et al., 2022). MacroGard® is a highly branched β-(1,3),(1,6)-glucan compared to the straight-chain β-1,3-glucan in paramylon. While MacroGard® β-glucan is the cell wall component of baker's yeast, paramylon is a storage polysaccharide in the cytoplasm of the microalga (Russo et al., 2017). Furthermore, paramylon is a high MW β-glucan (about 500 kDa) compared to yeast β-glucan (about 175kDa) (Machuca et al., 2022, Russo et al., 2017). Therefore, these two glucans of different structure and origin can have differential effects on the immune cells of animals.

Upon recognition of the β-glucans by PRRs, dectin-1 dependent signalling can increase $FOXP3^+$ T cells; as inferred from *in vitro* studies (Karumuthil-Melethil et al., 2014). The intestinal $CD103^+$ DCs isolated from yeast β-glucan fed mice was found to stimulate the expansion of IL-10 producing T cells (Gudi et al., 2019, Karumuthil-Melethil et al., 2014). Furthermore, the glucan product can significantly increase the abundance of bacteria belonging to the phylum Verrucomicrobia that included many carbohydrate fermenting types, with a subsequent increase in the faecal short-chain fatty acids - acetic acid, propionic acid and butyric acid (Gudi et al., 2020). Conversely paramylon can induce the proliferation of DCs and macrophages of anti-inflammatory phenotypes (Yasuda et al., 2020, Xie et al., 2021b). Akin to yeast β-glucan, paramylon increased the percentage of $FOXP3^+$ Treg and $IL\text{-}10^+$ T cells but reduced the percentage of IFNγ secreting Th1 cells in the gut of mice (Taylor et al., 2020). In addition, it has been reported that paramylon modulates the immune response by reducing the production of pro-inflammatory cytokines (IL-17, IL-6, and IFN-γ) in a mice model of arthritis (Suzuki et al., 2018). The microalga β-glucan can also promote the polarization of macrophages to their M2 phenotype in the liver of a mice model of LPS-induced inflammation (Xie et al., 2021b), thereby aiding in resolving the inflammation.

Other types of polysaccharides can also protect the intestine from inflammation. Among them is alginate that is mainly found in the matrix and cell walls of multi-cellular brown algae and bacteria (Lu et al., 2022) . Species belonging to the genera *Laminaria,*

Lessonia, *Macrocystis*, and *Sargassum*, are the major commercial sources of alginates, accounting for about 40% of the dry cell weight. Alginate oligosaccharides (AOS) produced by chemical or enzymatic digestion of alginates exhibit greater bioactive potential due to their lower molecular weight, better solubility, and bioavailability. 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 with different (M/G) ratios and degree of polymerization (Mrudulakumari Vasudevan et al., 2021). The TLR4 present on macrophages can recognize AOS and affect the downstream signalling pathways such as mitogen-activated protein kinase (MAPK) and NF-κB (Fang et al., 2017, Zhao et al., 2020). An *in vitro* study using murine intestinal cells reported that AOS is recognized by mannose receptor and this property was lost upon inhibition of the mannose receptor by its specific siRNA (Zhao et al., 2020). The ability of AOS to counter inflammation was attributed to the attenuation of nitric oxide and prostaglandin E2 (PGE2) production and inactivation of the NF-κB and MAPK pathways in a report on mice macrophage cell line (Bi et al., 2018). Another report on busulfan-induced damage of murine small intestine cells revealed that AOS assist in increasing the density of microvilli (improved absorption) and number of desmosomes on cell junctions (augmented barrier integrity), decreasing the protein expression of Caspase-8 (stalled apoptosis), and restoring the expression of marker genes associated with Paneth cells and goblet cells (Zhao et al., 2020). Dietary AOS also helped in increasing the villi length, goblet cell number and MUC2 expression (Wan et al., 2018) and stimulating the abundance of bacteria associated with SCFA production (Gupta et al., 2019). Furthermore, the antioxidant effects of AOS have been reported based on the enhanced activity of antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) in human umbilical vein endothelial cells (Jiang et al., 2021). The bioactivity of AOS is dependent on its MW (Lu et al., 2022); AOS <1kDa was effective in eliciting lysozyme activity, peroxidase activity, phagocytic capacity and total nitric oxide synthase activity compared to those having MW 1-2 or 2-4 kDa (Wang et al., 2014). Additionally, AOS of <1 kDa was efficient in scavenging superoxide, hydroxyl,

and hypochlorous acid radicals compared to 1 to 10 kDa product (Zhao et al., 2012). *In vivo* studies to understand the effect of MW of AOS on its anti-inflammatory potential have not been explored in detail.

The studies mentioned above showed that AOS and β-glucan are recognized by their specific receptors and the pathways evoked by them could be different. Hence, there is a need for further efforts to understand their effects using high-throughput techniques.

1.7. Approaches for studying functional additives that counter inflammation

1.7.1. Transcriptomics

Transcriptomic studies are important to understand alterations in the complete set of transcripts in a specific cell, tissue, or organism (Martin et al., 2016). RNA-Seq-based transcriptome profiling is rapidly being adopted in biological research and in this thesis, it has been employed to understand the responses associated with intestinal inflammation in zebrafish. Through a similar approach with Atlantic salmon fed soya saponin-containing diets, the alteration of several solute transporters and immune genes of the epithelial barrier and enrichment of steroid biosynthesis were reported by Kiron et al. (2020). The upregulation of the cholesterol biosynthesis pathway contributed to maintaining intestinal homeostasis in yellow perch (*Perca flavescens*) juveniles fed a soybean-based diet (Kemski et al., 2020). Likewise, intestinal transcriptome profiling indicated the upregulation of immune and inflammation-related pathways such as phagosome, natural killer cell-mediated cytotoxicity and NF-κB signalling pathways and the downregulation of genes related to biosynthesis of unsaturated fatty acids and cholesterol metabolism in hybrid grouper (*Epinephelus fuscoguttatus* ♀ × *Epinephelus lanceolatus* ♂) fed a SBM diet (He et al., 2020). Studies on zebrafish intestinal transcriptome have also reported the alteration of genes related to immune, lipid and cholesterol metabolism, all linked to soybean feeding (Valenzuela et al., 2021, Xie et al., 2021a). However, only few studies on zebrafish have used this

approach to understand the efficacy of bioactives-based mitigation strategies against soybean induced intestinal inflammation (Xie et al., 2021a, Li et al., 2022c, López Nadal et al., 2023). Besides, the overall effect of soybean-induced inflammation on the development of zebrafish has not been investigated in detail.

1.7.2. Metabolomics

Metabolomics is a technique for selective and non-selective chemical analysis of metabolites in biological specimens such as cells, tissues or biofluids. The method can provide insights about, among others, the nutritional impact on hosts (Roques et al., 2020). Moreover, the metabolomic profile of plasma can reflect systemic perturbations caused by intestinal inflammation (Roques et al., 2020). Only a few studies have been conducted using fish plasma to understand diet-induced alterations and they have revealed the changes in the metabolome of the liver and intestine of fishes fed soybean-incorporated diet. Amino acid metabolism was altered in the liver of red drum (*Sciaenops ocellatus*) fed a soybean diet; inferences were based on upregulated metabolites linked to glycine, serine, and threonine pathway and glycerophospholipid metabolism. However, due to variability in plasma metabolites, the authors were not able to detect any significant differences (Casu et al., 2017). Dietary SBM altered the serum metabolites in cobia (*Rachycentron canadum*), especially those related to energy metabolism (Schock et al., 2012). Alteration in the energy metabolism has also been reported in humans with inflamed intestine and perturbed plasma metabolites of amino-acid and fatty-acid metabolism (Aldars-García et al., 2021, Scoville et al., 2018). However, so far, no study has investigated the plasma metabolites of a diet-induced intestinal inflammation model in zebrafish.

1.7.3. Behavioural analysis

Zebrafish is an emerging model for behavioral studies and is widely used to understand the effects of drugs/chemicals on behavior (Basnet et al., 2019). At around 4 days postfertilization (dpf), following the development of swim bladder, zebrafish larvae start to swim freely, and their swimming behavior is modulated by both internal and

external stimuli (Kopp et al., 2018). With the use of high throughput tracking system, we can monitor the activity of an individual larva and split a particular movement into several measurable parameters like total distance travelled, total duration of movement, heading angle, turn angle, average velocity, angular velocity; all these parameters can be compared to understand the effect of different treatments on zebrafish behavior (Maeda et al., 2021). The light-dark locomotion test is adopted widely to study the behavioral response of zebrafish larvae to sudden changes in illumination. The alternating light and dark conditions prompt zebrafish to follow a specific pattern of movement: while a transition from a light to a dark regimen increases locomotion, a dark to light transition decreases its movement (Rokszin et al., 2010). This locomotor behavior depends on the coordination of eye and brain functions. Other tests like exploratory biting, T-maze test and diving response test are also used to understand zebrafish behavior (Benvenutti et al., 2021, Fontana and Parker, 2022). Diet-induced changes can also affect the locomotor behavior in animals. For example, a high-fat, low-fiber diet reduced the locomotor activity of mice and a probiotic diet (with *Lactobacillus rhamnosus* IMC 501) increased the movement of zebrafish (Shi et al., 2020) (Borrelli et al., 2016). Furthermore, intestinal inflammation can also affect animal behavior as reported for a chemically-induced colitis mice model with stress-associated behavior (Komoto et al., 2022). However, to my knowledge there are no studies that have investigated behavioral changes associated with soybean-induced intestinal inflammation in zebrafish.

2. OBJECTIVES

For an in-depth understanding of diet-induced inflammation, the appropriate strategy is to evaluate the changes in the intestine of the already established SBM-fed zebrafish model. The underlying immune-related responses can be revealed by studying the intestinal transcriptome of the fish. Furthermore, the systemic effects of SBM-associated inflammation can be investigated by assessing the behavioural and plasma metabolomic alterations in the model. Such information is essential to use the zebrafish model for investigating the efficacy of therapeutic agents against intestinal inflammation (**Figure 5**). *The hypothesis of this PhD project is that dietary oligo- and polysaccharides may alter the intestinal transcriptomic, metabolomic and behavioural response in a zebrafish model of SBM-induced inflammation*.

Accordingly, the specific objectives are:

1) To elucidate the impact of an SBM diet and yeast β-glucan on the intestinal transcriptome of juvenile zebrafish (**Paper I**).

2) To understand extra-intestinal effects associated with an SBM diet and the inflammation alleviation and developmental defects reducing efficacy of algal β-glucan in a larval zebrafish inflammation model (**Paper II**).

3) To evaluate the impact of alginate oligosaccharides on the intestinal transcriptome and plasma metabolome in an adult zebrafish inflammation model (**Paper III**)

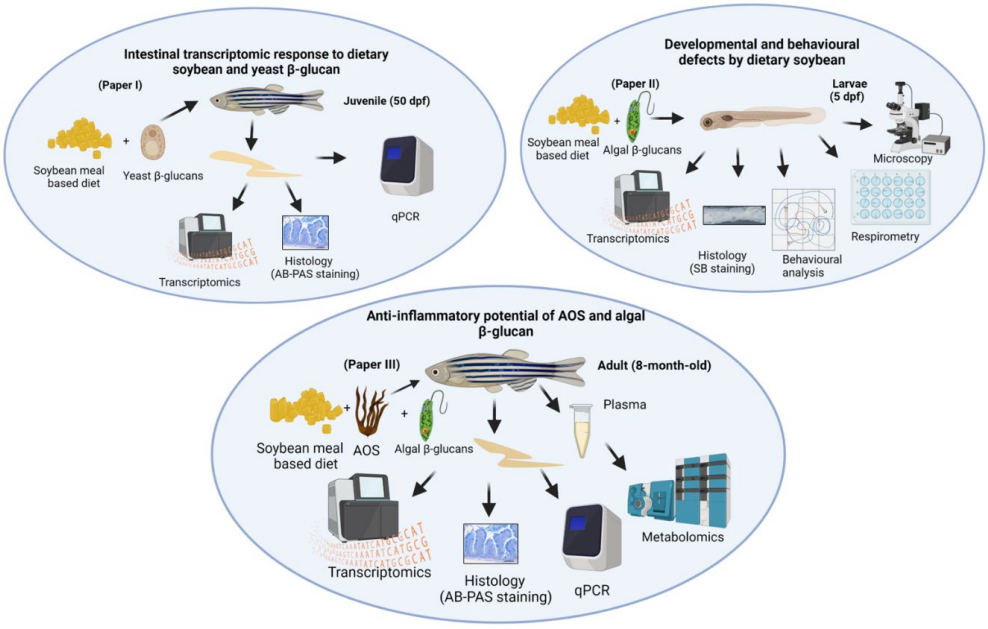


Figure 5. Overview of the different studies in the thesis. *Created with BioRender.com*

3. GENERAL DISCUSSION

The cellular and extracellular components of the intestinal mucosa can prevent the entry of pathogens and resolve unfavourable conditions such as inflammation. Diet is an important environmental factor that can regulate intestinal inflammation. Certain dietary components can trigger an intestinal inflammatory response whereas others can mitigate inflammation by maintaining immune homeostasis. However, there is limited knowledge on the mechanisms connected to diet component-linked inflammation and processes associated with the preventative properties of functional feed additives. Appropriate nutritional models could help not only to explore these mechanisms, but also to understand the extraintestinal effects linked to inflammation.

In this PhD project, transcriptomic and histological analyses were employed to elucidate SBM-induced alterations in the intestine of zebrafish (**Paper I**). Additionally, the intestinal transcriptome was evaluated to understand the response of the fish to a well-known anti-inflammatory compound, a yeast β-glucan. In the second study, behavioural analysis was performed along with transcriptome sequencing to understand how intestinal inflammation can affect the development and behaviour of zebrafish fed a SBM incorporated diet (**Paper II**). The study also looked at the efficacy of algal β-glucan to mitigate specific behavioural aberrations associated with inflammation in zebrafish, triggered by SBM. In the third study the intestinal transcriptome and plasma metabolome were analysed to evaluate the anti-inflammatory potential of alginate oligosaccharides and algal β-glucan in zebrafish fed a SBM diet (**Paper III**).

3.1. Soybean-induced inflammatory responses in the intestine of zebrafish

Effective enteritis models like the mice enteritis models have been developed in zebrafish by immersing the fish in DSS or through intrarectal injection of TNBS and oxazolone. Although these chemicals can generate inflammatory responses in the intestine of zebrafish, excessive physical damage of the intestinal mucosa can cause

mortality (Oehlers et al., 2013). Extensive epithelial abrasion caused by inflammation-inducing chemicals can damage the intestinal mucosa to evoke injury-associated responses. Hence, it will be difficult to separate inflammatory responses from the injury-associated ones. An acceptable approach would be to develop and test inflammation models that rely on dietary factors. For example, SBM can be used as an agent to develop an intestinal inflammation model in zebrafish. Previous studies have found that a 50% inclusion of SBM in the diet can induce enteritis in zebrafish (Solis et al., 2020). Hence, a diet containing 50% SBM was employed to elucidate SBM-induced alterations in the larval (**Paper II**), juvenile (**Paper I**), and adult zebrafish models (**Paper III)**. The attributes of the SBM-induced intestinal inflammation model are altered barrier-related genes, immune cell recruitment, elevated inflammatory marker genes and inflammation-linked histomorphological features. The intestinal barrier related genes namely *mucin5ac* (*muc5ac*), *mucin 5d* (*muc5d*), *chloride channel 2c* (*clcn2c*) and *clathrin, light chain B* (*cltb*) were altered in the SBM fed fish (**Paper I)**. An inflammatory condition can have an effect on mucins, which are glycoproteins present in mucus. For instance, deficiency of MUC2 makes the host prone to intestinal infections (Zarepour et al., 2013). In the first study (**Paper I**), the expression of *muc5d* (*muc2*-like) was significantly downregulated in zebrafish fed SBM. Therefore, the SBM diet-induced reduction in the *muc5d* gene expression is likely signalling a barrier breach and eventual inflammatory response in zebrafish intestine. The histological analysis in **Paper I** also indicated a weak barrier with fewer goblet cells in the soybean group, suggesting a dysregulated mucus production in zebrafish. Increased expression of matrix metalloprotease genes (*mmp9*, *mmp13a*) in the SBM-fed fish (**Paper III**) also indicates barrier disruption (Al-Sadi et al., 2021, Vandenbroucke et al., 2013). The protein, CLCN2C, that is localized near the tight junction proteins plays a significant role in maintaining epithelial barrier function (Nighot et al., 2017). Its overexpression increases occludin, which is essential for preventing tight junction permeability (Nighot et al., 2017). Hence, a loss of CLCN2C was found to increase the severity of DSS-induced colitis (Nighot et al., 2013). The expression of *clcn2c* was downregulated in zebrafish

fed a SBM diet that exhibited inflammation characteristics (**Paper I**). Furthermore, the observed upregulation of *clathrin, light chain B* (*cltb*), a major cytosolic protein that plays a key role in intracellular trafficking of membrane proteins, is known to promote clathrin-mediated endocytosis of adherens and tight junction proteins (Ivanov et al., 2004). Alteration in the endocytosis of junction proteins affect the intestinal permeability (Nighot and Ma, 2021, Nighot and Blikslager, 2012). Defects in the barrier function caused by intestinal structural changes can increase penetration of luminal antigens and the associated chemokine-induced recruitment of neutrophils (Xu et al., 2018). Several inflammation-linked GO terms like leukocyte chemotaxis and leukocyte migration were enriched by the upregulated immune genes (*mmp13a*, *coro1a*, *il22*, *ccl34a.4*, *CD5*9) in SBM-fed fish (**Paper III**). A SBM diet increased the number of granulocytes/neutrophils (Sudan black positive) in the intestine of zebrafish larvae (**Paper II**). An upregulation of genes associated with neutrophil recruitment–myeloperoxidase (*mpx*) and *chemokine (C-X-C motif) ligand 8a* (*cxcl8a*)–in the adult zebrafish fed SBM was reported in **Paper III.** The neutrophil marker *mpx* was found to be involved in the production of ROS in the mucosa of IBD patients (Chami et al., 2018) as well as in the inflamed intestine of zebrafish (Liu et al., 2014). Stimulated neutrophils activate their nicotinamide adenine dinucleotide phosphate oxidase to generate superoxide anion which eventually forms hydrogen peroxide that is used by myeloperoxidase to produce hypochlorous acid, the bactericidal ROS. An increase in ROS can negatively affect the protein-folding capacity of mitochondria resulting in an accumulation of misfolded proteins (Inigo and Chandra, 2022). Adenosine triphosphate (ATP)-dependent proteases participate in mitochondrial protein remodelling, folding and degradation to maintain organelle homeostasis (Feng et al., 2021, Baker et al., 2011). Enrichment of ATP-dependent peptidase activity was also noted, because of the upregulated mitochondrial matrix genes, namely, *LON peptidase N-terminal domain and ring finger 1* (*lonrf1*) and *caseinolytic mitochondrial matrix peptidase chaperone subunit Xb* (*clpxb*). These genes which are involved in the degradation of misfolded or damaged proteins (Li et al., 2023,

Feng et al., 2021), possibly indicates an effect of SBM on the mitochondrial protein-folding environment (**Paper I**). Furthermore, mitochondrial dysfunction has been associated with the development of inflammation (Novak and Mollen, 2015). The plasma metabolomic profile of zebrafish revealed the higher abundance of inflammation-linked metabolites such as itaconic acid, and lower abundance of taurochenodeoxycholic acid as well as the activation of the arginine biosynthesis pathway in adult fish fed an SBM containing diet (**Paper III**). Itaconic acid is considered a biomarker of inflammation as M1 macrophages of mammals are known to produce substantial amounts of itaconate following interferon-γ- or lipopolysaccharide-induced activation (Diotallevi et al., 2021). Secondary bile acids such as taurochenodeoxycholic acid are microbiota-associated metabolites and studies have reported a reduction in secondary bile acids in IBD patients reviewed by Kriaa et al. (2022). Additionally, alterations in arginine metabolic pathways have been reported during intestinal inflammation (Li et al., 2022b). In all the studies performed for this PhD thesis, SBM diets left a trail of inflammation hallmarks in the intestine and the plasma of zebrafish (**Paper I, II and III**), indicating the suitability of this model for examining diet-linked morpho-physiological alterations.

Although consistent inflammation-associated changes were observed at the molecular level (gene expression profile and plasma metabolome), a distinct barrier breach could not be validated through micromorphological observations (**Paper I and III**). For example, the lamina propria width in the juvenile and adult zebrafish were not affected by the SBM-based diet (**Paper I and III).** SBM (50% inclusion level) did not induce any changes in the intestinal morphology of zebrafish larvae (Solis et al., 2020, Hedrera et al., 2013). Our results point to the fact that 50% SBM is not enough to produce distinct inflammation-associated histological features in the intestine of zebrafish (**Paper I and III**).

The inflammatory response evoked by an SBM containing diet is likely to be dependent on the life stage of zebrafish. For example, juvenile zebrafish displayed a subdued inflammatory response (**Paper I**) compared to the adult stages (**Paper III**) in terms of

the number of differentially expressed immune genes in the intestine that are linked to inflammatory response. Susceptibility to intestinal inflammatory diseases in humans is known to progress with age (Sanada et al., 2018). Aging is associated with disruptions in gut homeostasis and is linked to altered intestinal stem cell proliferation, epithelial barrier function, perturbed gut microbiota composition and microbial metabolites and dysregulation of immune response (Walrath et al., 2021). Based on the results from **Papers I and III**, it can be broadly stated that the impact of soybean as an inflammatory agent may be age/organ-maturity dependent. It is critical to discriminate between the stages for studying intestinal inflammation, as the gene expression responses are different in juvenile and adult stages of zebrafish. Furthermore, the diversity and composition of zebrafish intestinal microbiota changes with the developmental stage (Stephens et al., 2016). Therefore, the distinct responses to SBM-induced inflammation are likely due to a specific microbiota profile. Future functional studies can delineate the role of the microbiota and the gene expression responses behind SBM-induced intestinal inflammation.

3.2. Metabolomic changes and extraintestinal effects associated with soybean meal intake

Soybean meal is a complex food ingredient with many constituents including anti-nutritional factors such as phytoestrogens and soyasaponin which can affect other physiological processes. In the SBM-fed fish, reproductive process-related GO terms such as reproductive process, male gamete generation, spermatogenesis were enriched based on the downregulation of several genes like *testis specific, 10* (*tsga10*), *spermatogenesis associated* 4 (*spata4*), *spata22*, *tudor domain containing* 9 (*tdrd9*), *tdrd12*, *follicle stimulating hormone receptor* (*fshr*), *gonadal somatic cell derived factor* (*gsdf*) (**Papers I** and **III**). These results are in agreement with a previous transcriptomic study which has revealed the enrichment of GO terms related to reproduction in the intestine of zebrafish fed SBM (Li et al., 2022c). Similar effects of SBM on reproductive performance and endocrine disruption were reported in human and fish studies (Bagheri et al., 2013, Patisaul, 2017). The isoflavones present in SBM have a structure

like those of endogenous oestrogens, facilitating their binding to oestrogen receptors in tissues including the intestine and thus altering the transcription of genes related to reproduction. These observations relevant to specific aspects of reproductive physiology were noted only in studies performed in juveniles (50 dpf) and adult zebrafish (8 months) (**Paper I** and **III**). King and co-workers have reported that sex-specific genes in zebrafish start to express at around 28 dpf (King et al., 2020). Based on the results linked to reproductive processes that have a bearing on behaviour and metabolism point to the need to develop a holistic zebrafish model of inflammation with processed SBM which is devoid of isoflavones and other reproduction disruptors.

SBM feeding can also influence cholesterol homeostasis. Disturbance in cholesterol metabolism could be due to the inadequate supply of cholesterol from a diet containing more SBM and less fishmeal (Kortner et al., 2014), as cholesterol is mainly derived from ingredients of animal origin. Cholesterol metabolism can be disrupted by soy isoflavones and saponins in SBM that can lower the intake of dietary cholesterol (Kortner et al., 2013, Gu et al., 2014). Cholesterol is an important component of cell membranes and a precursor of steroid hormones and bile acids (Sonal Sekhar et al., 2020). Steroid biosynthesis appeared as one of the enriched KEGG pathways in zebrafish larvae fed 50% SBM (**Paper II**). In addition, based on the upregulated genes, several GO terms like cholesterol metabolic process, sterol biosynthesis process were enriched in zebrafish larvae (**Paper II**). Inadequate cholesterol intake triggers endogenous cholesterol biosynthesis and suppresses cholesterol excretion through the biliary route (Gu et al., 2014, Kemski et al., 2020). The expression of solute carrier *slc51a*, that is involved in the absorption of bile acid was upregulated in the intestine indicating an effort to reduce bile excretion (Ballatori et al., 2013) (**Paper I**). There was also a decrease in the abundance of secondary bile acid metabolite taurochenodeoxycholic acid in the plasma of SBM-fed group (**Paper III**) indicating an alteration of bile acid metabolism. Previous transcriptomic studies have reported alterations in the genes related to cholesterol and bile acid metabolism in the intestine of fishes fed SBM (Kortner et al., 2013, Kemski et al., 2020). GO terms such as ER part,

and nuclear outer membrane- ER membrane network were enriched in the intestine of the SBM-fed group (**Paper III**). Furthermore, the expression of several ER-related genes like *lipase maturation factor 2b* (*lmf2b*), *receptor accessory protein 2* (*reep2*), *ELOVL fatty acid elongase 4a* (*elovl4*), *ELOVL fatty acid elongase* 6 (*elovl6*), *ER lipid raft associated* 2 (*erlin2*) were altered in SBM-fed fish (**Paper I and III**). The ER is the main organ in which cholesterol is synthesized and is responsible for cholesterol homeostasis (Röhrl and Stangl, 2018). The results in Papers I and III likely indicate the negative effect of dietary SBM on cholesterol homeostasis and ER functioning. Since a large proportion of cholesterol resides in the membrane as components of lipid rafts and disturbance of the lipid raft organization occurs during inflammation (Bowie et al., 2012), and this aspect can be explored in future studies.

Dietary SBM also affected the overall development of zebrafish larvae. The results in **Paper II** also indicated enrichment of GO terms related to developmental processes like regulation of gastrulation, formation of primary germ layer, somite development and positive regulation of organelle organization in the larvae fed SBM, based on several downregulated genes such as hairy-related 6 (*her6*), *N-alpha-acetyltransferase 50, NatE catalytic subunit* (*naa50*), COPI coat complex subunit beta 1 (*copb1*)*, growth regulating estrogen receptor binding 1* (*greb1*), *transportin* 1 (*tnpo1*), *Kruppel like factor 2a* (*klf2a*), *churchill domain containing 1*(*churc1*). SBM feeding impaired the development of swim bladder in larvae (**Paper II**) and reduced the head to trunk angle of the larvae (**Paper II**). Larvae with uninflated swim bladders will have skeletal deformities (Trotter et al., 2001). To my knowledge, none of the previous studies have reported the adverse effect of SBM on swim bladder development, though there is evidence of SBM-induced bone defects. Carnovali et al. (2022) reported that dietary SBM affected ossification (bone formation) in zebrafish larvae, by reducing intra-membranous mineralization in head by 17% and trunk by 47%. The observed reduction of head to trunk angle could be due to reduced mineralization in trunk resulting in skeletal deformities (**Paper II**). Carnovali et al. (2021) had earlier reported that SBM consumption could lead to the development of osteoporosis (decrease bone density)

like phenotype in the scale border of adult zebrafish. Inflammatory bowel disease patients are at high risk of osteoporosis due to different factors including increased production of proinflammatory cytokines (IL-1β, IL-6, IL-8, TNF-α). A severe systemic inflammation can elevate bone resorption without formation of osteoblasts (Chedid and Kane, 2020). Similarities in the impact of intestinal inflammation on bone development in zebrafish and humans indicate that the fish model can be used to study such adverse systemic responses.

SBM intake not only affects the swim bladder development, but also influences locomotor and feeding behaviour (Schwebel et al., 2018). Zebrafish larvae are adversely affected when their swim bladder does not function properly resulting in lack of buoyancy (Trotter et al., 2001). This can increase the metabolic demand (Schwebel et al., 2018), as indicated by the higher consumption of oxygen in the SBM-fed group (**Paper II**). The locomotor behaviour of zebrafish larvae fed SBM was also affected, as inferred from parameters like distance travelled, velocity and movement (**Paper II**).

Other impairments include their negative effect on eye size and sensory perception, as indicated by velocity time plot analysis of the SBM-fed group (**Paper II)**. Interestingly, several genes related to light perception including *visual system homeobox 1* (*vsx1*), *recoverin a* (*rcvrna*) and *guanylate cyclase activator 1b* (*guca1b*) were downregulated in SBM-fed fish group. These genes are important for terminal differentiation of retinal cells and cone photo response recovery (Ohtoshi et al., 2004, Zang and Neuhauss, 2018). Enrichment of pathways such as retinol metabolism and phototransduction pathway was reported in **Paper II**. The phototransduction pathway is connected to retinal photoreceptors, namely rods and cone cells which are active at low and high light intensities, respectively. These cells convert light stimulus into electrical signals which are then perceived by the nervous system (Koch and Dell'Orco, 2015). A previous study reported that retinoic acid and the associated alteration of gene expression could lead to abnormal eye development, visual impairment and altered behaviour in zebrafish larvae (Le et al., 2012). The eye size of zebrafish larvae that exhibited signs of SBM feeding-induced inflammatory response (based on the number of neutrophils and

proinflammatory cytokine expression in the intestine) was smaller compared to larvae fed a control diet (**Paper II**). IBD patients suffer from different eye complications like eye conjunctivitis, photophobia and ocular pain (Mintz et al., 2004, Troncoso et al., 2017). Previous studies have found that $CD103^+$ DCs promote immune tolerance via the production of retinoic acid and Treg cells. **Paper II** reports the upregulation of several genes such as *UDP glucuronosyltransferase 1 family, polypeptide A1*(*ugt1a1*), *ugt1a5*, *ugt1a4*, *cyp3a65* (cytochrome P450, family 3, subfamily A, polypeptide 65) that are involved in retinoic acid pathway. Such an upregulation in zebrafish fed a SBM diet is likely indicating the relationship between retinoic acid and inflammation. Retinoic acid can induce the expression of gut homing receptors (α4β7 and CCR9) on effector T cells as well as on regulatory T cells, the former population is required for Th1 and Th17 cell response (Hall et al., 2011). Another study reported that increased production of retinoic acid by intestinal macrophages can promote their polarization to inflammatory phenotype (Sanders et al., 2014). It should be noted that by metabolising retinol, dendritic cells can supply the bioactive form of vitamin A to macrophages to evoke antimicrobial responses (Kim et al., 2019). The metabolite ethylmalonic acid was abundant in the plasma of zebrafish fed an SBM diet (**Paper III**). Similarly, ulcerative colitis patients and mice models were found to have a higher abundance of ethylmalonic acid (Keshteli et al., 2019, Kohashi et al., 2014, Shiomi et al., 2011). Additional reports also suggest that high levels of ethylmalonic acid in tissues and body fluids are associated with brain mitochondrial dysfunction and increased oxidative damage in the cerebral mitochondria of rats (Schuck et al., 2015, Ritter et al., 2015). Furthermore, subcutaneous injection of ethylmalonic acid induced behavioural deficits in developing rats, as demonstrated by learning disabilities, memory loss and increased lipoperoxidation in brain hippocampus (Schuck et al., 2009). The increased abundance of ethylmalonic acid in the plasma of zebrafish fed SBM-based diet is likely to be a factor associated with behavioural problems (locomotor behaviour) observed in larvae (**Paper II)**. These extraintestinal manifestations arising from the intake of a diet containing 50% SBM, indicates that it can compromise the proper development and

behaviour of zebrafish (**Paper II and III**). From the results presented in this thesis it can be concluded that besides causing intestinal inflammation, SBM in diets influences several functions including reproduction, cholesterol homeostasis, eye development, and behaviour of zebrafish.

3.3. β-glucans and oligosaccharides as dietary supplements to reduce soybean-induced inflammatory response

Natural indigestible carbohydrates, known as prebiotics, are considered as effective agents for the treatment of IBD as they have the ability to regulate intestinal inflammation by maintaining immune homeostasis (Yuan et al., 2022). These bioactive carbohydrates can reduce intestinal inflammation by establishing beneficial bacteria that facilitate the production of SCFAs (McLoughlin et al., 2017). The potential of two bioactive products, β-glucans (**Paper I, II and III**) and AOS (**Paper III**) to alleviate SBM-induced intestinal inflammation was examined in this PhD project. It has been reported that the bacteria derived from zebrafish intestine can produce three main SCFAs (acetate, propionate, and butyrate) (Cholan et al., 2020). Plasma metabolomic analysis revealed that the AOS-fed fish had significantly higher levels of 2-hydroxybutyric acid (2-HB) compared to SBM-fed group (**Paper III**). A previous study also reported an increase of 2-HB in the serum of mice fed a polysaccharide (from *Astragalus*) (Li et al., 2022a). AOS can stimulate the proliferation of bacteria belonging to the genus *Alloprevotella* (Wang et al., 2020) and its abundance was positively correlated with the concentrations of both butyric acid and 2-HB (Wu et al., 2021b, Wang et al., 2020). Furthermore, dietary β-glucan has been linked to increased production of butyrate in mice (Miyamoto et al., 2018). Butyrate helps to thwart inflammation, reduce neutrophil migration, and maintain the mucosal barrier integrity (Chen et al., 2018, Li et al., 2021). In addition, butyrate, and 2-HB can regulate proinflammatory cytokines (Li et al., 2022a, Segain et al., 2000). The anti-inflammatory effect of butyrate was also reported in zebrafish, based on the fewer recruitment of neutrophils and M1 macrophages to wounds (Cholan et al., 2020). In the present thesis the two functional additives modulated several inflammation-related processes that were altered by SBM,

probably through the production of SCFAs. The additives also provided better barrier protection - AOS supplemented diet stimulated the development of goblet cells compared to SBM diet (**Paper III**). According to a previous *in vitro* study (using the human colon cell line), oligosaccharides can influence the mucosal barrier integrity by modulating the functions of intestinal goblet cells, by affecting mucins and trefoil proteins (Bhatia et al., 2015). Furthermore, more mucus cells per villi suggests that more intestinal cells are undergoing goblet cell differentiation to strengthen the barrier (**Paper III**). β-glucan feeding can significantly reduce the lamina propria width in zebrafish juveniles, indicating a positive effect on the mucosal barrier (**Paper I**). The ability of the tested polysaccharides to reduce the inflammatory response has been described in all three **Papers**. For example, the expression of several inflammatory marker genes (*mpx*, *cxcl8a*, *mmp9*) were downregulated by β-glucan and AOS-fed fish, while the expression of these genes was upregulated in SBM-fed group (**Paper III**). There were fewer granulocytes in the intestine of β-glucan group compared to the SBM-fed group and these cells (mainly neutrophils) are known to migrate quickly to an inflammatory site (Fuentes-Appelgren et al., 2014). β-glucan intake downregulated the expression of genes such as *janus kinase 1* (*jak1*), *leptin receptor* (*lepr*), *jagunal homolog 1-A* (*jagn1a*), *interleukin 13 receptor, alpha 2* (*il13ra2*), and this was associated with an enrichment of the GO terms leukocyte differentiation and cytokine mediated signaling (**Paper II**). Interestingly, AOS intake was associated with the downregulation of the expression of genes like *CD59 molecule* (*cd59*), *mpx*, *NLR family, CARD domain containing* 3 (*nlrc3*), *matrix metallopeptidase 25b* (*mmp25b*), causing an enrichment of the GO terms inflammatory response, humoral immune response and complement activation **(Paper III**). This indicates that the dietary oligo- and polysaccharides can modulate the expression of several inflammation-related genes triggered by the SBM diet, albeit through distinct mechanisms. It can be speculated that such carbohydrates can modulate immune responses probably via their prebiotic properties viz., fermentation of dietary polysaccharides and/or resultant metabolites, which in turn reduce inflammatory responses. These compounds might also have

accomplished immune modulation via another route; through direct interaction with the host immune system as they are recognized by different PRRs present on several immune cells. It can be concluded that these dietary carbohydrates can significantly mitigate the intestinal inflammatory response evoked by an SBM diet. Transcriptomic analyses undertaken for this PhD project did not reveal any alteration of specific immune receptors that recognize these bioactive compounds. It is likely that the doses of the tested products were not enough to detect the changes in these specific receptors.

The immune modulatory effect of these polysaccharides can have a beneficial effect on the overall physiology and development of zebrafish. Both the β-glucan- and AOS-fed fish had longer intestinal villi (**Paper I and III**), as reported in previous studies (Wan et al., 2018, Wu et al., 2021a). Nutrient absorption can be higher in animals with longer intestinal villi, which in turn gets translated into better growth and health (Wan et al., 2018, Ramos et al., 2017). Nevertheless, the tested polysaccharides did change the villi length, but the change could not be associated with a better growth performance in zebrafish. It is imperative to perform growth trials using zebrafish of age 20–40 dpf because it is within this range that energy from diet is completely allocated for growth (Gómez-Requeni et al., 2010). However, the feeding experiments were conducted when zebrafish were in juvenile (from 50 to 80 dpf) and adult stages (above 90 dpf) (**Paper I and Paper III**) and early larval stage (from 5-15 dpf) (**Paper II**) and this could be the possible reason for not detecting the feed additive-caused growth enhancement. Nevertheless, a positive effect of dietary polysaccharides on the overall development and behavior was observed in **Paper II**. For example, head to trunk angle and swim bladder area were significantly increased in the algal β-glucan- fed group compared to the SBM-fed group (**Paper II**). A previous report has stated that β-glucans can exert anti-osteoporotic impacts such as preventing bone loss, stimulation of bone formation and fracture healing (Ariyoshi et al., 2021). The interaction between osteoclasts (cells involved in bone resorption) and osteoblasts (cells promoting bone formation) and the action of multiple immunoreceptors present on innate immune

cells are important for the bone remodeling (Humphrey and Nakamura, 2016). For example, helper T cells can damage multiple joints by enhancing the expression of receptor activator of NF-κB ligand (RANKL) mediated by proinflammatory cytokines (Boyce and Xing, 2008). Previous studies have reported that β-glucans inhibit bone loss via varied mechanisms including suppression of RANKL (Ariyoshi et al., 2021, Aizawa et al., 2018). The associated mechanistic processes have been revealed in an *in vitro* study: yeast β-glucan suppressed RANKL formation via Dectin-1 receptor, attenuated the osteoclast related gene expression and inhibited RANKL‑mediated NF-κB signalling in mouse bone marrow cells (Hara et al., 2021). Therefore, it is possible that β-glucans might have inhibited the formation of osteoclasts leading to a positive effect on skeletal development in zebrafish larvae. An article in mice reported the ability of β-glucan to enhance the migration rate of human corneal epithelial cells to repair the corneal epithelial wounds and to reduce the acute inflammatory reaction in eyes (Choi et al., 2013). In **Paper II**, it was reported that β-glucan can restore the eye size of zebrafish larvae fed SBM diet. This indicates that the anti-inflammatory and tissue remodeling effect of β-glucan might also have a positive effect on eye development.

Locomotor behavior, as indicated by parameters like distance travelled, average velocity, movement, and velocity vs. time plot of the light-dark phase, was improved in zebrafish larvae fed β-glucan compared to larvae fed SBM. Previous studies have indicated the efficacy of β-glucan in enhancing the cognitive behavior in mice; by inducing the beneficial effects along the gut-brain axis i.e., by reducing the microglia activation and inflammatory cytokines in the hippocampus, altering gut microbiota profile and enhancing intestinal mucus production (Shi et al., 2020, Hu et al., 2022). Although, the effects of dietary β-glucan on brain functions and gut microbiota profile were not studied for this PhD project, **Paper II** reported improvement in locomotor behavior of zebrafish larvae fed β-glucan. Furthermore, the abundance of pantothenic acid also known as vitamin B5 (VB5) was higher in the plasma of algal β-glucan-fed fish. VB5 is obtained from diet or intestinal microbiota (Magnúsdóttir et al., 2015). An inverse correlation between dietary VB5 intake and the serum CRP concentration

(marker of inflammation) was observed in humans (Jung et al., 2017). Several intestinal commensal bacteria such as *Bacteroides fragilis* and *Prevotella copri* possess vitamin B5 biosynthesizing capacity (Magnúsdóttir et al., 2015, Yoshii et al., 2019) and it is likely that algal β-glucan can stimulate the proliferation of these gut microbes. VB5 is also associated with improved brain function and is essentially required for the synthesis of acetyl-CoA which is important for producing neurotransmitters like acetylcholine in the brain (Ismail et al., 2020). Furthermore, cerebral deficiency of VB5 is associated with several neurodegenerative diseases (Xu et al., 2020). Therefore, it can be inferred that an improved larval behavior in zebrafish can possibly be due to its prebiotic action. Overall, functional oligo-/polysaccharides can have protective effects on the intestine (**Paper I, II and III)**, and beneficial effects on the development and behavior of zebrafish (**Paper II)**.

3.4. Source/structure of the oligo- and polysaccharides influence their functional capacity

Functional oligo-/polysaccharides are produced from different sources like yeasts, mushrooms, macroalgae, microalgae and bacteria. These compounds may have different molecular weights, branching patterns, chain conformations and functional groups (Ullah et al., 2019). The structural differences in the bioactive carbohydrates affect their immune modulation ability. The potential of β-glucans derived from two different sources–yeast (*Saccharomyces cerevisiae*) (**Paper I**) and microalgae (*Euglena gracilis*) (**Paper II** and **III**)–to provide protection against soybean-induced intestinal inflammation was assessed in this PhD project. Furthermore, the anti-inflammatory potential of two oligosaccharides (AOS derived from the macroalga *Laminaria* sp.) that differed in terms of the percentage of low molecular weight fractions were evaluated (**Paper III**) to delineate their modes of action.

The yeast- and alga-derived β-glucans, β-(1,3),(1,6) and β-(1,3) respectively, differ mainly in terms of branching pattern. The former exhibits side chains that branch exclusively from the 6-position of the backbone, while the latter has a linear form that

does not have any branches (Han et al., 2020). These structural differences can affect their anti-inflammatory potential.

Yeast β-glucan with side chains downregulated the genes connected to the GO terms small GTPase-mediated signal transduction, guanyl nucleotide binding, guanyl and intracellular cellular signal transduction. On the other hand, another study reported that β-(1,3)-glucan recognition by Dectin-1 activated small GTPase (Choraghe et al., 2020). Small GTPases (Guanine nucleotide-binding proteins or G-proteins) are important in signal transduction, and they regulate diverse cellular responses including leukocyte-endothelial interactions and formation of epithelial junctions (Lu et al., 2013). Previous studies have found that small GTPases affect the functions of inflammatory phenotypes (Johnson and Chen, 2012, Chu et al., 2018). For example, members of small GTPase regulate diverse functions of neutrophils like adhesion, chemotaxis, and recruitment during inflammation. SBM intake caused the enrichment of small GTPase-mediated signal transduction by upregulated genes such as *ras homolog family member Ub* (*rhoub*),*RAB25, member ras oncogene family* (*rab25b*)and *ADP-ribosylation factor-like 4Ca* (*arl4ca*) (**Paper I**). These genes regulate epithelial cell polarity, intracellular membrane transport and the function of tight junction proteins (Lapierre et al., 2011, Fujii et al., 2015, Zihni and Terry, 2015). Because yeast β-glucans countered inflammation and there was evidence of suppression of small GTPases, it could be speculated that β-(1,3),(1,6) that was employed in the current study likely uses the same pathway as the β-(1,3) reported by Choraghe et al. (2020). Additional studies are needed to clarify the role of small GTPases in maintaining the intestinal homeostasis in zebrafish.

As for the unbranched β-(1,3) algal glucan-fed fish, several GO terms linked to negative regulation of proteolysis and endopeptidase inhibitor activity were enriched as a result of the alteration of genes such as *serpin peptidase inhibitor, clade B (ovalbumin), member 1* (*serpinb1*), *serpin peptidase inhibitor, clade B (ovalbumin), member 1, like 3* (*serpinb1l3*), *TIMP metallopeptidase inhibitor 4, tandem duplicate* 2 (*timp4.2*), *TIMP metallopeptidase inhibitor 2b* (*timp2b*), *mmp13a*, *complement component 7a* (*c7a*)

(**Paper II and Paper III**). The upregulated gene *serpinb1* produced by macrophages and neutrophils, is an important inhibitor of enzymes like elastase, proteinase-3, cathepsin G secreted by neutrophils (Choi et al., 2019) (**Paper II**). Algal β-glucan also increased the expression of genes associated with blocking of extracellular matrix-degrading metalloproteases (*timp4.2, timp2b*) (**Paper II and III**). Tissue inhibitors of metalloproteases (TIMPs) regulate diverse processes such as extracellular matrix deposition, tissue remodeling and wound healing (Cabral-Pacheco et al., 2020). Therefore, the alterations in the expression of the *timp* genes suggest that algal β-glucan might help to prevent tissue damage by inflammatory phenotypes such as neutrophils and macrophages that had their signatures during SBM-induced inflammation (**Paper II** and **III**). The algal feed additive was associated with an enrichment of the GO terms leukocyte differentiation and cytokine mediated signaling, (based on downregulated genes) (**Paper II**). Among the downregulated genes was *janus kinase 1* (*jak1*), which is important in mediating inflammatory cytokine signaling An inhibition of JAK1-mediated inflammatory signaling is targeted to counter IBD in humans (Schwartz et al., 2017). β-glucan is reported to be an inducer of innate immune memory in mononuclear phagocytes which causes an increased immune responsiveness to secondary challenges primarily via epigenetic alterations in immune cells. Moreover, β-glucan–induced innate immune memory in macrophages can suppress NLR family pyrin domain containing 3 inflammasome–facilitated caspase-1 activation and IL-1β secretion, thus inhibiting the inflammation pathway (Camilli et al., 2020). Therefore, enrichment of several GO terms (histone acetyltransferase complex, histone modifications, chromatin organization) related to epigenetic modifications, reported in **Paper II,** points to the possibility that algal β-glucan induced immune modulation is likely due to epigenetic modifications in immune cells; this warrants further research. In addition to the abovementioned source-specific changes in the intestine transcriptome, β-glucans could affect the micromorphology of the organ. For instance, yeast β-glucan prevented the lamina propria widening and increased the

villus height of the intestine compared to the SBM diet (**Paper I**) while such changes were not evident for algal β-glucan (**Paper II**).

It is known that the immune modulating ability of β-glucans depends on the branching pattern (Miyazaki et al., 1979). These functional polysaccharides with a branching ratio between 0.2 to 0.33 are effective immunomodulators (Han et al., 2020) and its sidechains can increase the binding affinity to dectin-1 (Adams et al., 2008). However, it has also been pointed out that large side chains can also interfere with each other, reducing the binding affinity of β-glucans. For example, the immune stimulation capacity of algal β-(1,3)-glucans (*E. gracilis*) increased with increasing concentrations while that of yeast β-(1,3)(1,6)-glucans (*S. cerevisiae*) decreased with their concentration (Sonck et al., 2010). The same study also reported that algal compound activated the ROS-production by monocytes and neutrophils compared to yeast product. Therefore, the distinct intestinal transcriptomic responses of yeast and algal β-glucans observed in the present study (**Paper I,** and **II, III**) might be due to the different branching patterns of β-glucans derived from two different sources.

Two oligosaccharides (AOS - AL and AH) were compared in this study based on the intestinal transcriptome, plasma metabolome and histomorphological changes in zebrafish (**Paper III)**. The AL (SBM + low molecular weight AOS) diet compared to the AH (SBM + high molecular weight AOS) diet evoked distinct responses in the intestine of zebrafish. Several GO terms like inflammatory response, complement activation and humoral immune response were enriched by the downregulated DEGs (like *cd59*, *c7a, mpx*, *ccl36.1*, *nlrc3*, *gpr142 and mmp25b*) in the AL group. On the other hand, only one GO term, namely negative regulation of immune system process was enriched by the downregulated DEGs (*lgals9l6*, *cd59 glycoprotein-like*) in the AH group. Histological analysis also revealed the increase in goblet cell counts and villi length in the AL diet-fed fish. Intestinal epithelial cells are sources of complement proteins and proper regulation of complement activation is essential to prevent intestinal epithelial cell damage (Sina et al., 2018). Increased activation of complement system is associated with intestinal inflammation (Ning et al., 2015). The gene *c7a* is part of the membrane

attack complex (MAC), and downregulation of this component might prevent complement activation. The protein CD59 restricts the activation of the complement system and the associated assembly of MAC, the decrease in epithelial expression of CD59 in IBD patients renders the epithelial cells more susceptible to complement lysis (Scheinin et al., 1999). Conversely, an increased expression of *cd59 glycoprotein-like* was found in the soybean fed group (Paper **III**). An increased production of CD59 was also found in human colon cancer cells (Zhang et al., 2018). The downregulation of *cd59* that was observed in zebrafish fed the AL diet compared to the SBM fed group could be indicating the ability of the AOS to maintain homeostasis. Therefore, the suppression of several processes related to inflammation by the downregulated DEGs in the intestine of the AL group suggests the capacity of AL group to reduce complement-mediated inflammation induced by the SBM containing diet. Furthermore, qPCR-based analysis revealed the increased expression of the antioxidant enzyme *catalase* in the AL group which suggests that it can possibly reduce oxidative stress generated during intestinal inflammation, as reported earlier (Ansar et al., 2020). On the other hand, *lgals9l6* that codes for protein galactoside-binding, soluble, 9 (galectin 9)-like 6, an ortholog of human LGALS9 (galectin 9/Gal-9) was downregulated in the AH group. *Gal-9*, a β-galactoside binding lectin with a carbohydrate recognition domain, is expressed in human crypt cells and its expression is decreased in IBD patients (Sudhakar et al., 2020). Furthermore, a direct comparison of the AL group with AH group revealed the downregulation of immune genes *ccl36.1* and *crp6* in the former group. The downregulated gene *crp6* is an ortholog of human CRP, a biomarker of systemic inflammation (Ishida et al., 2021, Vermeire et al., 2004). The present study on zebrafish concluded that the difference in the percentage of the low molecular weight fraction of AOS can affect their immune modulatory potential. The possible explanation is that low molecular weight polysaccharides are more soluble and have greater fermentability (Sarbini et al., 2013). It has also been reported that lower molecular weight polysaccharides and alginate have better prebiotic potential (Ramnani et al., 2012) and enhances the radical scavenging and

immunomodulatory capacities (Harada et al., 2021). Furthermore, their anti-inflammatory activity can increase when these low molecular weight AOS are degraded by the gut bacteria into α-L-guluronic acid and β-D-mannuronic acid, which also possess anti-inflammatory properties (Zhang et al., 2021, Xing et al., 2020). Thus, the bioactive carbohydrates derived from different sources and having different branching patterns can generate distinct transcriptomic and histological alterations in the zebrafish intestine. Furthermore, even if the oligosaccharides are from the same source, the molecular weight can determine their biofunctionality.

4. CONCLUSION

This thesis provided novel insights into the transcriptomic, metabolomic and behavioural changes in a zebrafish model of diet-linked intestinal inflammation. SBM included in the diet (at 50%) altered the expression of several inflammation- and barrier-related marker genes in the intestine of zebrafish. Transcriptome sequencing further indicated that SBM intake affected the expression of genes connected to several processes including ATP-dependent peptidase activity, steroid biosynthesis, endoplasmic reticulum part, reproduction, phototransduction, and retinoic acid metabolism. Metabolome analysis revealed that SBM can decrease the taurochenodeoxycholic acid levels but increase the abundance of itaconic acid and ethylmalonic acid and activated the arginine biosynthesis pathway in the plasma of zebrafish. Histological analysis revealed that SBM can increase the recruitment of granulocytes and reduce the goblet cell number in the intestine. Furthermore, SBM could adversely affect the eye development, swim bladder area and head trunk angle in zebrafish. In addition, SBM increased oxygen consumption and reduced locomotor behaviour activity in zebrafish.

β-glucans from yeast and microalga reduced the expression of genes related to small GTPase-mediated signal transduction and endopeptidase activity, respectively. Dietary algal β-glucans downregulated the genes connected to leukocyte differentiation and cytokine-mediated signalling. On the other hand, low molecular weight macroalgal oligosaccharide reduced the expression of genes related to complement activation, inflammatory response and humoral response in the intestine of zebrafish fed a SBM diet. Plasma metabolomic analysis revealed an increased abundance of pantothenic acid by dietary algal β-glucan, while dietary AOS increased the content of a short chain fatty acid in the plasma of zebrafish. Histological analysis revealed that dietary yeast β-glucan reduced lamina propria width in the intestinal mucosa whereas algal β-glucan reduced the number of granulocytes. On the other hand, the low molecular weight AOS in the diet increased the goblet cell number in the intestine. Both yeast β-glucan

and AOS increased the villi height of the intestinal mucosa of zebrafish. Furthermore, supplementing SBM diet with algal β-glucan counteracted the developmental defects and restored the locomotor behaviour activity in zebrafish.

Thus, this PhD project has given insights into SBM-induced inflammatory features and the distinct modes of actions of β-glucan and AOS to counteract intestinal inflammation.

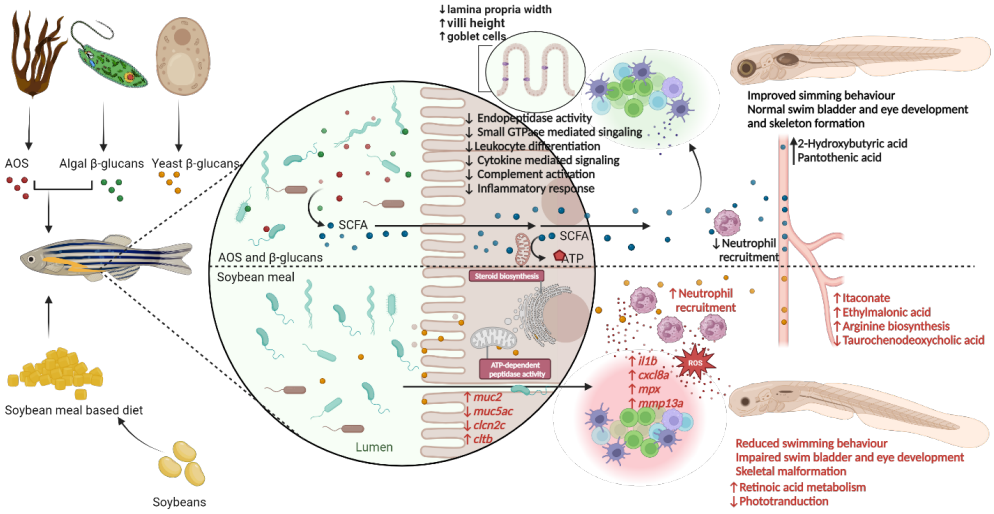


Figure 6. Overview of the key findings of the thesis. *Created with BioRender.com*

5. CONTRIBUTION TO THE FIELD

The studies performed for this PhD project provided novel knowledge about the developmental aberrations associated with SBM-induced intestinal inflammation in zebrafish. **Paper II** is the first publication that reported the effect of the intake of SBM on oxygen consumption and behaviour. The three studies have gathered adequate evidence to state that 50% SBM fed zebrafish can be a useful model species to unravel deeper insights into the effect of intestinal inflammation on behaviour and metabolism.

Plasma metabolomic analyses revealed alterations in metabolites accompanying the SBM-induced intestinal inflammation. **Paper III** represents the first report on the plasma metabolomic landscape of zebrafish. The information about the plasma metabolites (taurochenodeoxycholic acid, itaconic acid, ethylmalonic acid) and the enrichment of the arginine biosynthesis pathway reported in **Paper III** will be useful to devise strategies to mitigate intestinal inflammation.

Distinct differences in the mode of action of β-glucans (branching pattern) and AOS (molecular weight), based on the intestinal transcriptome and histomorphology of the intestine provide information for other investigators to decide the test products for future research. Furthermore, this information is critical for promoting the use of different bioactive oligo- and polysaccharides for specific benefits.

Our results provide in-depth information about the reliability of zebrafish as a model of intestine inflammation and its suitability to assess therapeutic agents against inflammatory diseases in humans.

6. FUTURE PERSPECTIVES

The PhD project results have revealed that dietary soybean can affect the behaviour of zebrafish. However, we did not investigate the effect of soybean-induced intestinal inflammation on the gut microbiota-brain axis. Previous studies have linked diet-induced behavioural changes to bacterial profiles which in turn affect the gut-brain axis. Using germ-free and gnotobiotic zebrafish, future studies should investigate the contribution of intestinal microbiota towards SBM-induced behavioural changes. Such studies will help elucidate the link between intestinal inflammation and behavioural alterations to develop effective therapeutic strategies against intestinal disorders as well as brain dysfunctions.

For this PhD project, only the transcriptome of whole intestine tissue or whole larvae was studied. Hence, future research should obtain an overview of the cell types that are predominantly affected by SBM or functional feed additives. Single cell RNA-Seq could be exploited to understand the diet-induced changes in the expression profile of different cell populations. This information reveals the particular cell types affected during inflammation and those that are active during its resolution.

β-glucan is reported to be an inducer of trained immunity (in mononuclear phagocytes) that primarily takes place through epigenetic alterations in immune cells. Several GO terms related to epigenetic modifications were altered in the present study. In the future, in depth studies linking epigenetic modifications and immune modulation by dietary β-glucan should be investigated using high throughput techniques like whole genome bisulphite sequencing and reduced-representation bisulphite sequencing.

This thesis revealed the differential modes of action employed by dietary β-glucans and AOS to exert their anti-inflammatory properties. It would be interesting to explore the possible synergetic effect of AOS and algal β-glucan and the associated transcriptomic and metabolomic alteration in the zebrafish model.

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RESEARCH ARTICLE

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Zebrafish intestinal transcriptome highlights subdued inflammatory responses to dietary soya bean and efficacy of yeast β-glucan

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Abstract

Anti-nutritional factors in dietary components can have a negative impact on the intestinal barrier. Here, we present soya bean-induced changes in the intestine of juvenile zebrafish and the effect of yeast β-glucan through a transcriptomic approach. The inclusion of soya bean meal affected the expression of several intestinal barrier function-related genes like *arl4ca*, *rab25b*, *rhoub*, *muc5ac*, *muc5d*, *clcn2c* and *cltb* in zebrafish. Several metabolic genes like *cyp2x10.2*, *cyp2aa2*, *aldh3a2b*, *crata*, *elovl4*, *elovl6*, *slc51a*, *gpat2* and ATP-dependent peptidase activity (*lonrf*, *clpxb*) were altered in the intestinal tissue. The expression of immune-related genes like *nlrc3*, *nlrp12*, *gimap8*, *prdm1 and tph1a*, and genes related to cell cycle, DNA damage and DNA repair (e.g. *spo11*, *rad21l1*, *nabp1b*, *spata22*, *tdrd9*) were also affected in the soya bean fed group. Furthermore, our study suggests the plausible effect of yeast β-glucan through the modulation of several genes that regulate immune responses and barrier integrity. Our findings indicate a subdued inflammation in juvenile zebrafish fed soya bean meal and the efficacy of β-glucan to counter these subtle inflammatory responses.

KEYWORDS

intestinal barrier, small GTPase, soya bean meal, yeast β-glucan, zebrafish

1 | INTRODUCTION

The intestinal epithelium acts as a selective barrier, and the epithelial cells evoke appropriate responses to microbial signals and antigenic factors (Soderholm & Pedicord, 2019). Normal functioning of the associated mucosal barrier, which includes the epithelial layer and lamina propria, is vital to carry out digestion and nutrient absorption by the intestine (Farré et al., 2020). A compromised mucosal barrier increases the permeability of the intestine, and such a condition allows undesirable luminal antigens to cross the barrier, leading to dysbiosis and activation of inflammatory response in the tissue. Damage to intestinal tissue can eventually result in the disturbance of the overall gut physiology (Farré et al., 2020).

Certain dietary components can alleviate issues related to intestinal disorders, whereas some others trigger intestinal inflammation (Hou et al., 2014; Khoshbin & Camilleri, 2020). Specific dietary components like soya saponins, the anti-nutritional factor in soya bean meal, can bind to the intestinal epithelial membrane cholesterol.

This subsequently leads to the formation of pores and makes the membrane permeable to luminal antigens (Böttger & Melzig, 2013). Hence, soya bean anti-nutritional factors are regarded as intestinal dysfunction-provoking agents. These dietary components are ideal to create an intestinal inflammation model in zebrafish (Coronado et al., 2019; Solis et al., 2020).

Most of the studies related to soya bean-induced inflammation in zebrafish have been conducted using the larval stages. Previous studies have reported that dietary soya bean meal can affect gut permeability and increase the expression of key inflammatory cytokines in the larval intestine (Hedrera et al., 2013; Solis et al., 2020). It should be noted that the intestine of the larval zebrafish does not represent a fully developed organ in terms of cell proliferation (Li et al., 2020), and the functional adaptive immune system matures during advanced larval stages (Brugman, 2016; Brugman et al., 2014). The global gene expression profile in post-larval stages of zebrafish fed soya bean will provide information related to the immune and metabolic aspects connected to soya bean-induced aberrations.

Intestinal disorders are connected to many mediators and signalling pathways (Newton & Dixit, 2012). Hence, it would be ideal to use anti-inflammatory feed additives that have the potential to alter such pathways (Bravo-Tello et al., 2017; Romarheim et al., 2013). β-glucan, a natural polysaccharide composed of glucose monomers linked by β (1,3) glycosidic bonds, is known to have anti-inflammatory properties (Ji et al., 2019; Liu et al., 2015). β-glucans derived from different sources like yeasts, seaweeds, mushrooms, algae and bacteria vary in their branching patterns, molecular weights, chain conformations and functional groups (Jin et al., 2018). These structural differences cause variations in their bioactivity. MacroGard®, a commercially purified yeast β-1,3/1,6-glucan obtained from the cell wall of *Saccharomyces cerevisiae*, is a commonly used and investigated feed additive (Raa, 2015). However, the underlying mechanism of the anti-inflammatory effect of dietary yeast β-glucan is unclear. We aimed to develop a diet-induced inflammation model in zebrafish juveniles using dietary soya bean meal as an inflammation-inducing agent. A transcriptomic approach was employed to elucidate soya bean meal-induced alterations in the intestine of a juvenile zebrafish model. We further examined the effects of a combination of MacroGard® and soya bean meal on the intestinal transcriptome, to understand the anti-inflammatory effect of yeast β-glucan.

2 | MATERIALS AND METHODS

2.1 | Zebrafish husbandry

Healthy AB zebrafish juveniles (n = 144) were used for the experiment. Zebrafish were bred at Nord University, Norway, according to standard protocols (Westerfield, 2000). The eggs were maintained in E3 medium and incubated at 28°C in an incubator until hatching, that is 2–3 days post-fertilization. From 5 to 14 days post-fertilization, the larvae were fed *ad libitum* on *Artemia nauplii* and commercial micro diet Zebrafeed® (Sparos Lda, Olhão, Portugal) of <100 μm particle size. From 15 days post-fertilization (advanced larval stage) onwards, they were fed only microdiets of 100–200 μm particle size (Zebrafeed®, Sparos Lda, Olhão, Portugal).

At 30 days post-fertilization, the fish were transferred to a freshwater flow-through system (Zebtec Stand Alone Toxicological Rack, Tecniplast, Varese, Italy) with 3.5-L tanks. They were acclimatized on a commercial zebrafish diet (CZ) of 300 μm particle size. Fifty-day-old juvenile zebrafish (Kimmel et al., 1995) weighing 50–60 mg were used for the experiment. These fish were randomly distributed into 12 tanks (12 fish per tank). The water temperature in the tanks was 28°C, and the water flow rate was 2.5 L/h. The dissolved oxygen in the tanks ranged between 7 and 8 ppm (oxygen saturation above 85%). A 14L:10 D photoperiod was maintained throughout the 30-day feeding experiment.

2.2 | Diet preparation and feeding experiment

Sparos Lda. prepared the three diets that were fed to the experimental fish (Figure S1). The reference zebrafish diet containing high-quality marine protein served as the control. The control diet (CZ, control zebrafish diet) was formulated with a high level (59%) of premium-quality marine protein sources (fishmeal, fish protein hydrolysate, shrimp meal), and the primary lipid sources were fish oil and soya lecithin. The second diet (CP, plant-based diet) had 50% soya bean meal and 47% basal ingredients; this diet was intended to induce a pro-inflammatory effect. The CP diet also contained a higher level of saturated fat source and 3% cholesterol. The third diet (PM, plant-based diet containing MacroGard®) was identical to CP but was further supplemented with 1.66% of a cell wall baker's yeast (*Saccharomyces cerevisiae*) β-glucan (MacroGard®, Biorigin, Antwerp, Belgium). This inclusion level corresponded to a β-glucan supplementation of 1,000 mg/kg feed.

Diets were manufactured by SPAROS Lda. All powder ingredients were mixed and ground (<50 μm) in a micropulverizer hammer mill (SH1, Hosokawa-Alpine, Germany). Subsequently, the oils were added to the mixtures, which were humidified with 25% water and agglomerated by a low-shear cold extrusion process (ITALPLAST, Italy). The resulting pellets of 0.6 mm were dried in a convection oven for 4 hr at 45°C (OP 750-UF, LTE Scientifics, United Kingdom) and mechanically sieved to guarantee the desired particle size. Diets were refrigerated throughout the trial. Fish were fed daily at 5% body weight (offered manually as three rations at 08:00, 13:00 and 18:00), and each experimental diet was offered to fish held in 4 replicate tanks.

2.3 | Proximate analysis of the experimental diets

Analysis of the experimental feeds was carried out with analytical duplicates (Table 1), following, in most cases, the methodology described by AOAC (Williams, 1984). Dry matter was determined by drying at 105°C for 24 hr and total ash by combustion (550°C during 6 hr) in a muffle furnace (Nabertherm L9/11/B170, Germany).

TABLE 1 Proximate composition (dry matter basis, %) of the experimental diets

	CZ	CP	PM
Dry matter	92.76 ± 0.08	92.72 ± 0.06	92.73 ± 0.06
Protein	66.76 ± 0.05	54.89 ± 0.06	54.91 ± 0.04
Lipid	13.40 ± 0.05	13.54 ± 0.04	13.53 ± 0.05
Ash	13.14 ± 0.04	8.46 ± 0.05	8.49 ± 0.03
Energy (MJ/Kg)	21.44 ± 0.01	21.55 ± 0.02	21.58 ± 0.01

The crude protein (N × 6.25) was analysed by a flash combustion technique followed by a gas chromatographic separation and thermal conductivity detection with a Leco N analyzer (Model FP-528, Leco Corporation, USA). The evaluation of the crude lipid was done by petroleum ether extraction (40–60°C) using a Soxtec™ 2055 Fat Extraction System (Gerhardt, Germany) with prior acid hydrolysis with 8.3 M HCl, and the gross energy was determined in an adiabatic bomb calorimeter (Werke C2000, IKA, Germany).

2.4 | Sampling

At the end of the 30-day feeding trial, the fish were killed by immersing in a lethal dose of 200 mg/L of tricaine methane sulphate (Argent Chemical Laboratories) buffered with an equal amount of sodium bicarbonate. After recording the weight of each fish using Scout® STX weighing balance (OHAUS, Parsippany), they were dissected on cold plates to collect the distal intestine. The tissue obtained was frozen in finely powdered dry ice (−78.5°C) and then transferred to a −80°C freezer for storage until use. Distal intestine samples ($n = 4$) from each treatment group were used for the transcriptomic analysis. Similarly, 4 distal intestine samples ($n = 4$) were taken from each group for assessing the histomorphology.

2.5 | RNA extraction, library construction and sequencing

To extract total RNA, the frozen intestine samples were briefly homogenized in QIAzol lysis reagent (Qiagen, Crawley, UK) at 6,500 rpm for 2 × 20 s in a Precellys 24 homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France). RNA was extracted from the tissue homogenate using Direct-zol™ RNA MiniPrep (Zymoresearch) following the manufacturer's instructions. The RNA concentration, purity and quality were determined using NanoDrop™ 1,000 (Thermo Fisher Scientific) and Tape Station 2,200 (Agilent Technologies). RNA samples (RIN value >7) were used to construct RNA-Seq libraries. The preparation of libraries from total RNA was done using the NEBNext Ultra™ RNA Library Prep Kit (NE Biolabs) with the poly (A) mRNA magnetic isolation module following the manufacturer's protocol. Briefly, 1 μg of the total RNA was used for library preparation, and after Poly(A) enrichment, mRNA was fragmented to obtain fragments of 100–200 nt length. Next, we synthesized the first and second strand of cDNA, which was then purified, end-repaired and used for adaptor ligation followed by barcoding using NEBNext Multiplex Oligos (NE Biolabs). PCR enrichment was done with 9 cycles, and the amplified libraries were purified using AMPure XP beads (Beckman Coulter, Inc.). The barcoded libraries were then pooled and loaded at 1.4 pM on the Illumina NextSeq 500 sequencer (Illumina) with the NextSeq 500/550 High Output Kit (v2.5, 75 cycles) for 75-bp single-end sequencing at the Nord University genomics platform (Bodø, Norway).

2.6 | Bioinformatic analysis

Adapter sequences were trimmed from the raw reads using the fastp software (Chen et al., 2018) with default parameters. Further, the quality of the reads was assessed using the fastQC command line, and reads were filtered based on the Phred quality score (Q ≥ 30). They were then aligned to the reference zebrafish genome and transcriptome that were downloaded from NCBI (release 100) using HISAT2, version 2.2.1, which uses an indexed reference genome for alignment (Kim et al., 2015). The reads were annotated using featureCounts (Liao et al., 2014) to obtain the read counts that belong to each gene. Differential expression of the genes across the treatment groups was determined by DESeq2 (Love et al., 2014). Transcripts with $|\text{Log}_2$ fold change$| \geq 1$ (|fold change| ≥ 2) and an adjusted p-value (q-value) of <0.05 (Benjamini–Hochberg multiple test correction method) were considered significantly differentially expressed and used for gene ontology analysis. Enrichment of KEGG pathways and gene ontology was performed with the software DAVID (database for annotation, visualization and integrated discovery) version 6.8 (Jiao et al., 2012). The packages ggplot2, pheatmap, GOplot and enhancedVolcano in R were employed to visualize the data.

2.7 | Expression of selected genes-qPCR study

Selected differentially expressed genes from the transcriptome data were employed to confirm the reliability of the RNA-Seq data; their relevance is presented in Table S1. The same samples used for RNA-Seq ($n = 4$) were employed for qPCR-based verification, and reactions were run on triplicates. One μg of total RNA from each sample was reverse transcribed using the QuantiTect reverse transcription kit (Qiagen), according to the manufacturer's instructions. The cDNA was further diluted 10 times with nuclease-free water and used as a PCR template. The PCR was conducted using the SYBR green in LightCycler® 96 Real-Time PCR System (Roche Holding AG, Basel, Switzerland). We designed the primers for the selected genes using the Primer-BLAST tool in NCBI. The primers were then 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 in Table S2. Relative expression of selected genes was determined based on the geometric mean of reference genes (*eef1a* and *rpl13α*), and we employed the primers that were reported previously (Tang et al., 2007). The data were checked for assumptions of normality (Shapiro–Wilk) and homogeneity of variance

(Bartlett's test). Based on the outcome of the assumption check, the statistical difference was determined by Student *t*-test or Welch two-sample *t*-test for two group comparisons and analysis of variance (ANOVA) or Kruskal–Wallis test for 3 groups.

2.8 | Histological analysis

Distal intestinal samples ($n = 4$) from each group were fixed in 3.7% (w/v) phosphate-buffered formaldehyde solution (pH 7.2) at 4°C for 24 hr. Standard histological procedures were employed for dehydration, processing and paraffin embedding. The paraffin blocks thus prepared were sectioned using a microtome (Microm HM3555, MICROM International GmbH). Four-micrometre-thick longitudinal sections were cut and mounted on SuperFrost® slides (Menzel, Braunschweig, Germany), and a robot slide stainer (Microm HMS 760×, MICROM International GmbH) was used to stain the slides with Alcian Blue–Periodic Acid Schiff's reagent (AB-PAS, pH 2.5). First, all acid mucins were stained blue with Alcian blue, and in the subsequent PAS reaction, only the neutral mucins were stained magenta. Light microscopy photomicrographs were taken with the Olympus BX61/Camera Color View IIIu (Olympus Europa GmbH) and the photo program Cell P (Soft Imaging System GmbH). The ImageJ software was used for scoring the tissue microarchitecture. To understand the histopathological changes, we measured 5 parameters of the histological architecture of the intestine. Villi length, epithelium width, the width of lamina propria and goblet cell size were measured, and goblet cell numbers were counted from 4 fish per group (Figure S2). Statistical differences were identified using the independence test with exact distribution (Zeileis et al., 2008). Significant differences are reported for $p < 0.05$, while trends in differences are described for $p < 0.1$.

3 | RESULTS

3.1 | RNA sequencing data quality and statistics

Four hundred and twelve million raw reads were retrieved from 12 samples, and after adapter trimming and quality filtering, 409 M reads with a Phred quality score of Q ≥ 30 were obtained. Among these, 302 M reads were uniquely mapped to the zebrafish genome and 71 M reads were mapped to multiple locations in the genome. Overall, the mapping percentage of filtered reads was 91.1% (Table S3).

3.2 | Differentially expressed genes in the intestine of soya bean meal fed zebrafish

To understand the effect of dietary soya bean meal on zebrafish intestinal responses, we compared the intestinal transcriptome of the soya bean meal fed (CP) group with that of the control (CZ) group. We retrieved 73 differentially expressed genes, of which 29 were upregulated and 44 were downregulated (|Log2 fold-change| ≥ 1, *q*-value <0.05, Figure 1, Table S4). The principal component analysis plot reveals the differential clustering of the CP and CZ groups, and the first principal component (PC1) explained 72% variability in the data (Figure 2a). The volcano plot (Figure 2b) shows the separation of differentially expressed genes based on Log_2 fold changes.

Genes related to the immune response were differentially expressed in the CP group compared to the CZ group (Figure 1). *Nod-like receptors* (NLRs), namely, *nlrc3* and *nlrp12*, were upregulated in the CP group by 7.4- and 2.8-fold, respectively. GTPase genes like *adp-ribosylation factor-like 4Ca* (*arl4ca*), *rab25 member ras oncogene family* (*rab25b*) *and ras homolog family member Ub* (*rhoub*) were also upregulated. We found a striking downregulation of *gtpase imap family member 8-like* (*gimap8*), by 8.9-fold. Genes like *PR domain containing 1c with ZNF domain* (*prdm1c*) that are related to immune regulation were downregulated in the CP group.

Many genes involved in metabolic and transport functions were also differentially expressed in fish fed the CP diet. The lipid metabolism gene, *carnitine O-acetyltransferase a* (*crata*), was upregulated by 7.4-fold. Cytochrome P450 encoding genes, namely, *cyp2x10.2*, *cyp2aa2*, were upregulated by 4.2- and 3.8-fold, respectively. Similarly, fatty acid elongation gene *elovl fatty acid elongase 6* (*elovl6*) was upregulated by 3.6-fold, while *elovl fatty acid elongase 4* (*elovl4b*) was downregulated by 5-fold. $NAD(P)^+$-dependent enzyme *aldehyde dehydrogenase 3 family, member A2b* (*aldh3a2b*), *solute carrier family 51 alpha subunit* (*slc51a*) and ATP-dependent proteases genes like *LON peptidase N-terminal domain and ring finger* 1 (*lonrf1*), and *caseinolytic mitochondrial matrix peptidase chaperone subunit b* (*clpxb*) were upregulated, while *tryptophan hydroxylase-1a* (*tph1a*) and *glycerol-3-phosphate acyltransferase* (*gpat2*) were downregulated in the CP group. Barrier function-related genes, namely, *mucin 5AC* (*muc5ac*), *guanylin* (*guca2a*) and *clathrin light chain B* (*cltb*), were upregulated in the CP group, while *mucin 5d* (*muc5d*) and *chloride channel 2c* (*clcn2c*) were downregulated in the CP group. Interestingly, we also found cilia-related gene, namely, *dynein axonemal assembly factor 3* (*dnaaf3*) as the most upregulated gene with a 10.5-fold change. On the other hand, another cilia-related gene, *regulatory factor X 3* (*rfx3*), was downregulated in the CP group. Several genes such as *cohesin subunit* genes (*stag2*, *rad21l1*), *serine/threonine-protein kinase 31-like* (*stk31*), *spo11 initiator of meiotic double-stranded breaks* (*spo11*), *speedy/ringo cell cycle regulator family member A* (*spdya*), *tudor domain containing* (*tdrd6*, *tdrd9*) and *nucleic acid binding protein 1b* (*nabp1b*) that are related to cell cycle and DNA damage and genes related to reproduction, namely, *testis-specific 10* (*tsga10*), *spermatogenesis associated* (*spata6l*, *spata22*) and *zona pellucida glycoprotein 3d* (*zp3d.2*), were downregulated in the CP group.

3.3 | Gene ontology (GO) enrichment analysis based on differentially expressed genes in soya bean meal fed fish

The analysis revealed the enrichment of several GO terms (Figure 3a) in the soya bean-fed fish (CP). The differentially upregulated genes

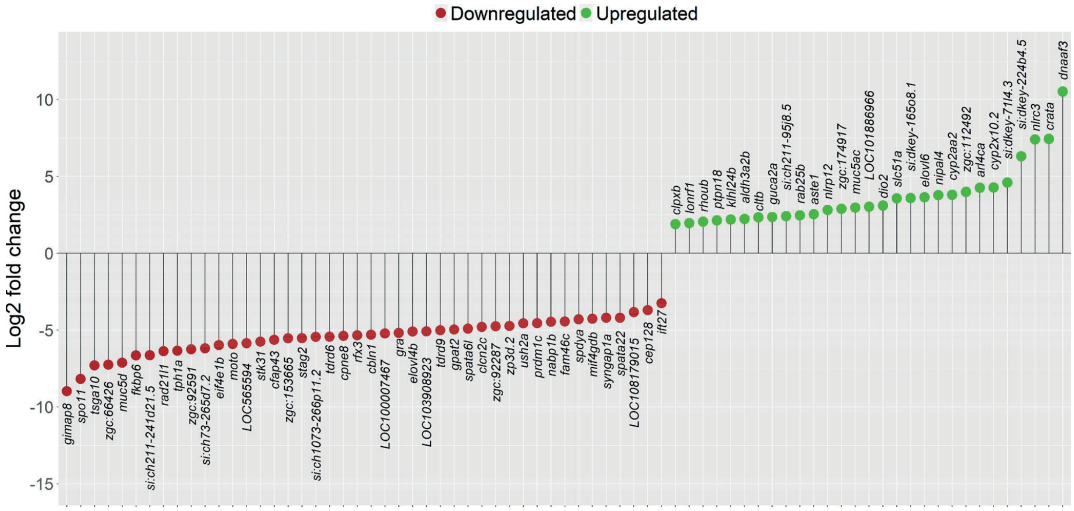


FIGURE 1 Differentially expressed genes in the intestine of the soya bean group (CP) compared to the control (CZ) group. An adjusted *p*-value below 0.05 and |Log2 fold change| ≥1 were employed in the function of DESeq2 (dots are plotted from left to right in the increasing order of Log2 fold change). Each treatment group consisted of four biological replicates

in the CP group caused the significant enrichment of oxidoreductase activity, ATP-dependent peptidase activity, oxidoreduction process and small GTPase-mediated signal transduction. On the other hand, the downregulated genes in the CP group caused the significant enrichment of GO terms such as reproductive process, cell cycle, nitrogen metabolism, DNA repair and metabolic process (Figure 3b).

3.4 | Inclusion of MacroGard® in soya bean meal-based feed altered the intestinal transcriptome in zebrafish

To analyse the effect of dietary glucan supplementation on zebrafish, we compared the data from the fish fed MacroGard® (PM) diet with those from fish fed the CP and the CZ diets. We identified 28 differentially expressed genes in the PM versus CP group: 9 were upregulated and 19 were downregulated (|Log2 fold-change| ≥ 1, *q*-value < 0.05, Figure 4, Table S5). Of these differentially expressed genes, 12 were uncharacterized. The PCA plot illustrates the separation of the PM and CP group along with PC1 (Figure 5a). Hierarchical clustering (Figure 5b), and the volcano plot (Figure S3) shows a clear separation of differentially expressed genes in the PM group compared to the CP group.

Among the upregulated genes in the PM versus CP group were neutrophil protease *elastase 2* (*ela2*), *actin-related protein 2/3 complex subunit 4 like* (*arpc4l*) and autophagy-related gene *receptor-interacting serine-threonine kinase 2* (*ripk2*). The most downregulated (by 8-fold) genes in the PM group was *chemokine C-C motif ligand 36* (*ccl36.1*). The other downregulated genes were anti-viral genes, namely, *sterile alpha motif domain-containing protein 9 like* (*samd9*) and *interferon-induced protein with tetratricopeptide repeats 9* (*ifit9*), metabolic gene *alpha* (*1,3*) *fucosyltransferase gene 2* (*ft2*), *iodothyronine deiodinase 2* (*dio2*), GTPase gene *ADP-ribosylation factor 4b (arf4b)* and *pleckstrin 2 (plek2)*.

Comparison of the PM and CZ groups revealed 23 differentially expressed genes, of which 8 were upregulated and 15 were downregulated (|Log2 fold-change| ≥ 1, *q*-value < 0.05, Figure 6, Table S6). Of these differentially expressed genes, 12 were uncharacterized. The principal component analysis plot reveals differential clustering of the PM and CZ groups; the first principal component (PC1) explained 61% variability in the data (Figure 7a). The volcano plot (Figure 7b) shows the separation of differentially expressed genes based on Log_2 fold changes. CP and PM versus CZ and CP versus PM comparisons revealed some common differentially expressed genes (Figures 4 and 6). The upregulated gene, *ifi30 lysosomal thiol reductase (ifi30)* (upregulated by 7.6-fold) and the downregulated genes, *gtpase imap family member 4-like* (*gimap4*), *coiled-coil domain-containing protein 134* (*ccdc134*), *interferon-induced very large gtpase 1 (vlig-1)* and *enoyl-acyl-carrier-protein reductase, mitochondrial-like* (*mecr*), were detected only by the PM versus CZ comparison.

3.5 | Gene ontology (GO) enrichment analysis based on differentially expressed genes in MacroGard® fed fish

The downregulated genes in the PM compared to the CP group revealed the significant enrichment of several GO terms like small

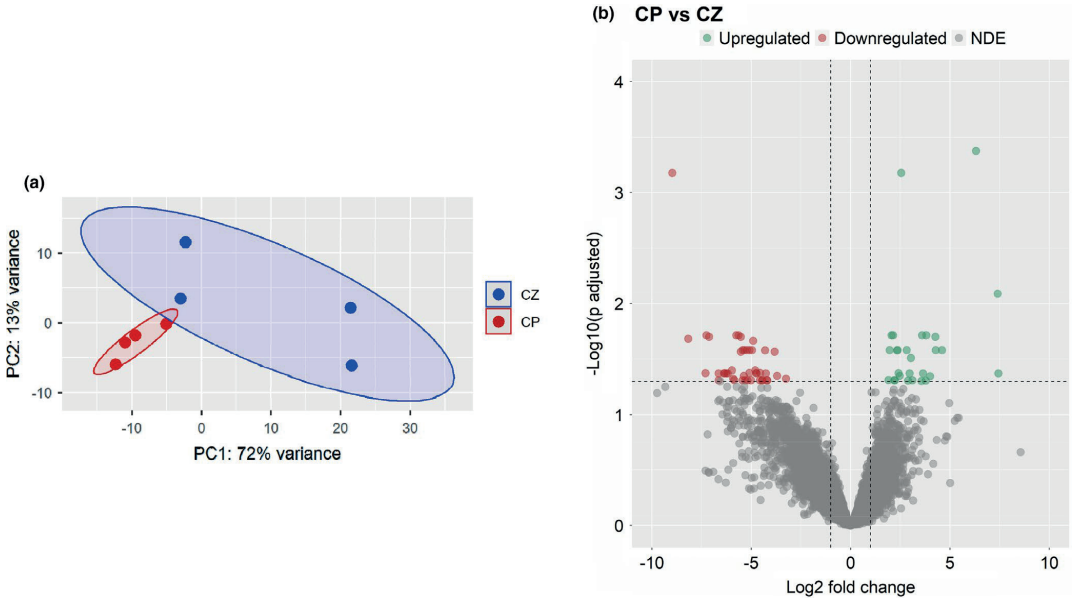


FIGURE 2 Intestinal transcriptome-based differences in the soya bean group (CP) compared to the control (CZ) group. Principal component analyses (a) and Volcano plot (b) of the differentially expressed genes in the CP group compared to the CZ group. Volcano plot shows the differentially expressed genes (red dots) with an adjusted *p*-value below 0.05 and |Log2 fold change| ≥1. Each treatment group consisted of four biological replicates

GTPase-mediated signal transduction, intracellular signal transduction, response to stimulus, carbohydrate derivative binding, GTP binding, guanyl ribonucleotide binding and guanyl nucleotide binding (Figure 8). GO term analysis did not reveal any enrichment based on the upregulated genes in the PM group. Five of the abovementioned GO terms (based on downregulated genes from PM versus CP groups) were also enriched by the downregulated genes from the PM versus CZ comparison, small GTPase-mediated signal transduction, GTP binding, guanyl ribonucleotide binding and guanyl nucleotide binding and intracellular cellular signal transduction (Figure S4). None of the differentially expressed genes caused a significant enrichment of KEGG pathways.

3.6 | Verification of transcriptomic data

We profiled the relative expression of 10 genes mentioned in the Table S1 (Figure 9). Overall, the expression of the selected genes was in agreement with transcriptomics data (Figure 10).

3.7 | Soya bean meal and MacroGard® inclusion altered the intestinal architecture in juvenile zebrafish

Statistical analysis of the goblet cell numbers revealed a decreasing trend ($p < 0.1$) in the CP group compared to the CZ group (Figure 11a, b). An apparent widening of the lamina propria could also be observed in the CP group compared to the CZ group, but the difference was not statistically significant (Figure 11a, b; $p > 0.05$). The width of lamina propria in the PM group was significantly reduced by 33.91% compared to the CP group. The CP group had shorter villi (based on a trend indicated by $p < 0.1$) compared to the PM group (47.2% reduction) (Figure 11a, b, Figure S5, Table S7).

4 | DISCUSSION

Soya bean meal is used as an inflammatory agent to develop intestinal inflammation models. Previous studies using zebrafish larvae have found that a 50% inclusion of soya bean meal in their diet can induce enteritis, characterized by changes in the expression of inflammatory genes and intestinal permeability (Hedrera et al., 2013; Solis et al., 2020). In the present study, we also used 50% dietary soya bean meal to understand the effect of soya bean-induced inflammation in zebrafish juveniles; to our knowledge, there are not many studies that reported the effect in juveniles. Here, we report that the responses in juvenile zebrafish towards soya bean meal are not as strong compared to responses in larvae, and acute inflammatory signals were not evident; we did not observe any changes in the expression of key inflammatory genes. Nevertheless, our study revealed the dietary soya bean-induced changes in the expression of several immune and metabolic genes. Furthermore, we

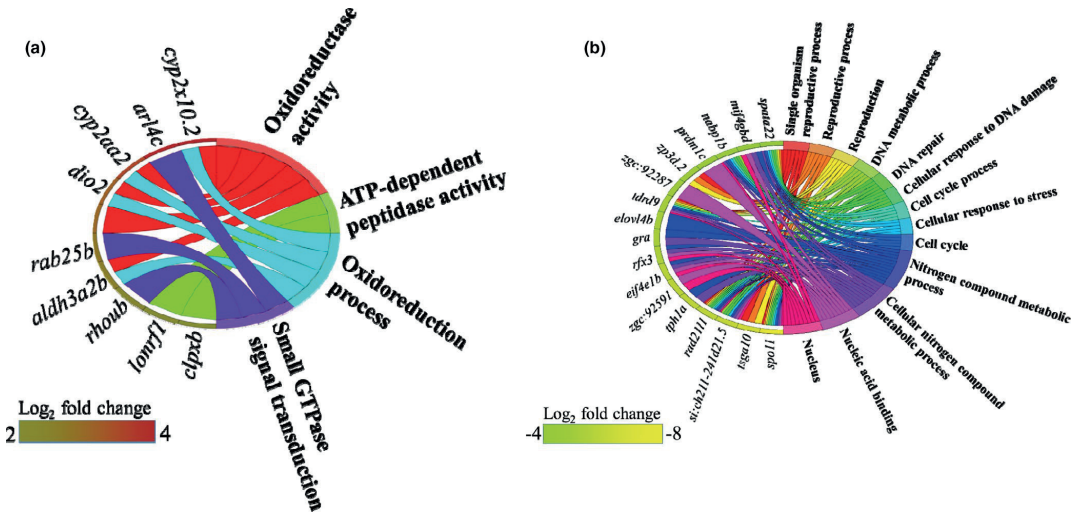


FIGURE 3 Chord diagram showing the link between the enriched GO terms in the soya bean (CP) group and the associated genes. The genes were upregulated (a) and downregulated (b) in the CP group compared to the control (CZ) group. The enriched GO terms are colour-coded, and differentially expressed genes contributing to this enrichment are shown on the left of the circle. The gradient colour bar intensity varies with the Log2 fold change (adjusted p-value < 0.05 and |Log2 fold change| ≥1)

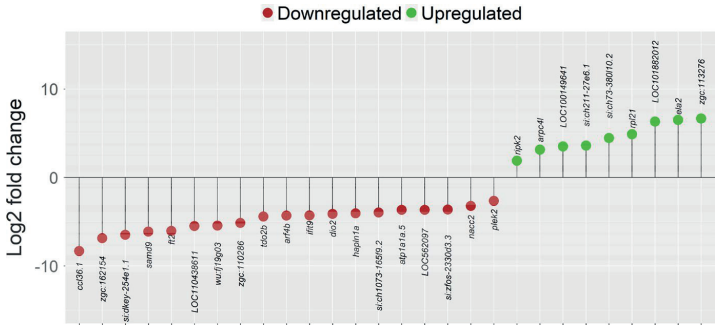


FIGURE 4 Differentially expressed genes in the intestine of the MacroGard® group (PM) compared to the soya bean group (CP). An adjusted p-value below 0.05 and |Log2 fold change| ≥1 were employed to generate the results (dots are plotted from left to right in the increasing order of Log2 fold change). Each treatment group consisted of four biological replicates

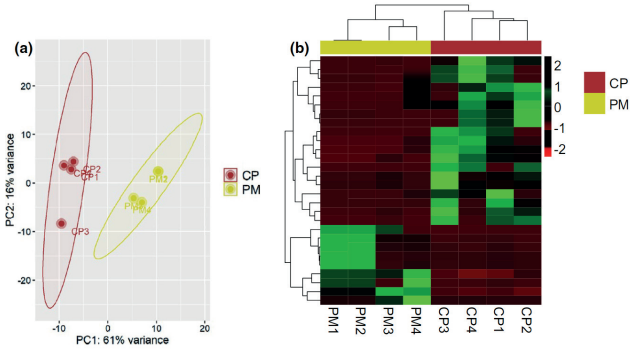


FIGURE 5 Intestinal transcriptome-based differences in the MacroGard® group (PM) compared to the soya bean group (CP). Principal component analyses (a) and heatmap (b) of differentially expressed genes in the PM group compared to the CP group. Each treatment group consisted of four biological replicates

investigated the intestinal transcriptomic response involved in counteracting the soya bean-induced alterations in zebrafish juveniles fed MacroGard®. We first compared the intestine transcriptome of the fish fed soya bean diet (CP) with those fed a commercial feed (CZ); the altered genes were related to, among others, oxidative processes, small GTPase, ATP-dependent proteases, DNA repair and cell cycle and the enriched GO terms included oxidoreductase activity, oxidoreduction process and small GTPase signal transduction, reproductive process, cell cycle, DNA repair and metabolic process. The comparison of intestinal transcriptome of the fish fed soya bean diet (CP) with those fed a MacroGard®-soya bean mixture (PM) revealed the changes that can be attributed to the barrier maintenance effect of the product. The inclusion of MacroGard® in the soya bean diet altered the expression of several barrier and immune related genes and the enriched GO terms were small GTPase-mediated signal transduction, intracellular signal transduction, response to stimulus and carbohydrate derivative binding.

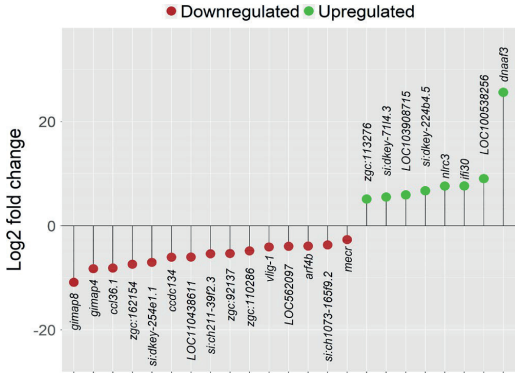
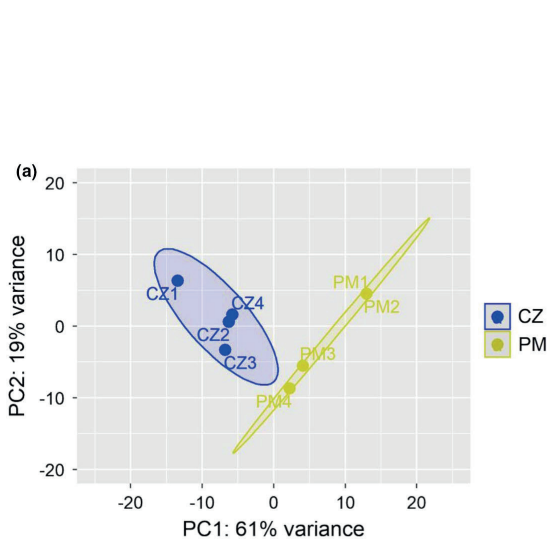


FIGURE 6 Differentially expressed genes in the intestine of the MacroGard® group (PM) compared to the control group (CZ). An adjusted *p*-value below 0.05 and |Log2 fold change| ≥ 1 were employed to generate the results (dots are plotted from left to right in the increasing order of Log2 fold change). Each treatment group consisted of four biological replicates

Ras proteins belong to a prominent family of small GTPases. These proteins are activated by extracellular stimuli and feeding soya bean meal to zebrafish upregulated genes such as *rhoub*, *rab25b* and *arl4ca* that are members of small GTPases. The former two genes are known to control epithelial cell polarity (Krishnan et al., 2013; Van Aelst & Symons, 2002). Furthermore, *rab25b* can regulate the expression of claudins, the epithelial tight junction proteins (Krishnan et al., 2013) and influence protein trafficking (Kessler et al., 2012). Overexpression of *rab25* is associated with microtubule-dependent transformation and integrin-dependent focal adhesion disruption (Lapierre et al., 2011). Overexpression of *rhoub* also disturbs epithelial focal adhesion and tight junctions (Chuang et al., 2007). The gene *rho* was upregulated in the intestine of salmon fed a plant-based feed (Johny et al., 2020). In addition, induction of *arl4ca* is known to stimulate intestinal epithelial tubule formation (Matsumoto et al., 2014). Furthermore, higher expression of *arl4ca* can enhance cellular



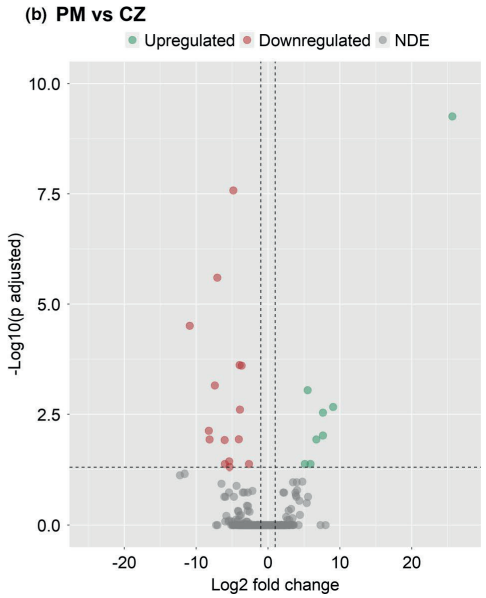


FIGURE 7 Intestinal transcriptome-based differences in the MacroGard® group (PM) compared to the control (CZ) group. Principal component analyses (a) and Volcano plot (b) of the differentially expressed genes in the PM group compared to the CZ group. Volcano plot shows the differentially expressed genes (red dots) with an adjusted *p*-value below 0.05 and |Log2 fold change| ≥1. Each treatment group consisted of four biological replicates

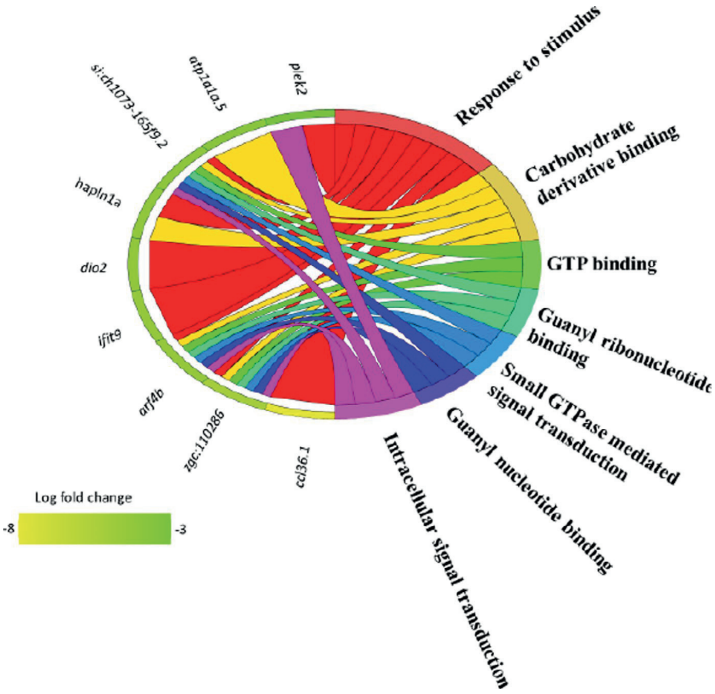


FIGURE 8 Chord diagram showing the link between the enriched GO terms in the MacroGard® group and the associated genes. The genes were downregulated in the PM group compared to the soya bean (CP) group. The enriched GO terms are colour-coded, and differentially expressed genes contributing to this enrichment are shown on the left of the circle. The gradient colour bar intensity varies with the Log2 fold change (adjusted *p*-value < 0.05 and |Log2 fold change| ≥1)

migration and progression of colorectal tumours (Fujii et al., 2015). Moreover, small GTPase alterations are linked to barrier function defects caused by intestinal epithelial and endothelial cytoskeletal rearrangement (López-Posadas et al., 2017). Specific functional studies are needed to investigate the role of small GTPases in maintaining the intestinal barrier in zebrafish.

Several other genes namely *muc5ac*, *muc5d*, *clcn2c* and *cltb* that are regulators of intestinal barrier function were altered in the soya bean fed group. Mucins are glycoproteins present in mucus, and gel-forming mucins, namely, *muc5ac*-like and *muc5d* (*muc2*-like), were significantly altered in zebrafish fed soya bean. During intestinal inflammation in humans, *muc2* was downregulated because of apoptotic defects (Wibowo et al., 2019). An upregulation of *muc5ac* in the intestine cells of humans by food-derived peptides has been reported previously (Martínez-Maqueda et al., 2012). In our study, *muc-5ac-like* (2.9-fold) was upregulated, and *muc5d* (7-fold) was downregulated in the CP group. A similar downregulation of *muc2-like* and upregulation of *muc-5b like* in the intestine of salmon fed wheat gluten (30%) has been reported earlier (Johny et al., 2020). In addition, *muc2* was downregulated in Atlantic salmon fed 20% soya bean meal (Sørensen et al., 2021). Further research is required to conclusively establish the response of mucin genes to plant-based diets. The *clcn2c* gene codes for a voltage-gated chloride channel, which plays a critical role in preserving the intestinal barrier. The protein, Clcn2c, is found near tight junctions, and its deficiency has been associated with increased endocytosis of occludins (Nighot & Blikslager, 2012), which enhances the severity of DSS-induced colitis and intestinal paracellular permeability (Ye et al., 2010). *clcn2* was downregulated in the intestine of Atlantic salmon fed soya bean products (Kiron et al., 2020) as observed in the present study. Furthermore, the observed upregulation of *cltb* is known to promote clathrin-mediated endocytosis of both adherens and tight junction proteins in intestinal epithelial cells (Ivanov et al., 2004). The expression of *cltb* increased during exposure to pathogens, inflammatory cytokines and dietary soya bean (Fukumatsu et al., 2012; Król et al., 2016; Utech et al., 2010). Therefore, the altered expression of *muc5ac*, *muc5d*, *clcn2c* and *cltb* in the CP group probably indicates aberrant barrier integrity in the intestine of zebrafish fed soya bean diet.

Changes in the barrier function are often linked to abnormalities in the paracellular or transcellular transport of ions. The genes, *guca2a*, *dnaaf3* and *rfx3* were altered in the soya bean fed group. The intestinal natriuretic peptide, *guca2a*, secreted into the lumen binds to *guanylate cyclase-C* (*gc-c*) receptor and inhibits sodium and water absorption and increases bicarbonate and chloride secretion via cGMP-dependent signalling pathways (Nakazato, 2001). The peptide also acts as a second messenger that affects smooth muscle contractibility (Ochiai et al., 1997). Reduced expression of *guca2a* and *gc-c* is known to damage intestinal epithelium and increase inflammation (Waldman & Camilleri, 2018) but increased expression of *gc-c* results in diarrhoea in humans (Fiskerstrand et al., 2012). Our finding of increased expression of *guca2a* corroborates the result linked to saponin feeding in salmon (Kortner et al., 2012). The gene, *dnaaf3*, encodes for an axonemal cytoskeletal motor protein of cilia, and *rfx3*

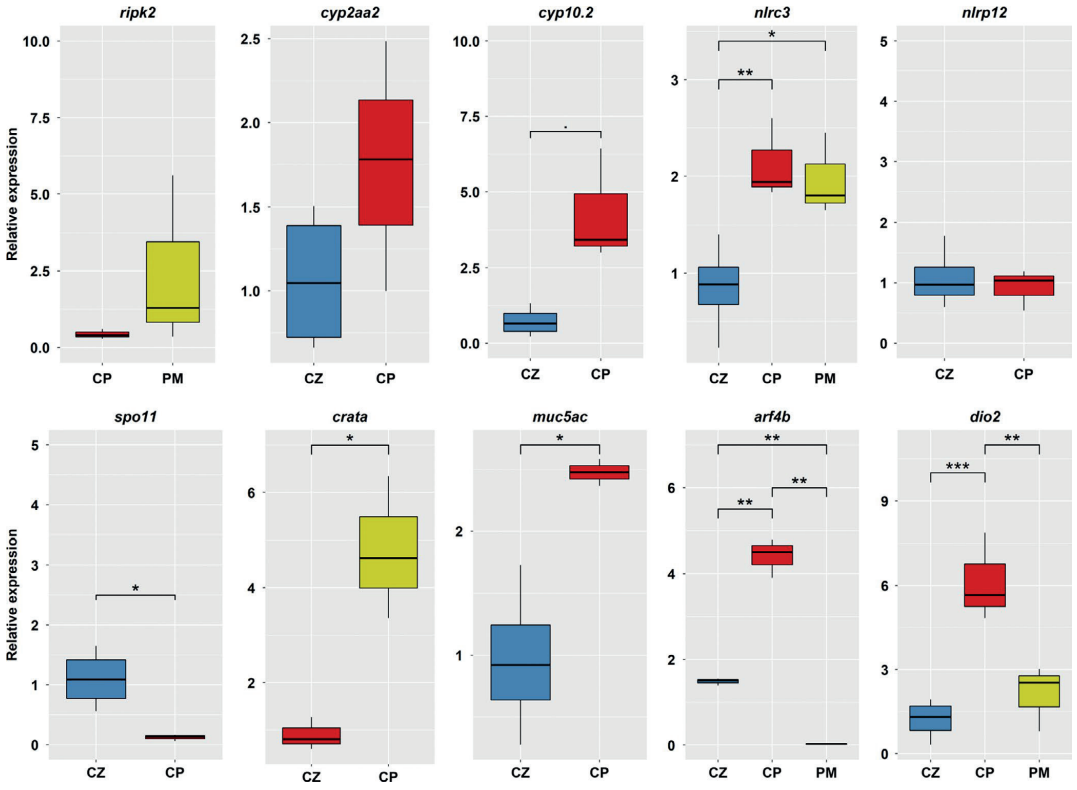


FIGURE 9 Relative gene expression of 10 selected genes in the intestine of the zebrafish fed control (CZ), soya bean (CP) and MacroGard® (PM) diets ($n = 4$). Asterisks *** indicate $p < 0.001$, ** indicate $p < 0.01$, * indicates $p < 0.05$, and • indicates $p < 0.1$

is a transcriptional factor that regulates the expression of axonemal dyneins; both these genes are involved in the motility of cilia (El Zein et al., 2009). Abnormality of *dnaaf3 and rfx3* is linked to dyskinesia (Mitchison et al., 2012), and abdominal wall dyskinesia is associated with uncontrolled motility of the muscle (Gupta & Kushwaha, 2017). Although a direct connection between these genes and intestinal functions has not been confirmed yet, intestinal motility increases during dietary cholesterol-induced alteration in zebrafish (Progatzky et al., 2014). In the present study, CP group contains 3% cholesterol, and cholesterol accumulation in immune cells is known to be a cause of intestinal inflammation (Progatzky et al., 2014).

Inflammation is associated with changes in the expression of key inflammatory genes like interleukins and other cytokines as observed in chemical-induced inflammation in adult zebrafish (Brugman et al., 2009; Geiger et al., 2013). However, we did not observe dietary soya bean-induced changes in the expression of inflammatory cytokines. In our study, the expression of several immune-related genes like *nlrc3*, *nlp12*, *gimap8*, *tph1a and prdm1c* were altered in the soya bean fed group. NOD-like receptor (NLRs) genes like *nlrc3* and *nlrp12* that are localized in immune cells and are known to inhibit inflammatory cytokine production (Allen, 2014) were upregulated in the CP group, indicating an attempt of zebrafish to cope with the soya bean-induced alterations. The gene *gimap8* was found to correlate with the recirculating B-cell numbers, and deletion of *gimap* can delay the apoptosis of mature T cells (Filén & Lahesmaa, 2010). In the present study, we observed an 8-fold reduction in *gimap8* in fish-fed soya bean diet. Another study also pointed out the downregulation of *gimap8*; in soya bean meal fed yellow perch (Megan Marie Kemski, 2018). On the other hand, in Atlantic salmon a 3-day feeding of soya bean meal diet led to alteration of two other *gimap* genes: *gimap4* (downregulated) and *gimap7* (upregulated) (Sahlmann et al., 2013). Another gene, *prdm1* (*blimp-1*), that is known to affect the development and differentiation of T and B cells was downregulated in the CP group. Deletion of *prdm1* in mice resulted in the loss of T regulatory cell functions (Ogawa et al., 2018) and progression of chronic intestinal inflammation (Salehi et al., 2012). The gene, *tph1*, is a mast cell-derived immune tolerance factor that regulates inflammatory response in the intestine (Nowak et al., 2012). While dietary administration of a probiotic *L. rhamnosus* increased the expression of *tph1a* in the gut

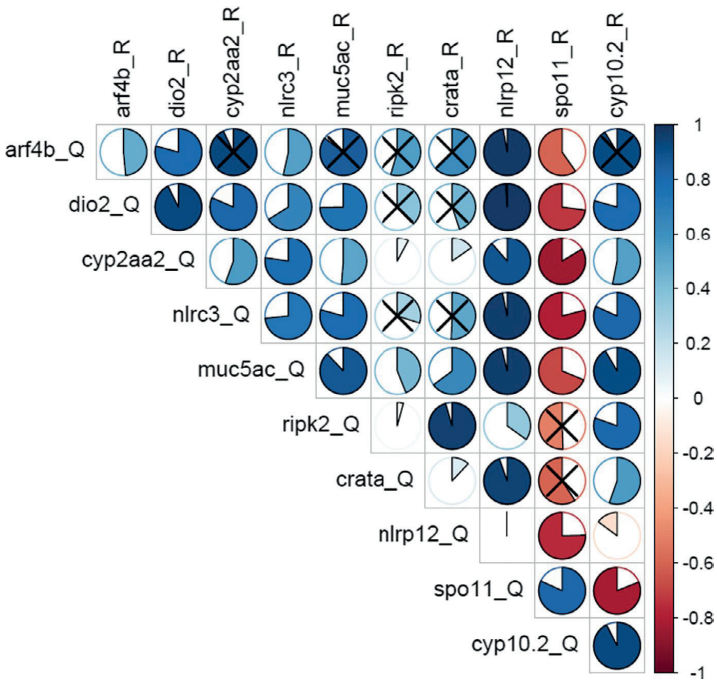


FIGURE 10 Correlation between the normalized counts from the RNA-Seq data and gene expression values from the qPCR data

of zebrafish (Borrelli et al., 2016), soya bean feeding, in our study, downregulated *tph1a*.

Soya bean feeding caused an enrichment of oxidoreductase activity driven by the upregulation of *cyp2x10.2*, *cyp2aa2*, *dio2 and aldh3a2b*. Plant-based feeds can induce reactive oxygen species, ROS (Zhang et al., 2020), and *cyp* is involved in the production of ROS (Veith & Moorthy, 2018). The increased expression of the *aldh3a2b* gene also implies oxidative stress (Singh et al., 2013). Diet-induced oxidative stress brought about by terrestrial plant ingredients has been reported frequently in teleosts (Olsvik et al., 2011). However, the extent to which an organism copes with stress is largely dependent upon its inherent ability to produce antioxidant enzymes (Birnie-Gauvin et al., 2017). An increase in ROS negatively affects the protein-folding capacity of mitochondria resulting in an accumulation of misfolded or misassembled proteins (Scherz-Shouval & Elazar, 2007). ATP-dependent proteases participate in mitochondrial protein remodelling, folding and degradation to maintain organelle homeostasis (Baker et al., 2011). The enrichment of ATP-dependent peptidase activity by the upregulated mitochondrial matrix genes, namely, *lonrf1* and *clpxb*, which are involved in the degradation of misfolded or damaged proteins (Baker et al., 2011), probably indicates an effect of dietary soya bean on the mitochondrial protein-folding environment. Furthermore, mitochondrial dysfunction during DSS-induced intestinal inflammation has been reported previously (McQueen et al., 2019) and mitochondrial dysfunction has been associated with the development of inflammation (Novak & Mollen, 2015).

Soya bean products have also been shown to be potent arrestors of the cell cycle machinery, and this ability is being investigated to promote their use as anticancer agents (Zhang et al., 2013). Dysregulation of the intestinal cell cycle has consequences for intestinal homeostasis (McKernan & Egan, 2015). Dietary change-induced cell cycle alterations in the intestine of zebrafish have been previously reported (Peyric et al., 2013). Several genes such as, *spo11*, *rad21l1*, *nabp1b and spata22* that are related to cell cycle were downregulated in the CP group. The downregulation of the genes related to the cell cycle is likely to be a consequence of exposure to bioactive compounds present in the soya bean meal. Chronic DSS mice inflammation model has revealed the associated reduction in the intestinal villi height, but the authors have not attributed the aberration to cell cycle but to enterocyte apoptosis (Parker et al., 2019). Some of the genes that were associated with the enriched GO term cell cycle were also associated with another GO term DNA repair due to the significantly downregulated genes *spata22*, *spo11*, *rad21l1*, *tdrd9 and nabp1b*. DNA integrity is preserved by cells through the activation of an evolutionarily conserved network known as DNA damage response (DDR), and DDR in a cell is activated to repair lesions in DNA and to ensure genome stability (Campos & Clemente-Blanco, 2020). The cellular responses are also affected by oxidative stress (Barzilai & Yamamoto, 2004). The increase of oxidoreductase activity and oxidoreduction processes by the upregulated genes in the CP group might be pointing to oxidative stress as mentioned previously. This endogenous factor can jeopardize genome stability (Campos & Clemente-Blanco, 2020). Furthermore, the knockdown

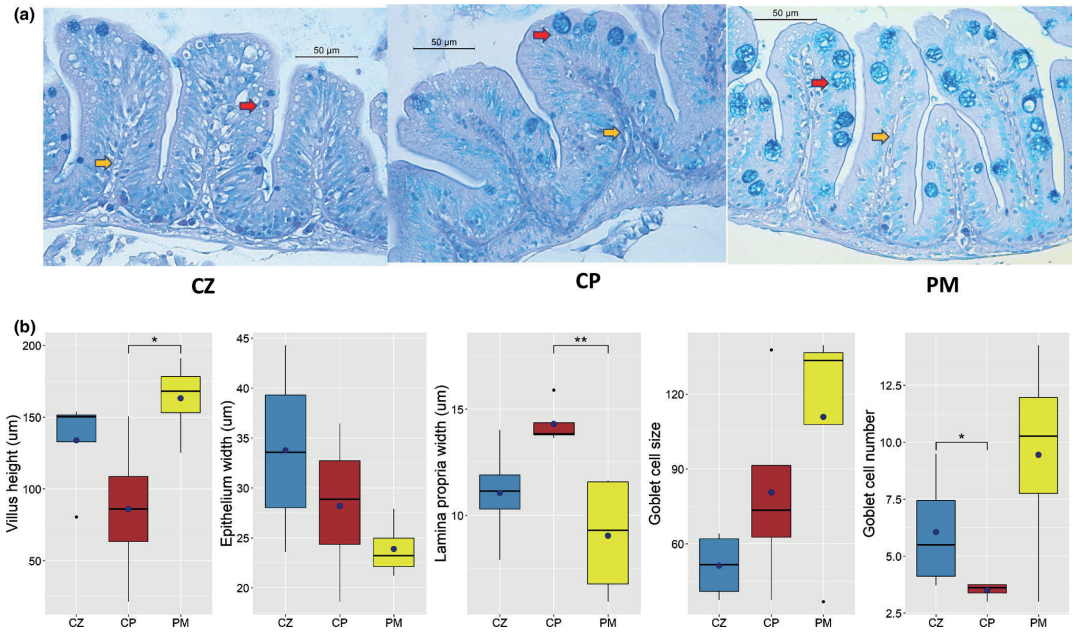


FIGURE 11 Differences in micromorphology of the distal intestine of zebrafish fed different feeds. Representative histological images (a) and box plots (b) showing the changes in the tissue architecture of the distal intestine of zebrafish stained with AB-PAS. The control group (CZ) has more absorptive vacuoles. MacroGard® group (PM) has a narrower lamina propria compared to the soya bean group (CP). Goblet cells (red arrow) and lamina propria (yellow arrow) are shown in image (a). Scale bar = 50 µm

of *tdrd9* promotes DNA double-strand break (DSB) in tumour positive cell lines (Guijo et al., 2017), while Spo11 is the catalytic unit of meiotic DSB formation (Keeney, 2008). On the other hand, the genes *rad21*, *spata 22* and *nabp1* are essential for DSB repair (Boucher et al., 2015; Hays et al., 2017; Xu et al., 2010). Therefore, the alteration in the expression of genes related to cell cycle, DNA damage and DNA repair machinery by soya bean meal in zebrafish is worth further investigation.

Lipid metabolism in the intestine is a complex process that involves the absorption of lipid species into the enterocytes and their ensuing transport. Besides, the energy demands of enterocytes are heavily dependent on fatty acid (FA) oxidation. The gene *crata* is involved in the transfer of FAs from the cytoplasm to mitochondria for β oxidation. Short- and long-term studies in rats have revealed that dietary soy proteins can cause a sustained reduction in the insulin: glucagon ratio (Tovar et al., 2002). This reduction has been further linked to increased β oxidation through increased expression of *crata* gene (De Santis et al., 2015). Soya bean meal also affects the absorption of lipids in the intestine through alteration of the bile physiology in fish (Murashita et al., 2018). The high affinity of the amino acids of soya protein to bile acids prevents the reabsorption of the latter (Choi et al., 2002). We observed an upregulation of *slc51a* in the CP group, and the solute carrier is essential for intestinal bile acid absorption (Ballatori et al., 2013). Glycerol-3-phosphate acyltransferase (*gpat*) is the first rate-limiting enzyme involved in the de novo biosynthesis of the glycerolipid pathway. In an in vitro study, soya isoflavone downregulated *gpat* in HepG2 cell line (Shin et al., 2007), whereas intraperitoneal injection of the same product upregulated *gpat* in the liver of rainbow trout (Cleveland & Manor, 2015). We observed a downregulation (by 4.9-fold) of *gpat2* in the CP group. ELOVL family of fatty acid elongases catalyses the formation of long-chain fatty acids; *elovl4* can catalyse the synthesis of both very-long-chain saturated fatty acids and very-long-chain polyunsaturated fatty acids (VLC-PUFA, with chain lengths ≥28 carbons) (Deák et al., 2019). *elovl4* elongates PUFAs, and higher intake of LC-PUFA has an inverse relationship with the IBD onset (Ananthakrishnan et al., 2014). On the other hand, *elovl6* is known to promote high fat diet-induced inflammation (Matsuzaka et al., 2012) and enhance macrophage recruitment to lipid deposits in aortic blood vessel in mice (Saito et al., 2011). A previous study has also indicated an upregulation of *elovl6* in the intestine of fish-fed soya bean (Kemski et al., 2020).

Soya bean-derived bioactive compounds resemble the structure of endogenous oestrogens, which can bind to oestrogen receptors present in a wide range of tissues (Dahlman-Wright et al., 2006). Several downregulated genes (*tsga10*, *spata22*, *tdrd9*, *zp3d.2a*) in the CP group caused a significant enrichment of the reproductive process-related GO terms. Previous studies have reported an effect of soya bean meal on reproductive performance and endocrine

disruption in fishes (Bennetau-Pelissero et al., 2001; Ng et al., 2006). The downregulation of genes related to the reproductive process in the CP group indicates possible effects on the reproductive performance of zebrafish. Zebrafish become sexually mature around 90 dpf, and when our experiment was terminated, the fish were 80 dpf. It should be noted that the effect of soya bean feeding on reproductive performance was not the main objective of our study. Nevertheless, we assume that soya bean feeding might have suppressed some processes due to the alteration of the associated genes; during the experimental period, the fish may have entered the reproductive developmental phase.

After understanding the effects of soya bean on intestinal tissue, we delineated the effectiveness of MacroGard® in counteracting the adverse effects of soya bean, for example barrier function disruption. We compared the MacroGard® fed group (PM) with both the CP and CZ groups. Among the many natural anti-inflammatory substances, MacroGard® effectively suppresses inflammatory cytokines and maintains mucosal barrier integrity and gut microbiota balance (Bacha et al., 2017; Carballo et al., 2019).

Soya bean feeding for 30 days did not alter the inflammatory cytokines in zebrafish. On the other hand, certain genes that could affect barrier functions were altered in zebrafish fed soya bean. Based on the observed alterations of genes in the intestine of zebrafish fed soya bean meal, we presume that the ingredient is likely to affect the barrier integrity in the fish. Intestinal disorders are accompanied by increased intestinal barrier permeability which facilitates microbes to cross the protective mucosal layer resulting in dysbiosis (Tsuboi et al., 2015). The gene *ripk2* is known to promote autophagy (Lupfer et al., 2013), and overexpression of *ripk2* was correlated with increased ability of cells to resist viral and bacterial infections (Zou et al., 2016). We have observed an upregulation of *ripk2* in the PM group. In the epithelial cells, actin filament turnover is essential for adherens and tight junction assembly, which in turn regulates the barrier integrity. The gene *actin-related protein (Arp) 2/3 complex (arpc4l)*, which is known to nucleate the branches of actin filaments (Amann & Pollard, 2001), was upregulated in the PM group. Furthermore, *arp2/3* complex deficiency is associated with cellular abnormalities and more susceptibility to inflammation (Kahr et al., 2017). Although it is believed that the paralogs of *arf4* are present in the actin filaments, the localization and functions of *arf4b* are still unknown (Marwaha et al., 2019). Hence, we are unable to explain the downregulation of *arf4b* in the PM group.

Chemokines are low molecular weight proteins that guide specific leukocytes like macrophages or neutrophils to appropriate sites during intestinal dysfunction (Wang et al., 2009). In the PM group, the chemokine motif ligand gene *ccl36.1* was the most downregulated gene. There are 2 duplicates for *ccl36* in zebrafish, with no orthologues of the gene found in human and mouse genomes. Furthermore, the specific role of *ccl36.1* has not been reported yet. *ccl36.1* was downregulated in flounder embryonic cells infected with viral haemorrhagic septicaemia virus (Hwang et al., 2021). The gene *ela2* encodes for serine protease in neutrophil and monocyte granules; the inflammatory condition is associated with an upregulation of the gene and alteration of the serine protease can impair barrier integrity (Pham, 2008). On the other hand, the proteolytic processing of chemokines by neutrophil elastase can decrease the activity of chemokines (Ryu et al., 2005; Valenzuela-Fernández et al., 2002), compromising their chemotactic action on immune cells. β-glucan can affect the chemotactic migration of leukocytes and the activity of cytokines (LeBlanc et al., 2006) and the alteration in the expression of these genes by β-glucan suggests a homeostatic role in modulating the inflammatory response in the tissue.

Several genes like *ft2*, *dio2*, *plek2*, *ifit9 and samd9* were downregulated in the PM group. Although the main function of the oxidoreductase selenoenzyme, *dio2*, is to catalyse the conversion of T4 to T3 (activated form of thyroid hormone), the gene plays a significant role in monocyte and macrophage functions via cytokine secretion (Van der Spek et al., 2020). An increased expression of *dio2* was observed both in murine liver and murine macrophages after an inflammatory stimulus (Kwakkel et al., 2014). The expression of *dio2* was downregulated in the PM group, while a comparison of CP versus CZ groups revealed the upregulation of the gene in the CP group, indicating the efficacy of dietary β-glucan in counteracting the soya bean induced response. *Pleckstrin* (*plek2*) is involved in the pro-inflammatory cytokine secretion and the phosphorylation of this protein increases proinflammatory cytokine secretion by macrophages in diabetes mellitus patients (Ding et al., 2007). Furthermore, knockdown of *plek2* resulted in decreased cytokine production in the human macrophage (Ding et al., 2007). The gene *ft2* (*fut2*) encodes the enzyme α(1,3) fucosyltransferase that catalyses the addition of terminal α(1,2)-fucose residues on intestine epithelial cells, and these residues act both as an attachment site and carbon source for intestinal bacteria. Knockdown of *fut2* in the intestine epithelial cell line of piglets decreased the adhesion of *E.coli* (Wu et al., 2018). *Interferon induced protein with tetratricopeptide repeats* (*ifit*) and the cytoplasmic protein *sterile alpha motif domain-containing protein 9-like* (*samd9*) are stimulated after virus exposure. The gene *ifit* was upregulated in the inflamed tissues of inflammatory bowel disease patients (Andreou et al., 2020; Niess et al., 2015). The expression of *samd9* is positively regulated by inflammatory cytokines IFN-γ and TNF-α (Hershkovitz et al., 2011).

Among the differentially expressed genes, we detected some common genes in the CP versus CZ and PM versus CZ comparisons; this similarity could be because of the inclusion (50%) of soya bean meal in the two diets (CP and PM). *ifi30 lysosomal thiol reductase* (*ifi30/gilt*) that is expressed on antigen-presenting cells either facilitates major histocompatibility (MHC) class-II restricted-antigen presentation via the reduction of disulphide bonds in the antigen or enables cross presentation (Hastings, 2013). Furthermore, β-glucan is recognized by the immune receptors present on the antigen-presenting cells, causing an increased expression of MHC-II (Sonck et al., 2011). The upregulation of *ifi30* in the PM group may partly be explained by the recognition and processing of β-glucan by antigen-presenting cells in the intestine. Although the expression of *vlig-1* is induced by interferons, the functional significance of its downregulation is yet to be revealed (Haque et al., 2021). The gene *ccdc134*

serves as an immune cytokine promoting CD8(+) T-cell activation (Huang et al., 2014), and hence, the downregulated expression of *ccdc134* in the PM group suggests an immunomodulatory response following β-glucan feeding.

The abovementioned alterations of genes by MacroGard® likely point to the immunomodulatory and barrier safeguarding effects of the additive. Furthermore, GO terms namely GTP binding and small GTPase mediated signal transduction were suppressed because of the downregulation of certain genes in the PM group. On the other hand, small GTPase signal transduction was enriched by the upregulated genes in the CP versus CZ comparison. These two results probably indicate the impact of soya bean feeding on the barrier function and the counteracting effect of MacroGard® through intestinal barrier function modification during soya bean meal feeding. Widening of the lamina propria is a hallmark of inflammation (Brugman, 2016), and a manifestation of compromised intestine barrier function (Laukoetter et al., 2008). Although there was a widening of lamina propria in the CP group compared to CZ, this difference was not statistically significant. Ingestion of 50% soya bean meal by zebrafish larvae also did not induce any changes in the intestinal morphology (Hedrera et al., 2013; Solis et al., 2020). On the other hand, dietary soya bean meal at 20% inclusion led to the development of intestinal inflammation in chinook salmon, Atlantic salmon (Booman et al., 2018) and common carp (Urán et al., 2008). The gene expression analysis in the present study indicated an aberrated barrier function due to alteration of *arl4ca*, *rab25b*, *rhoub*, *clcn2c*, *cltb* and *guca2a*. Furthermore, our results indicated fewer goblet cells in the CP group, suggesting a dysregulated mucus production (Brugman et al., 2009). This reduction in the goblet cell number can be linked to the alteration of the mucin genes *muc5ac* and *muc5d* in the CP group. It is noteworthy that MacroGard® feeding significantly reduced the lamina propria width in zebrafish juveniles compared to those fed CP diet, indicating a positive effect of β-glucan on the intestinal barrier (Liu et al., 2015).

Intestinal inflammation in zebrafish is often linked to a shift in microbial communities (Brugman et al., 2009). Furthermore, it has been observed that zebrafish intestinal microbiota changes in terms of diversity and composition from larval to the juvenile stage (Stephens et al., 2016). Therefore, it may be speculated that the resistance to soya bean-induced inflammation may be due to either a specific microbiota profile or mature adaptive immune system of the juvenile zebrafish compared to the larvae. Hence, future studies can investigate how exposure to dietary soya bean meal influences the juvenile zebrafish intestinal microbiota. Furthermore, functional studies are required to establish the mechanisms that may ultimately explain the role of the functional adaptive immune system and/or microbiota in countering inflammation in juvenile zebrafish.

5 | CONCLUSION

The intestinal transcriptome of juvenile zebrafish fed soya bean-based feed revealed the modulation of several genes related to barrier function, oxidative stress, mitochondrial protein folding, cell cycle, DNA damage and DNA repair. Furthermore, dietary soya bean meal affected the expression of several immune and reproductive genes. Although 30-day feeding of 50% soya bean meal did not alter the expression of key pro-inflammatory genes, mild inflammatory signals were evident in zebrafish. Nevertheless, MacroGard® in the soya bean-based feed indicated a positive effect on the mucosal barrier with the histomorphological changes supporting the gene expression results. Since we observed only subtle inflammatory responses of soya bean feeding, future studies should employ higher incorporation levels of soya bean meal or consider using antinutritional factors like saponin to induce inflammation in zebrafish juveniles. In addition, the reason behind the considerable resistance of juvenile zebrafish to soya bean-induced enteritis needs to be verified.

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CONFLICT OF INTEREST

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

AUTHOR CONTRIBUTIONS

VK, JD and SR designed the study. JD prepared feeds for the experiment. SR and AG performed the feeding experiment. VK and JF provided reagents and materials for the experiments. SR, AG and PS performed the laboratory work. AG and SR performed the bioinformatic analysis. SR and VK wrote the manuscript. All authors read, revised and approved the manuscript.

ETHICS STATEMENT

The approval for the conduct of this study was obtained from the Norwegian Animal Research Authority, FDU (Forsøksdyrutvalget ID-22992). We adhered to the rules and regulations regarding the research on experimental animals, FOR-2015–06–18–761. Also, the biosafety rules and regulations stipulated by the Health, Safety and Environment (HSE) system of Faculty of Biosciences and Aquaculture, Nord University, were followed during the experiment.

DATA AVAILABILITY STATEMENT

The data sets generated from this study can be found in the Sequence Read Archive (NCBI) with the accession number PRJNA689790.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Developmental defects and behavioral changes in a diet-induced inflammation model of zebrafish

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Soybean meal evokes diet-induced intestinal inflammation in certain fishes. Although the molecular aspects of soybean-induced intestinal inflammation in zebrafish are known, the impact of the inflammatory diet on fish behavior remain largely underexplored. We fed zebrafish larvae with three diets - control, soybean meal and soybean meal with β-glucan to gain deeper insight into the behavioral changes associated with the soybean meal-induced inflammation model. We assessed the effect of the diets on the locomotor behavior, morphological development, oxygen consumption and larval transcriptome. Our study revealed that dietary soybean meal can reduce the locomotor activity, induce developmental defects and increase the oxygen demand in zebrafish larvae. Transcriptomic analysis pointed to the suppression of genes linked to visual perception, organ development, phototransduction pathway and activation of genes linked to the steroid biosynthesis pathway. On the contrary, β-glucan, an anti-inflammatory feed additive, counteracted the behavioral and phenotypic changes linked to dietary soybean. Although we did not identify any differentially expressed genes from the soybean meal alone fed group vs soybean meal + β-glucan-fed group comparison, the unique genes from the comparisons of the two groups with the control likely indicate reduction in inflammatory cytokine signaling, inhibition of proteolysis and induction of epigenetic modifications by the dietary glucan. Furthermore, we found that feeding an inflammatory diet at the larval stage can lead to long-lasting developmental defects. In conclusion, our study reveals the extra-intestinal manifestations associated with soybean meal-induced inflammation model.

KEYWORDS

soybean meal, zebrafish, behavior, β-glucan, inflammation

Introduction

Intestinal inflammation is a significant health problem that affects a considerable portion of the world population (1). A myriad of genetic and environmental factors have been associated with the onset of the disease (2) and intestinal inflammation can adversely affect the functions of other organs. For instance, chronic intestinal inflammation can lead to the development of several forms of psychiatric disorders (3–5). Mice models have been employed to understand the aftereffects of intestinal inflammation (6). For example, stress-associated behavior of chemically-induced colitis model (7, 8) and compromised cognitive ability after the consumption of high-fat inflammatory diet (9).

Similar to mice, zebrafish is a model organism widely used to understand intestinal inflammation (10, 11). This model replicates inflammation hallmarks like increased intestinal permeability, immune cell recruitment and alteration in the microbiota profile (12–14). An inflammation model of zebrafish has been developed by feeding zebrafish larvae at 5 days post-fertilization (dpf) with a soybean meal-based diet (12, 15). The success of the model could be due to triggering of an inflammatory reaction in the intestine of zebrafish larvae by saponins, which are antinutritional factors in soybean meal (12). This model is used extensively and mainly to understand the molecular aspects of diet-induced inflammation (15, 16). However, the extra-intestinal manifestations of this model have not been extensively studied. We believe that zebrafish is an ideal model to study diet-induced changes in swimming behavior.

Zebrafish larvae start to swim freely at around 4 dpf and their swimming behavior is modulated by both internal and external stimuli (17). At this early stage, larvae have a narrow repertoire of discrete stereotyped movements, which can be assessed to understand their behavior. With the use of automated movement tracking systems, it is now possible to monitor the activity of an individual and split a particular movement into several measurable parameters like distance travelled, movement, heading and turn angle which can be assessed to understand behavioral changes. In the present study, we analyzed the locomotor behavior of zebrafish larvae to understand the behavioral changes associated with a well-established soybean meal-induced inflammation model (12, 15). Additionally, we exploited transcriptomics data to gain deeper insight into the underlying molecular aspects of behavioral changes in the larval zebrafish model. Since it is well-known that β-glucan can impart anti-inflammatory effects (18, 19), we tested the efficacy of a commercial product to abate soybean meal-induced behavioral changes.

Materials and methods

Zebrafish husbandry

Adult zebrafish (AB strain) were maintained in a recirculatory aquaculture system at Nord University, Norway, following standard protocols (20). Zebrafish eggs were obtained by naturally breeding sexually mature males and females. Fish in five tanks were used for breeding, and in each of these tanks there were 15 males and 30 females. They were community bred and 300-400 eggs were obtained from each tank. These eggs were kept in larval rearing tanks (3.5 L) which were part of a freshwater flow-through system (Zebtec Toxicological Rack, Tecniplast, Varese, Italy), hereafter called system water. The eggs were randomly distributed into 18 rearing tanks, with 100 eggs in each tank. The water temperature in the tanks was 28 ± 0.5°C, the water flow rate was 1 L/h, and dissolved oxygen was 7-8 ppm (oxygen saturation > 85%). A 14L:10D photoperiod was maintained throughout the experimental period.

Test diets and feeding study

Five-day-old larvae (five days post fertilization, 5 dpf) were used for the study and the test diets were prepared by SPAROS Lda (Olhão, Portugal). The control diet, CT, was a fish meal-based diet with high-quality marine protein. Soybean-based diet (SBM) contained 50% of the test component to induce a pro-inflammatory effect (12, 15). The β-glucan diet (BG) was supplemented with 2.5% (w/w) of the product Aleta™ (derived from the microalga *Euglena gracilis*; Kemin, Des Moines, USA) in the SBM diet (Supplementary Table 1). Each experimental fish group (6 tanks/group) was assigned to the respective test diet (< 100 µm particle size) from 5 to 14 dpf. From 15 dpf to 30 dpf, the larvae in the SBM, BG and CT groups were offered the control diet (100-200 µm particle size) (Supplementary Figure 1). The larvae were hand-fed four times a day *ad libitum*, i.e., at 08:00, 12:00, 16:00 and 20:00. The feeding study that started with 5-day-old larvae ended at 30 dpf.

Sampling

At 15 and 30 dpf, larvae were analyzed under the microscope. In addition, their behavior was assessed, and oxygen consumption was recorded. Samples were collected at 15 dpf for transcriptomics. The larvae were immersed in a lethal dose of 200 mg/L of tricaine methanesulfonate (Argent Chemical Laboratories, Redmond, WA, USA) buffered with an

equal amount of sodium bicarbonate. Five larvae were pooled to obtain one sample and 6 replicate samples were prepared from each treatment group. The samples were snap-frozen in liquid nitrogen and then stored at -80°C until further analyses.

Microscopic examination

Larvae from each group (*n*=9-10) were randomly selected for the microscopic examination. For the study, the larvae were immobilized on a cavity glass slide using 3.5% (w/v) methylcellulose (Sigma Aldrich, Saint Louis, USA). Images were captured using a stereomicroscope (SZX12, Olympus, Shinjuku, Japan) equipped with an Olympus SC50 camera (Olympus Soft Imaging Solutions, Münster, Germany). Key morphological traits like standard length, snout-vent length, head-trunk angle, swim bladder area and eye area (21, 22) were measured using the *ImageJ* software (23).

Locomotor behavior test

Larval locomotor behavior was assessed using the DanioVision system (Noldus Information Technology, Wageningen, the Netherlands). The assessment was performed as described in our previous study (24). The larvae were first acclimatized to 24 well plates for one hour at 28°C in an incubator (Sanyo MIR-154, Osaka, Japan). The analysis was carried out three times in the DanioVision system (*n*=20). The temperature of the well plates (28 ± 1°C) was maintained using the DanioVision temperature control unit. The 20 min behavior analysis included a 5 min dark period followed by a 5 min light period and then a second cycle of 5 min of darkness followed by 5 min of light period. The video recordings were analyzed using the EthoVision® XT 16.0 software (Noldus Information Technology) to assess the distance moved, velocity, movement, angular velocity and heading angle.

Sudan black staining

Zebrafish larvae (*n*=24-25; 15 dpf) were fixed overnight in 4% formaldehyde in PBS at 4 °C. After the fixation step, larvae were washed with cold PBS containing 0.1% Tween 20 (PBT) (Sigma Aldrich, Saint Louis, USA), and incubated in Sudan Black stain (Sigma Aldrich) for 20 min. Then the samples were washed (3-4 times; each time 10 min) in 70% ethanol. Larvae were then rehydrated with PBT and mounted in 90% glycerol for viewing under a stereomicroscope (SZX12, Olympus). Thereafter, the images were captured using Olympus SC50 camera (Olympus Soft Imaging Solutions) and analyzed to quantify the granulocytes in the mid and posterior intestine.

Oxygen consumption analysis

Oxygen consumption of the larvae was determined using the Loligo® Microplate Respirometry System (Loligo Systems, Viborg, Denmark). Twenty-four hours before the measurement, the instrument was calibrated with oxygen-saturated and oxygen-depleted system water at 28°C. The oxygen-depleted water was first prepared by dissolving 20 g of sodium sulfite (Sigma Aldrich) in 1 liter of system water. Then larvae from each treatment (*n*=12) were placed in the two independent 24-well plate sensor dishes (PreSens, Regensburg, Germany), with each well containing one larva. Thereafter, the 24-well plate sensor dish was submerged in a tank containing system water. During the 2 hour-long respiration measurement, the tank and plates were kept at 28°C in a climate chamber. The oxygen saturation was recorded using the software MicroResp® version 1.0.4 (Loligo).

RNA isolation, library preparation and mRNA sequencing

Total RNA was extracted from the frozen samples using the Direct-zol™ RNA MiniPrep kit (Zymoresearch, CA, USA) following the manufacturer's instructions. The RNA concentration and integrity were determined using the Invitrogen Qubit 3.0 fluorometer (ThermoFisher Scientific, USA) and Tape Station 2200 (Agilent Technologies, Santa Clara, CA, USA). RNA from the samples (*n*=6) with RIN value ≥7 were used for RNA-Seq. Library preparation and sequencing were done by Novogene Europe, Cambridge, United Kingdom. Messenger RNA was purified from total RNA using poly-T oligo-attached magnetic beads. After fragmentation, the first strand cDNA was synthesized using random hexamer primers followed by the second strand cDNA synthesis. The libraries were end repaired, A-tailed, adapter ligated, size selected, amplified, and finally purified. The libraries were quantified using Qubit and real-time PCR and bioanalyzer was employed to detect the size distribution. The barcoded libraries were then pooled and loaded on the Illumina NovaSeq 6000 Sequencing system (Illumina, San Diego, CA, USA) to obtain 150 bp paired end reads. For each sample, a minimum of 20 million paired raw reads were obtained with an average of 21.4 million reads per sample. Overall, the average mapping percentage of filtered reads was 88% (Supplementary Table 2).

Transcriptome data analyses

The quality of raw reads was assessed using the *FastQC* command line, and reads were filtered based on the Phred quality score (Q ≥ 30) using the tool *fastp* (25) (Supplementary Table 2).

The filtered reads were then aligned to the reference zebrafish genome downloaded from NCBI (release 106) using *HISAT2*, version 2.2.1 with default parameters (26). *featureCounts* version 1.5.3 was employed to obtain the read counts that belong to each gene (27). Differential expression of the genes across the treatment groups was determined by *DESeq2* (28). Transcripts with $|\text{Log}_2$ fold change$| \geq 1$ and an adjusted p-value < 0.05 (Benjamini-Hochberg multiple test correction method) were considered significantly differentially expressed. The gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed with the software *DAVID* (Database for Annotation, Visualization and Integrated Discovery) version 6.8 with p value of 0.05 and minimum gene count of 2 (27). GO term-gene networks were generated using *Cytoscape* version 3.8.2 (29). The packages *ggplot2*, *pheatmap*, *GOplot*, in R were employed to visualize the parameters of interest.

Statistical analysis

The behavioral, morphological and granulocyte data were checked for the assumptions of normality (Shapiro-Wilk) and homogeneity of variance (Bartlett's test). Parametric *t*-test and one-way ANOVA were performed where the normality assumptions were met. In the case of non-parametric data, statistical differences were identified using the Wilcoxon-Mann-Whitney test and Kruskal-Wallis test. Tukey's test (parametric data) and Dunn's test (non-parametric data) were employed to understand the statistical differences between treatments. We employed the gam function in the *mgcv* package of R to study the oxygen depletion in the three groups. In addition, we employed the *gganimate* package to create a gif to display the differences at 15 and 30 dpf separately. The angular data was analyzed using circular package in R and the statistical differences were detected using Watson U^2 test.

Results

Morphological changes, locomotor activity, granulocyte number and oxygen consumption

Microscopic evaluation helped us to understand the diet-induced changes in the morphology of zebrafish larvae (Figures 1A-D and Supplementary Figure 2). The standard length and snout-vent length of the treatment groups did not differ significantly (Figures 1E, F). However, we found a significant decrease in the eye area (Figure 1G) of the SBM group ($p < 0.01$) compared to the CT group. The eye area in the BG group was significantly increased ($p < 0.05$) compared to the SBM group. Also, there was a significant difference in the swim bladder area in the SBM group ($p < 0.001$) and BG group ($p < 0.05$) compared to the CT group. In the BG group, however, the effect was less pronounced, since swim bladder area was significantly increased ($p < 0.001$) compared to the SBM group (Figure 1H). We found a significant reduction in the head-trunk angle of the larvae from the SBM group ($p < 0.05$) compared with the CT group (Figure 1I). On the other hand, the head-trunk angle was increased in the BG group compared to the SBM group ($p < 0.01$).

The locomotor activity of the experimental larvae was evaluated by conducting a light-dark (LD) locomotion test. The total distance travelled (Figure 2A) by the SBM group was significantly reduced ($p < 0.001$) at 15 dpf. Average velocity (Figure 2B) and movement (Figure 2C) were also significantly ($p < 0.001$) reduced in the SBM group compared to both the CT and BG groups. The parameter angular velocity (Figure 2D) did not appear to be altered compared to the CT group ($p < 0.1$). However, the heading angle seemed to be slightly, but not significantly, altered in the SBM group (Figure 2E). The velocity vs. time plot (Figure 2F) of the light-dark phase experiment clearly indicated the loss in the dark-induced motion phenotype in the SBM group compared to the CT group. The BG group also showed a slightly altered activity after the first dark phase.

The larvae were stained with Sudan Black to examine the number of granulocytes in the intestine. The SBM diet-fed larvae had a greater number of granulocytes ($p < 0.001$) compared to the CT group. The BG group had lower number of granulocytes ($p < 0.05$) compared to the SBM group (Figures 3A, B).

We found an overall decrease in oxygen saturation with time in all the treatments. The oxygen saturation decreased at a significantly higher rate in the SBM and BG groups than in the CT group (Figure 4). The factors treatment and time were found to be significant ($p < 0.05$).

Suppression of genes linked to visual perception and organ development

To understand the underlying effects of the soybean feeding in zebrafish larvae, we compared the transcriptome of the SBM group with that of the CT group. The analysis revealed 707 differentially expressed genes ($|\text{Log}_2$ fold-change$| \geq 1$, adjusted p-value < 0.05) with 280 upregulated genes and 427 downregulated genes in the SBM group (Supplementary Table 3). The principal component analysis (PCA) plot shows the differential clustering of the SBM and CT groups along the first principal component (PC1), which explains 58% variability in the data (Figure 5A). Hierarchical clustering (Figure 5B) revealed a clear separation of differentially upregulated and downregulated genes in the SBM group compared to the CT

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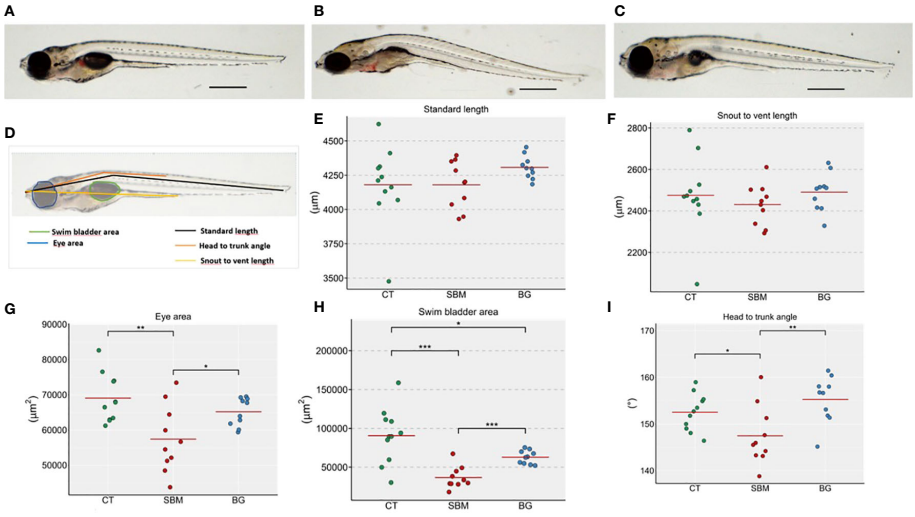

FIGURE 1
Diet-induced morphological changes in zebrafish larvae. Representative images of the zebrafish larvae fed the **(A)** CT **(B)** SBM and **(C)** BG diets **(D)**. Measurement strategy that was adopted to assess the morphological changes in zebrafish larvae. The measured parameters include **(E)** Standard length **(F)** Snout to vent length **(G)** Eye area **(H)** Swim bladder area **(I)** Head to trunk angle. Asterisks *** indicate p < 0.001, ** indicate $p < 0.01$, * indicates $p < 0.05$. Larvae were assessed at 15 dpf (n = 9-10 per group). Control - CT, soybean - SBM and β-glucan - BG. Scale bar = 500 µm.

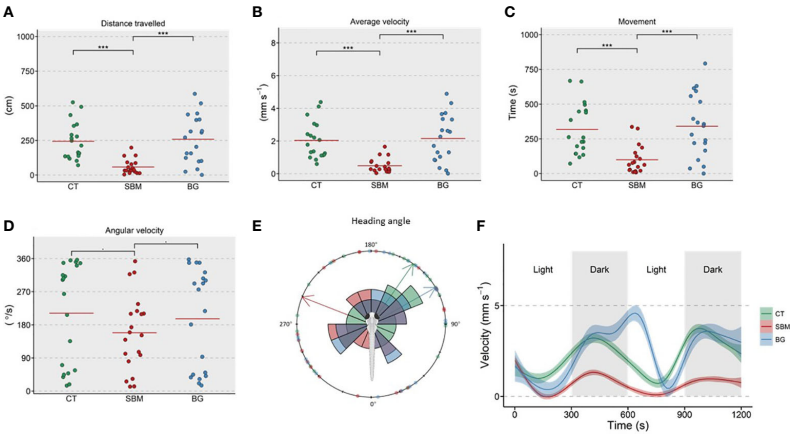

FIGURE 2
Changes in parameters linked to locomotor activity of zebrafish larvae. The measured parameters included **(A)** Distance travelled **(B)** Average velocity **(C)** Movement **(D)** Angular velocity and **(E)** Heading angle. Effect of alternating light-dark phases on the **(F)** velocity of zebrafish larvae. *** $p < 0.001$ and (.) $p < 0.1$. Larvae assessed at 15 dpf (n = 18-20 per group). Control - CT, soybean - SBM and β-glucan - BG.

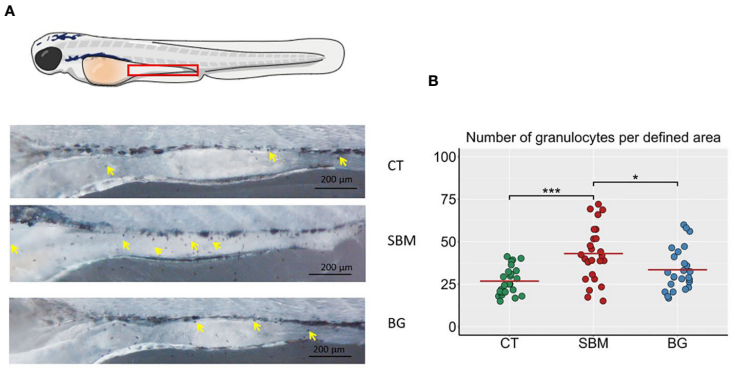

FIGURE 3
Localization of granulocytes in the intestine of zebrafish larvae. **(A)** Representative images of the intestine region of zebrafish larvae stained with Sudan Black to reveal the presence of granulocytes, yellow arrows indicate the granulocytes in the intestine region. **(B)** Quantification of Sudan-Black^{+} cells in the intestine region of zebrafish larvae. *** $p < 0.001$ and * $p < 0.05$. n = 24-25 per group. Control - CT, soybean - SBM and β-glucan – BG. Scale bar = 200 µm.

group. The downregulated genes-associated significantly enriched GO terms in the SBM group were related to developmental processes like regulation of gastrulation, formation of primary germ layer, somite development and positive regulation of organelle organization (Figure 6). Furthermore, several GO terms related to sensory perception like sensory organ development, eye development, sensory perception of light stimulus and camera type eye development

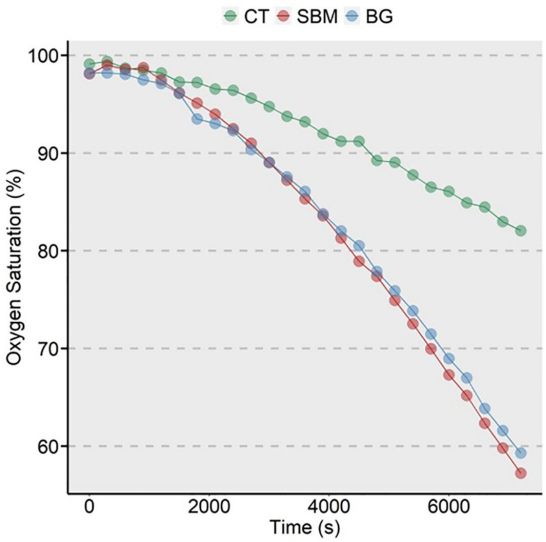

FIGURE 4
Oxygen consumption of zebrafish larvae from the 3 study groups. A generalized additive model using R package *mgcv* indicated significant differences ($p < 0.05$) in oxygen saturation. Larvae assessed at 15 dpf (n = 12 per group). Control - CT, soybean - SBM and β-glucan - BG.

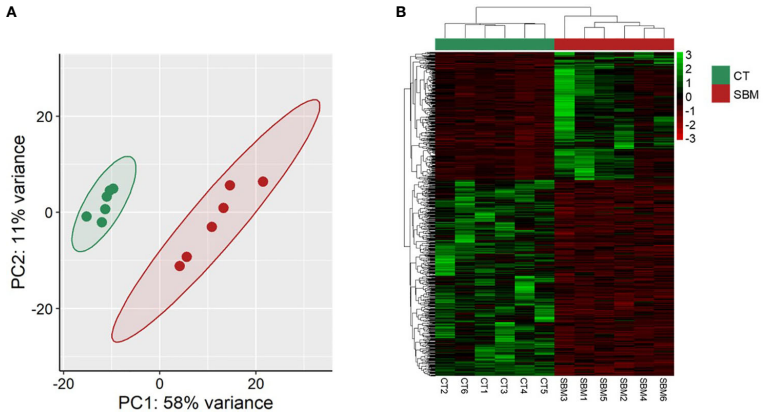

FIGURE 5
Transcriptome-based differences in zebrafish larvae from the soybean group compared to the control group. Principal component analyses **(A)** and heatmap **(B)** of the differentially expressed genes (DEGs) in the soybean (SBM) group compared to the control (CT) group. Transcripts with an adjusted *p*-value below 0.05 and |Log2 fold change| ≥ 1 were considered significantly differentially expressed. There are six biological replicates in each study group.

were also enriched based on the downregulated genes. KEGG enrichment analysis that considered the downregulated genes revealed the alteration of the phototransduction pathway in the SBM group compared to the CT group (Figure 7). On the other hand, upregulated genes-based KEGG pathway enrichment revealed the possible alteration of pathways like steroid biosynthesis, peroxisome proliferator activated lipid receptor (PPAR) signaling and metabolism of xenobiotics by cytochrome P450 (Figure 7). Similarly, upregulated genes-based GO enrichment analysis indicated the likely alteration of steroid biosynthesis process, sterol metabolic process, cholesterol metabolic process, fatty acid transport and lipid

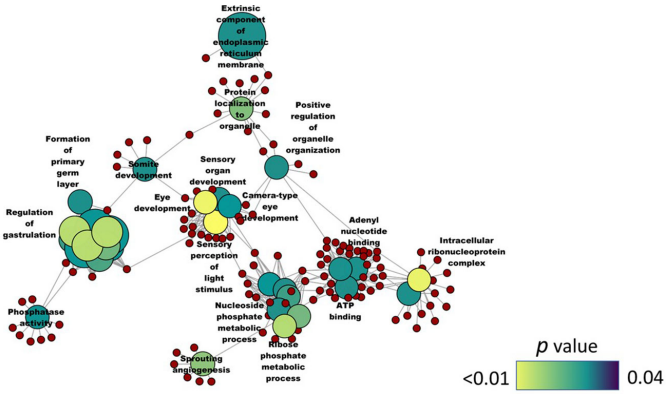

FIGURE 6
Network plot showing the link between the enriched GO terms. DEGs (downregulated; SBM vs CT) that were considered for the enrichment are indicated using red circles and only the non-redundant GO terms are shown in the cluster. The gradient color bar intensity varies with the *p* value and the sizes of the nodes of the GO terms increase with the associated fold change. There are six biological replicates in each study group. Control - CT, soybean - SBM.

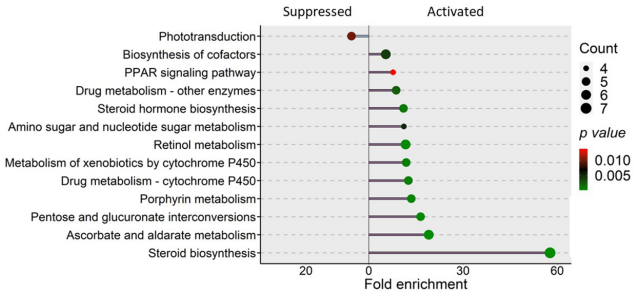

FIGURE 7
KEGG pathways that were enriched in the zebrafish larvae from the soybean group. Differentially expressed genes in the soybean (SBM) group compared to the control (CT) group were employed for the pathway enrichment analysis. The size of the circles is proportional to the gene count and gradient color bar intensity of circle correlates with the *p* value. There are six biological replicates in each study group.

homeostasis (Figure 8). Furthermore, there was a significant enrichment of GO terms (based on the upregulated genes in the SBM group) related to protein degradation, namely proteasome core complex, exopeptidase activity and aminoglycan catabolic process (Figure 8). Several immune, intestinal barrier and brain-related genes were altered in the SBM group compared to the CT group (Supplementary Figures 3A-C).

β-glucan supplementation-caused distinct changes in zebrafish larvae

Here we describe the DEGs from the BG vs CT comparison. As we did not detect any DEGs from the SBM vs BG comparison, unique (upregulated and downregulated) genes from the SBM vs CT and BG vs CT comparisons are given importance in this section.

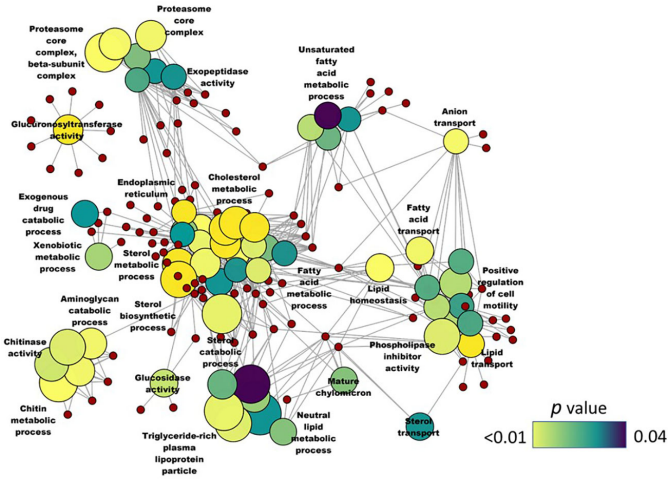

FIGURE 8
Network plot showing the link between enriched GO terms. DEGs (upregulated; SBM vs CT) that were considered for the enrichment are indicated using red circles and only the non-redundant GO terms are shown in the cluster. The gradient color bar intensity varies with the *p* value and the sizes of nodes of the GO terms increase with the associated fold change. There are six biological replicates in each study group. Control - CT, soybean - SBM.

In addition, we selected the genes linked to the immune system to gather more evidence on the effect of β-glucan. A comparison of the transcriptome of the BG group with that of the CT group revealed 736 DEGs ($|Log_2$ fold-change$| \geq 1$, adjusted p value < 0.05) with 537 downregulated genes and 199 upregulated genes (Supplementary Table 4). The PCA plot shows differential clustering of the BG and CT groups along PC1, which explains 59% variability in the data (Figure 9A). Hierarchical clustering (Figure 9B) revealed a clear separation of differentially upregulated and downregulated genes in the BG group compared to the CT group. BG vs CT transcriptomic comparison revealed several common genes which were also present in the SBM vs CT comparison, possibly due to the presence of soybean meal in the BG diet too. We used a Venn diagram to display the common and unique DEGs from the SBM vs CT and BG vs CT comparisons (Supplementary Figure 4); 343 common DEGs (239 downregulated and 104 upregulated) and 298 unique downregulated genes and 95 unique upregulated genes from the BG vs CT comparison. To reveal the efficacy of the β-glucan, we focused on the altered genes that are unique to the BG vs CT comparison. GO term enrichment by considering 95 unique upregulated genes included negative regulation of proteolysis, endopeptidase inhibitor activity and negative regulation of cellular protein metabolic process (Figure 10A). GO term enrichment based on the 298 unique downregulated genes in the BG group included cytokine mediated signaling pathway, leukocyte differentiation, histone acetyltransferase complex, histone acetylation, histone modification and regulation of G-protein coupled receptor protein signaling pathway (Figure 10B).

From the DEGs of the three comparisons, immune-, barrier- and brain-related genes were selected to understand the inflammation mitigation capacity of β-glucan. We observed that BG feeding restored the expression of several genes which were perturbed by SBM feeding (Supplementary Figures 3A-C). The expression of immune genes like *major histocompatibility complex class I UBA* (*mhc1uba*), *complement C3a* (*c3a.3*), *interleukin 2 receptor, gamma a* (*il2rga*), *macrophage stimulating 1 receptor a* (*mst1ra*) that was upregulated in the SBM group was downregulated in the BG group. The expressions of brain-related genes like *tachykinin receptor 1b* (*tacr1b*), *bradykinin receptor b1* (*bdkr1*) in the BG group were similar to those in the CT group.

A defective swim bladder persisted despite the cessation of soybean feeding

A switch to the control diet without soybean meal helped in restoring the phenotypic characteristics, locomotor activity and oxygen consumption of the SBM group. The locomotor behavioral analysis revealed that all the study groups performed similarly at 30 dpf (Supplementary Figures 5A-D), i.e., when the fish did not consume soybean meal-based diets. However, the head to trunk angle in the SBM group was significantly decreased ($p < 0.05$) compared to CT and BG groups. The swim bladder area in the SBM group was significantly smaller ($p < 0.001$) compared to the CT and BG groups. Notably, the swim bladder area in the BG group seemed

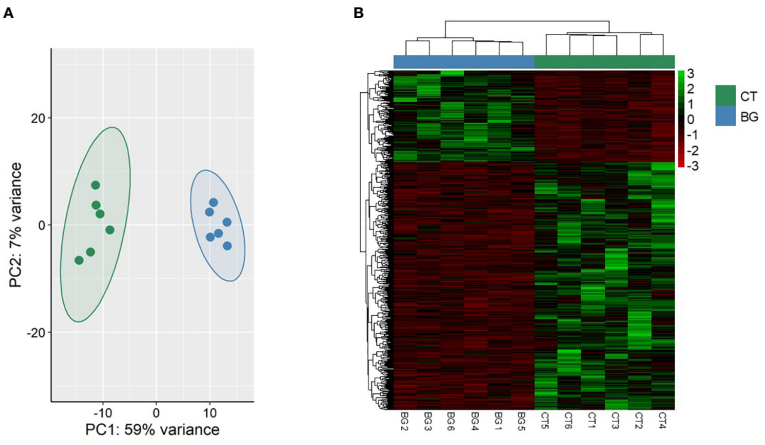

FIGURE 9
Transcriptome-based differences in the zebrafish larvae from the β-glucan group compared to the control group. Principal component analyses **(A)** and heatmap **(B)** of differentially expressed genes (DEGs) in the β-glucan (BG) group compared to the control (CT) group. Transcripts with an adjusted p below 0.05 and |Log2 fold change| ≥ 1 were considered as significantly differentially expressed. There are six biological replicates in each study group.

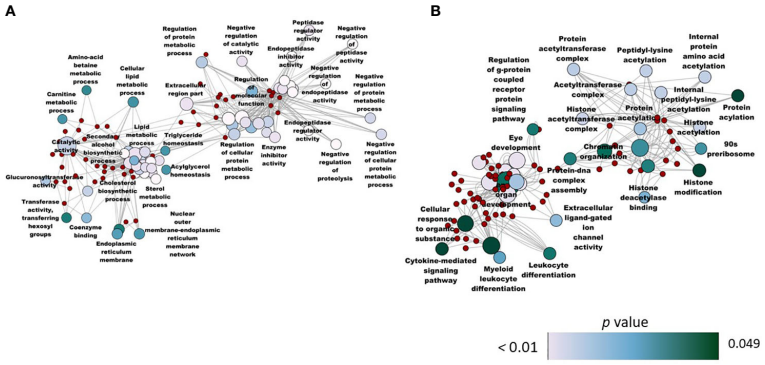

FIGURE 10
Network plot showing the link between the enriched GO terms. The enriched GO terms that were unique (from the BG and SBM vs CT comparisons) to the zebrafish larvae fed with the BG diet are shown in the figure, and 298 and 95 genes were significantly **(A)** upregulated and **(B)** downregulated, respectively, in BG vs CT comparison. Control - CT, soybean - SBM and β-glucan - BG.

to have returned to a level similar to the CT group (Supplementary Figures 5E, 6). The oxygen consumption in all the three treatments at 30 dpf did not differ significantly (Supplementary Figure 7), even though we observed a steep decline in oxygen saturation in all the three treatments, indicating a higher consumption of oxygen at 30 dpf.

Discussion

Soybean meal-based diet is effective in inducing intestinal inflammation in zebrafish. The attributes of inflammation in this model are increased intestinal permeability, immune cell recruitment and alteration in the microbiota profile (13–15). Behavioral changes accompanying diet-induced intestinal inflammation in animals have been reported previously (30, 31). In the present study, we have linked behavioral changes of zebrafish with alterations in the transcriptome to gain deeper insights into the impact of inflammatory and anti-inflammatory diets. Dietary soybean was found to reduce the locomotor activity, induce developmental defects and increase oxygen consumption of zebrafish larvae. Transcriptomic analysis indicated the soybean meal-induced suppression of the genes linked to visual perception, organ development, phototransduction pathway and activation of genes related to steroid biosynthesis pathway. On the contrary, β-glucan partly negated the behavioral and phenotypic alterations brought about by the soybean diet.

Soybean-induced alteration in locomotor behavior

We performed a light-dark locomotion test to understand soybean meal-induced behavioral changes in zebrafish larvae. Alternating light and dark conditions prompts zebrafish to follow a specific pattern of movement; while a transition from light-dark increases locomotion, a dark-light transition decreases its movement (32). The CT and BG groups displayed normal responses to light, as indicated by the velocity-time analysis. On the other hand, the behavior of the SBM group was abnormal, probably due to a defect in sensory perception, as indicated by the potential shift in sensory organ development, sensory perception of light stimulus and the significantly affected phototransduction pathway. This pathway occurs in the retinal photoreceptors, namely rods and cone cells which are active at low and high light intensities, respectively (33). These cells convert light stimulus into electrical signals which are then perceived by the nervous system. Several genes related to light perception including *visual system homeobox 1* (*vsx1*), *recoverin a* (*rcvrna*) and *guanylate cyclase activator 1b* (*guca1b*) were downregulated in the SBM group. The proteins coded by these genes are critical for retinal functions like terminal differentiation of retinal cells and cone photo response recovery (34, 35). The transcriptomic data also revealed the potential alteration of several pathways such as retinol metabolism, steroid biosynthesis, PPAR signaling based on the upregulated genes in the SBM group. Alterations in these

pathways have been linked with retinal impairments (36–38). In addition, SBM feeding reduced the eye area, indicating a possible impact on the overall eye development. Therefore, it can be speculated that the metabolic changes induced by the soybean-based diet might have affected retinal functions. The altered heading angle and angular velocity of the SBM group can also be linked to the impaired light perception. Furthermore, several locomotor behavioral parameters like distance travelled, velocity and movement were also decreased in the SBM group. Other feeding studies have also reported diet-induced changes in locomotion; while a high-fat, low-fiber diet reduced the motor activity of mice (30, 39) a probiotic diet (with *Lactobacillus rhamnosus* IMC 501) increased the movement of zebrafish (40). This indicates that locomotor behavior of animals can be altered by diet. Concerning the β-glucan diet, another study has indicated the efficacy of the compound in improving the cognitive ability in diet-induced colitis model of mice (9). The observations in the present study also likely indicate an improvement in locomotor behavior. Diet-induced behavioral changes can be due to microbiota alterations which in turn affect the gut-brain axis as observed in mice models (9). Although in the present study we did not investigate the microbiota changes, future studies should focus on the effect of diet-induced inflammation on the gut-brain axis.

Soybean-induced developmental defects and oxygen demand

We did not detect any significant differences in growth parameters like standard length and snout to vent length in zebrafish larvae belonging to the 3 study groups. The short duration of the experiment, i.e., 10 days may not be sufficient to reveal any significant differences in growth, as observed in a study on red seabream, *Sparus aurata* larvae (41). We found a significant reduction in swim bladder area and head-trunk angle in the SBM group. The swim bladder is an important organ to maintain buoyancy, and larvae with uninflated swim bladders develop complications such as spinal deformities and lordosis (38). GO terms such as regulation of gastrulation, somite development, positive regulation of organelle organization and formation of primary germ layer likely indicate developmental defects of the larvae in the SBM group. As soybean meal-induced inflammation causes morphological changes in the intestine (42), and the swim bladder develops as an evagination of the digestive tract, there is a possibility that dietary soybean meal is interfering with swim bladder inflation. Furthermore, metabolic demands are higher in zebrafish larvae with uninflated swim bladders and these larvae use additional energy to maintain buoyancy (38, 39). The altered locomotor behavior and high oxygen consumption that we observed in the SBM group is likely due to uninflated swim bladder (43, 44). Furthermore, feeding CT diet to 15-30 dpf larvae of the 3 groups indicated that the SBM-caused adverse effects on behavior-related parameters and oxygen consumption rate can be abated by stopping the SBM diet. Although the swim bladder area was increased in SBM group at 30 dpf as compared to 15 dpf, it was still significantly smaller compared to the other treatment groups. It seems that developmental defects such as uninflated swim bladder and reduced head-trunk angle persist even after stopping the SBM diet. Addition of β-glucan speeds up the recovery or lowers the effect of SBM on development. This suggests that proper nutrition during the critical developmental window is essential to avoid long-lasting effects on an organism (45).

KEGG pathway analyses revealed steroid biosynthesis (most enriched pathway based on *p* value and gene count) and steroid hormone biosynthesis as the enriched pathways, based on the upregulated genes in the SBM group. We also found enrichment of several GO terms like cholesterol metabolic process, sterol biosynthesis process, and sterol metabolic process. This can be because plant-derived food has no cholesterol and soybean contains phytosterols which can reduce dietary cholesterol absorption (46). Such a scenario can lead to stimulation of cholesterol biosynthesis, as reported in a study on fish fed soybean meal (47). Cholesterol is the key precursor of steroid hormone biosynthesis and cholesterol biosynthesis is an oxygen-intensive process that requires 11 molecules of O_2 per molecule of cholesterol (41). Furthermore, molecular oxygen is a prerequisite to most of the enzymatic reactions in the steroid biosynthesis pathway (45). Therefore, the higher consumption of oxygen could be attributed to the increased metabolic demands in the SBM and BG groups and steroid biosynthesis can be a causative factor.

Plausible effects of dietary β-glucan on zebrafish larval transcriptome

Several immune, intestinal barrier and brain-related genes were altered in the SBM and BG groups compared to the CT group. In this section we describe the common and unique genes from the SBM and BG vs CT comparisons. It was only in the BG group that the differential expression of *jak1*, *zbtb11*, *jagn1a*, *lepr*, *il13ra2*, *ccl34b.1* caused an enrichment of the GO terms, leukocyte differentiation and cytokine mediated signaling. Janus Kinase (JAK1) plays an important role in inflammatory cytokine signaling and inhibition of JAK1-mediated inflammatory pathways are effective therapeutic targets to counter intestinal inflammation (48, 49). Furthermore, genes such as *leptin receptor* (*lepr*), *jagunal homolog 1-A* (*jagn1a*) that are related to neutrophil development and migration (50, 51) and inflammation-associated cytokine like *il13ra2* (52) were differentially downregulated in the BG group. Granular cells (mainly neutrophils) are the first responders that migrate to an inflammatory site (53).The number of granulocytes in the BG group was lower compared to the SBM group, indicating the

dietary β-glucan-mediated lowering of inflammation. In addition, the BG group was associated with GO terms related to negative regulation of proteolysis and endopeptidase inhibitor activity, as a result of the upregulated genes such as *serpin peptidase inhibitor, clade B, member 1, like 3* (*serpinb1l3*), *TIMP metallopeptidase inhibitor 4, tandem duplicate 2 (timp4.2)*. Serpin family B member is produced by macrophages and neutrophils to restrict the activity of serine proteases and inflammatory caspases to suppress inflammation (54). Also, tissue inhibitors of metalloproteinases (TIMPs) regulate diverse processes such as tissue remodeling, wound healing and inhibition of matrix metalloproteinases (46). Therefore, the upregulation of *serpinb1, serpinb1l3* and *timp4* in the β-glucan fed group might possibly help in controlling the tissue damage caused by dietary soybean. Moreover, the enriched GO term, G-protein-coupled receptor (GPCR) based on the downregulated genes, *ubiquitin-specific protease 20* (*usp20*) and *phosducin* (*pdca*) in the BG group is likely pointing to the efficacy of the glucan to counter inflammatory pathways. The protein coded by *usp20* is involved in the Tumor necrosis factor (TNFα)-induced activation of Nuclear factor kappa-light-chain-enhancer of activated B cells protein (NF-κB) pathway through the stabilization of p62 protein (55). In addition, β-glucan is also a potent epigenetic modulator (50, 51). Several GO terms related to epigenetic modifications such as histone acetyltransferase complex, histone modifications, chromatin organization were enriched (based on downregulated genes) in the BG group. Histone acetyltransferases (HATs) transfer acetyl groups from donor-acetyl coenzyme A to lysine residues of the histone proteins to sustain an active transcription. HATs also act as a cofactor for NF-κB activation by acetylating its various promotor proteins (56) because a HAT knockout study reported reduced DSS-induced colitis in mice (57). The expression of immune genes like *mhc1uba*, *c3a.3*, *il2rga*, *mst1ra* were upregulated in the SBM group, but the CT and BG groups had similar mRNA levels. Two brain-related genes, *tachykinin receptor 1b* (*tacr1b)* and *bradykinin receptor b1* (*bdkr1*) that were upregulated by the SBM diet (58, 59) were found to be downregulated by the BG diet. Therefore, we speculate that β-glucan can also reduce intestinal inflammation induced by the soybean diet, plausibly by altering the expression of GPCRs, cytokine signaling and inducing epigenetic modifications.

Conclusion

Our study shows that dietary soybean meal reduces larval locomotor behavior, increases oxygen consumption, and induces developmental defects in zebrafish larvae. Transcriptomic analysis indicated soybean meal-induced suppression of genes related to the phototransduction pathway, organ development and activation of genes linked to the steroid biosynthesis pathway. Dietary β-glucan can likely alleviate the behavioral defects induced by the inflammatory diet and negate the aforementioned alterations in gene expression. Importantly, when zebrafish larvae receive an inflammation-sustaining dietary component, the developmental defects persist even after the withdrawal of the inflammatory diet. It would be interesting to explore if the gut microbiota has a role in the observed alterations. Hence, future studies should focus on the effect of dietary soybean-induced inflammation on the gut-brain axis.

Data availability statement

The datasets presented in this study can be found in online repositories. The name of the repository/repositories and accession number(s) are: NCBI, PRJNA867519.

Ethics statement

The animal study was reviewed and approved by the Norwegian Animal Research Authority, FDU (Forsøksdyrutvalget ID-22992).

Author contributions

SR, VK, SB and JD designed the study. JD prepared feeds, and SR and AG performed the feeding experiment. AG, SR and SV did the sampling. SR and SV performed the behavioral study and its data analysis. AG and SR performed the bioinformatic analysis. SR and VK wrote the manuscript. All authors reviewed the article and approved the submitted version.

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Conflict of interest

Author JD was employed by company SPAROS Lda.

The remaining 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/fimmu.2022.1018768/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Experimental design of the study.

SUPPLEMENTARY FIGURE 2
Microscopic images of the zebrafish larve at 15 dpf. Scale bar = 500 µm.

SUPPLEMENTARY FIGURE 3
Heatmaps of mean normalized counts of selected differentially expressed genes associated with the 3 study groups **(A)** Immune **(B)** Barrier and **(C)** Brain-related differentially expressed genes (DEGs) were selected from different treatment groups.

SUPPLEMENTARY FIGURE 4
Venn diagram to display the common and unique differentially expressed genes from the soybean vs control, and β-glucan group vs control comparisons.

SUPPLEMENTARY FIGURE 5
Changes in parameters linked to locomotor activity and morphology of zebrafish larvae. The measured parameters included **(A)** Distance travelled **(B)** Average velocity **(C)** Movement. Effect of alternating light-dark phases on the **(D)** Velocity of zebrafish larvae **(E)** Head to trunk angle **(F)** Swim bladder area. Larvae assessed at 30 dpf (n = 18-20 per group).

SUPPLEMENTARY FIGURE 6
Microscopic images of the zebrafish larvae at 30 dpf. Scale bar = 500 µm.

SUPPLEMENTARY FIGURE 7
Oxygen consumption of zebrafish larvae from the 3 study groups. Larvae assessed at 30 dpf (n = 12 per group). Control - CT, soybean - SBM and β-glucan - BG.

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Potential of algae-derived alginate oligosaccharides and β-glucan to counter inflammation in adult zebrafish intestine

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Alginate oligosaccharides (AOS) are natural bioactive compounds with anti-inflammatory properties. We performed a feeding trial employing a zebrafish (*Danio rerio*) model of soybean-induced intestinal inflammation. Five groups of fish were fed different diets: a control (CT) diet, a soybean meal (SBM) diet, a soybean meal+β-glucan (BG) diet and 2 soybean meal+AOS diets (alginate products differing in the content of low molecular weight fractions - AL, with 31% < 3kDa and AH, with 3% < 3kDa). We analyzed the intestinal transcriptomic and plasma metabolomic profiles of the study groups. In addition, we assessed the expression of inflammatory marker genes and histological alterations in the intestine. Dietary algal β-(1, 3)-glucan and AOS were able to bring the expression of certain inflammatory genes altered by dietary SBM to a level similar to that in the control group. Intestinal transcriptomic analysis indicated that dietary SBM changed the expression of genes linked to inflammation, endoplasmic reticulum, reproduction and cell motility. The AL diet suppressed the expression of genes related to complement activation, inflammatory and humoral response, which can likely have an inflammation alleviation effect. On the other hand, the AH diet reduced the expression of genes, causing an enrichment of negative regulation of immune system process. The BG diet suppressed several immune genes linked to the endopeptidase activity and proteolysis. The plasma metabolomic profile further revealed that dietary SBM can alter inflammation-linked metabolites such as itaconic acid, taurochenodeoxycholic acid and enriched the arginine biosynthesis pathway. The diet AL helped in elevating one of the short chain fatty acids, namely 2-hydroxybutyric acid while the BG diet increased the abundance of a vitamin, pantothenic acid. Histological evaluation revealed the advantage of the AL diet: it increased the goblet cell number and length of villi of the intestinal mucosa. Overall, our results indicate that dietary AOS with an appropriate amount of < 3kDa can stall the inflammatory responses in zebrafish.

KEYWORDS

microalgae, macroalgae, prebiotics, metabolomics, RNA seq, β-glucans, gut

1 Introduction

Inflammatory bowel disease (IBD) is a multifactorial disorder characterized by chronic and recurrent episodes of inflammation in specific segments of the intestine. IBD can be instigated by both genetic and environmental factors, but the rise in IBD cases over the last decade suggests the decisive role of diverse environmental factors in the pathogenesis of IBD (1). Moreover, the accelerated incidence of IBD in developing nations is correlated with a high intake of Western diet. Current approaches for the treatment of IBD include the use of different anti-inflammatory drugs (2). The remitting and relapsing nature of the disease necessitates prolonged use of such anti-inflammatory agents, leading to undesirable side effects (3). Diet is an important environmental factor that can be an alternative to drugs, since components such as prebiotics are known to regulate intestinal inflammation by maintaining immune homeostasis. These non-digestible carbohydrates are considered as establishers of beneficial bacteria that can produce bioactive metabolites, such as short-chain fatty acids that provide energy to enterocytes and maintain mucosal integrity (4).

Alginate oligosaccharides (AOS) are natural bioactive compounds and among other bioactivities have anti-inflammatory, antioxidant and prebiotic properties (5). They are produced through chemical or enzymatic digestion of alginates mainly extracted from brown algae. 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 with different M/G ratios and degrees of polymerization. The biological functions of AOS is dependent on the molecular weight (MW) (5). An *in vitro* study has reported that low molecular weight alginates enhance the radical scavenging and immunomodulatory capacities in the gut (6) and AOS < 1 kDa and 1.84 M/G can efficiently scavenge superoxide, hydroxyl, and hypochlorous acid radicals compared to AOS of MW 1 to 10 kDa (7). Furthermore, an *in vitro* study reported that AOS < 1kDa is effective in eliciting lysozyme activity, peroxidase activity, phagocytic capacity and total nitric oxide synthase activity compared to AOS of MW 1-2 kDa or 2-4 kDa (8). Inflammation suppressing ability of AOS has also been described previously; through attenuation of nitric oxide and prostaglandin E_2 production and inactivation of the nuclear-factor kappa B and mitogen-activated-protein-kinase signaling pathways, as reported for mice macrophage cell lines (9) and enhancement of the activity of antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT), as reported for human umbilical vein endothelial cells (10). Dietary AOS can also alter intestinal morphology and barrier function; by increasing the villi length, goblet cell number and mucin-2 (MUC2) expression (11). Furthermore, AOS supplemented diet ameliorated the inflammatory responses in a DSS-induced colitis mice model by reducing the infiltration of neutrophils and level of inflammatory markers (TNF-α, COX-2) and increasing the expression of tight junction proteins Zonula occludens-1 and Occludin (12). We have reported the ability of AOS to increase the abundance of bacteria associated with short chain fatty acid (SCFA) production (13). Most of the previous reports on the anti-inflammatory and antioxidant activities of AOS have been based on *in vitro* studies. Hence, it is essential to generate *in vivo* study-based evidence on the anti-inflammatory potential of AOS using an animal model. Furthermore, *in vivo* studies to understand the effect of molecular weight of AOS on its anti-inflammatory potential has not been explored in detail.

Algal β-(1, 3)-glucan is a known prebiotic derived from the unicellular alga *Euglena gracilis*. Paramylon is the storage polysaccharide in *E. gracilis* and it is a straight-chain β- (1, 3)-glucan (14). Previous studies have reported the anti-inflammatory potential of paramylon; oral administration of paramylon reduced the number of infiltrating $CD3^+$ T-lymphocytes, and decreased expression of *Ccl2* and *Il-11* in the gut of gastric cancer mice model (14). Furthermore, paramylon treatment activated M2 macrophages and downregulated the expression of inflammatory cytokines in the liver of mice (15).

In the present study, intestinal transcriptome and plasma metabolome of zebrafish were profiled to reveal the effects of dietary AOS (*Laminaria* sp.-derived, with varying amounts of < 3kDa fraction). We employed an adult zebrafish intestine inflammation model to understand the efficacy of the macroalga-derived oligosaccharides to counter inflammation. In addition, we investigated the changes caused by AOS and those imparted by a well-known anti-inflammatory product, algal β-(1, 3)-glucan (16).

2 Materials and methods

2.1 Experimental fish

Adult zebrafish (8-month-old AB strain) were used for the experiment. To obtain this stock, the parents were bred in five tanks at the zebrafish facility of Nord University, Norway, following a previously reported protocol (17). Fifteen males and 30 females in each of the five replicate tanks were community bred to obtain 300-400 eggs from each tank. These eggs were kept in E3 medium and incubated at 28 °C in an incubator until hatching i.e., at around 50 h post-fertilization. Larvae at 5-day post -fertilization (dpf) stage were fed *ad libitum* commercial micro diets (< 100 µm particle size, Zebrafeed®, Sparos Lda, Olhão, Portugal). From 15 dpf (advanced larval stage), they were transferred to a recirculatory system and fed micro diets of 100-200 µm particle size (Zebrafeed®). From 30 days post-fertilization, the fish were fed a zebrafish diet (Zebrafeed®) of 300 µm particle size. Upon reaching the 8^{th} month, 250 male zebrafish weighing 300-400 mg were transferred to a freshwater flow-through system (Zebtec Stand Alone Toxicological Rack, Techniplast, Varese, Italy) and acclimatized in 3.5 L tanks of the system. These fish were randomly distributed into 25 tanks (10 fish per tank). The water temperature in the tanks was 28°C; the water flow rate was 2.5 L/h and dissolved oxygen in the tanks ranged between 7-8 ppm (oxygen saturation above 85%). A 14L:10D photoperiod was maintained throughout the 30-day feeding experiment.

2.2 Diet preparation and feeding experiment

Sparos Lda. prepared the five experimental diets (Supplementary Table 1A): One control diet and four soybean-based diets. The control (CT) diet was a fish meal-based diet with high-quality marine protein. Soybean-based (SBM) diet contained 50% (w/w) soybean meal (defatted, protein content 44%) and 11% soy protein isolate; the former is expected to induce intestinal inflammation (16). The product Aquastem™ 300DR (derived from the microalga *Euglena gracilis*) from Kemin, Des Moines, USA was added (2.5%, w/w) to the SBM diet to prepare the β-glucan diet (BG). Likewise, the diets AL and AH were prepared by adding 0.962 and 0.658% (w/w) alginate oligosaccharide, AOS (derived from the macroalga *Laminaria*; Centre d'Etude et de Valorisation des Algues (CEVA), Pleubian, France). The AL diet had a lower overall MW, in particular a higher content of short-chain AOS (over 30% < 3kDa). In addition, AL had 3-10kDa (7%), 10-30kDa (22%) and 30-60kDa (40%) compared to the AH diet that had AOS < 3kDa (3%), 3-10kDa (5%), 10-30kDa (30%) and 30-60kDa (60%). AOS in both AL and AH diets were prepared from the same batch of purified alginates. Hence, they have the same M:G ratio (0.9) and M:G distribution along the polymer (Supplementary Table 1B). Thus, BG, AL and AH diets had all the ingredients of the SBM diet in addition to the respective test compound. The experimental fish were fed daily at 5% body weight (offered manually as three rations at 08:00, 13:00 and 18:00) for 30 days. Fish in 5 replicate tanks were allotted to each of the five study groups.

2.3 Sampling

At the end of the experimental period, the fish were sacrificed by immersing (5 min) in a lethal dose of 200 mg/l of tricaine methanesulfonate (Argent Chemical Laboratories, Redmond, WA, USA) buffered with an equal amount of sodium bicarbonate. Total length and weight of the individual fish from each treatment group were measured and the information is in Supplementary Figure 1. The fish were dissected to collect the posterior intestine (n = 5 per group) and snap-frozen in liquid nitrogen. These samples were later stored in a −80°C freezer until further analyses. Blood drawn by tail ablation (18) was collected in a heparinized tube and centrifuged at 5000 g for 10 min at 4°C to collect the plasma (n = 5 per group; 5 fish from each tank pooled). Intestine samples (n=6-9 per group) were taken to assess the histomorphology.

2.4 RNA isolation, mRNA sequencing and bioinformatic analyses

Total RNA was extracted from the frozen intestine samples using Direct-zol™ RNA MiniPrep (Zymoresearch, CA, USA), following the manufacturer's instructions. The RNA concentration and integrity were determined using Qubit 4 Fluorometer (Thermo Fisher Scientific, Waltham MA, USA) and Tape Station 2200 (Agilent Technologies, Santa Clara, CA, USA). RNA samples exhibiting RIN value ≥ 7 were used for qPCR and preparation of RNA-Seq libraries. Library preparation and sequencing of samples (n=5 for each diet group) were done by Novogene Europe (Cambridge, UK). Messenger RNA was purified from total RNA using poly-T oligo-attached magnetic beads. After fragmentation, the first strand cDNA was synthesized using random hexamers followed by the second strand cDNA synthesis. The libraries were end-repaired, A-tailed, adapter ligated, size selected, amplified, and finally purified. The libraries were quantified by Qubit and real-time PCR and size distribution was checked by bioanalyzer. The barcoded libraries were then pooled at equimolar concentrations and loaded on the Illumina NovaSeq 6000 Sequencing system (Illumina, San Diego, CA, USA) to obtain 150 bp paired end reads. For each sample, an average of 22 million filtered reads were obtained with a minimum of 19.8 million reads per sample. The average mapping percentage of the filtered reads was 86% (Supplementary Table 2). The bioinformatic analysis of the RNA-Seq data was performed following our previously described protocol (16). In brief, the quality of raw reads was assessed using the FastQC command line, and the tool fastp to filter the reads by considering the Phred quality score (Q ≥ 30). The filtered reads were then aligned to the reference zebrafish genome downloaded from NCBI (release 106) using HISAT2, version 2.2.1 with default parameters. Read counts that belong to each gene were generated using featureCounts version 1.5.3. Differential expression of the genes across the treatment groups was determined by DESeq2 and transcripts with $|\text{Log}_2$ fold change$| \geq 1$ and an adjusted p-value < 0.05 (Benjamini-Hochberg multiple test correction method) were considered significantly differentially expressed. The gene ontology (GO) enrichment analyses were performed using the software Database for Annotation, Visualization and Integrated Discovery version 6.8 with a p value of 0.05 and minimum gene count of 2. The packages ggplot2, pheatmap and GOplot in R were employed to present the data.

2.5 qPCR analysis

Genes related to intestinal inflammation, namely *interleukin-1b* (*il1b*), *matrix metalloproteinase-9* (*mmp-9*), *myeloid-specific peroxidase* (*mpx*), *interleukin-10* (*il10*), *chemokine (C-X-C motif) ligand 8a* (*cxcl8a*), *mucin2.1* (*muc2.1*), *mucin5ac* (*muc5ac*) and those of antioxidant enzymes *superoxide dismutase 1* (*sod1*), *glutathione peroxidase 1a (gpx1a), catalase* (*cat*) were selected for qPCR (n = 5 for each diet group) and each reaction was done with technical replicates. One µg of total RNA from each sample was reverse transcribed using the QuantiTect reverse transcription kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The cDNA was further diluted 10 times with nuclease-free water and used as a PCR template. PCR reactions were performed using the SYBR green in LightCycler® 96 Real-Time PCR System (Roche Holding AG, Basel, Switzerland) with the following conditions: initial denaturation at 95°C for 10 min, followed by 35 cycles of 95°C for 20 s, 60°C for 30 s and 72°C for 10 s. We designed the primers for the selected genes using the Primer-BLAST tool in NCBI. The primers were then 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 in Supplementary Table 3. Relative expression of selected genes was determined based on the geometric mean of three reference genes (*eef1a1l1*, *rpl13α* and *actb1*). The data were checked for assumptions of normality (Shapiro-Wilk) and homogeneity of variance (Bartlett's test). Based on the normality and equal variance assumption check results, the statistical difference was determined by Analysis of Variance (ANOVA) or Kruskal-Wallis test. The pair wise comparison between the treatments was done by Tukey's or Dunn test.

2.6 Intestinal histomorphometry

Distal intestine sample were fixed in 3.7% (v/v) phosphate-buffered formaldehyde solution (pH 7.2) at 4°C for 24 h. Standard histological procedures were employed for dehydration, processing, and paraffin embedding as described by Bancroft and Gamble (19). The paraffin blocks thus prepared were sectioned using a microtome (Microm HM3555, MICROM International GmbH, Walldorf, Germany). Four micrometer thick longitudinal sections were cut and mounted on SuperFrost® slides (Menzel, Braunschweig, Germany), and a robot slide stainer (Microm HMS 760×, MICROM International GmbH) was used to stain the slides with Alcian Blue-Periodic Acid Schiff's reagent (AB-PAS, pH 2.5). First, all acid mucins were stained blue with alcian blue, and in the subsequent PAS reaction, only the neutral mucins were stained magenta. Light microscopy photomicrographs were taken with the Olympus BX61/Camera Color View *III*u (Olympus Europa GmbH, Hamburg, Germany) and the photo program Cell P (Soft Imaging System GmbH, Munster, Germany). The ImageJ software (20) was used for analysing the tissue microarchitecture. To understand the histopathological changes, we measured five parameters of the intestine features: number of eosinophils, goblet cell number, goblet cell size, villi length and width of lamina propria.

2.7 Plasma metabolomics

Metabolomic profiling was carried out by MS-Omics (Vedbæk, Denmark). The analysis was carried out using a Thermo Scientific Vanquish LC (Thermo Fisher Scientific, Waltham, U.S.) coupled to Orbitrap Exploris 240 MS (Thermo Fisher Scientific). The company used an electrospray ionization interface as the ionization source. The analysis was performed in positive and negative ionization mode under polarity switching. The ultra-performance liquid chromatography was performed using a slightly modified version of the protocol described by Doneanu et al. (21). Peak areas were extracted using Compound Discoverer 3.2 (Thermo Fisher Scientific). Metabolites in the samples were identified at four levels; Level 1: identification by retention times (compared against in-house standards), accurate mass (with an acceptable deviation of 3 ppm), and MS/MS spectra, Level 2a: identification by retention times (compared against in-house standards), accurate mass (with an acceptable deviation of 3ppm). Level 2b: identification by accurate mass (with an acceptable deviation of 3 ppm), and MS/MS spectra, Level 3: identification by accurate mass alone (with an acceptable deviation of 3 ppm). The obtained metabolomic data were analyzed employing MetaboAnalyst 5.0 (22). The data were log-transformed and auto-scaled (mean-centered and divided by the standard deviation of each variable) before downstream analyses. Principal component analysis was performed using the *mixomics* package in R 4.2.1 to understand the differential clustering of the study groups. Metabolites with a $|\text{Log}_2$ fold change$| \geq 0.6$ and a p value of < 0.05 are reported as significantly altered metabolites. The packages ggplot2 and pheatmap in R 4.2.1 were employed to prepare the illustrations in this article.

3 Results

3.1 Soybean-based diet altered the expression of key genes related to inflammation

To gather evidence on soybean meal-induced inflammatory response in zebrafish, we examined the relative expression of selected inflammatory genes in the intestine of the fish fed a soybean meal-based diet for 30 days. The relative expression of *il1b* was significantly ($p < 0.05$) increased in the SBM and AH groups compared to CT group (Figure 1A). Furthermore, the SBM group had significantly higher expression ($p < 0.05$) of *mmp9* compared to the BG and AH groups (Figure 1B) and significantly ($p < 0.001$) higher expression of *mpx* compared to the CT group (Figure 1C). However, the expression of *mpx* in the BG, AL and AH groups was significantly lower compared to the SBM group. The expression of *cxcl8a* was significantly higher in the SBM group ($p < 0.001$) compared to CT, BG and AH groups (Figure 1D). In addition, we observed significant differences in the expression of the antioxidant genes *sod1* and *cat* (Figures 1E, F); the expression of *sod1* was significantly lower in the AH group compared to the CT, SBM and AL groups and the expression of *cat* was significantly higher in the AL group compared to the CT group. We did not detect any statistically significant differences in the expression of the mucin genes, *muc2.1* and *muc5ac*, the gene of the antioxidant enzyme, *gpx1a*, and the anti-inflammatory gene, *il10* (Figures 1G–J).

3.2 Intestinal transcriptome profile reflected soybean-induced inflammation

To gain a deeper understanding of the effects of soybean meal-induced inflammation, we analyzed the intestinal transcriptome of zebrafish fed the soybean meal-based diet. A comparison of the SBM group with the CT group revealed 141 differentially expressed genes (DEGs), of which 58 were upregulated and 83 were downregulated in the SBM group (Figure 2A and Supplementary Table 4). The principal component analysis (PCA) plot shows the differential clustering of the SBM and CT groups along the first principal component (PC1), which explains 56% variability in the

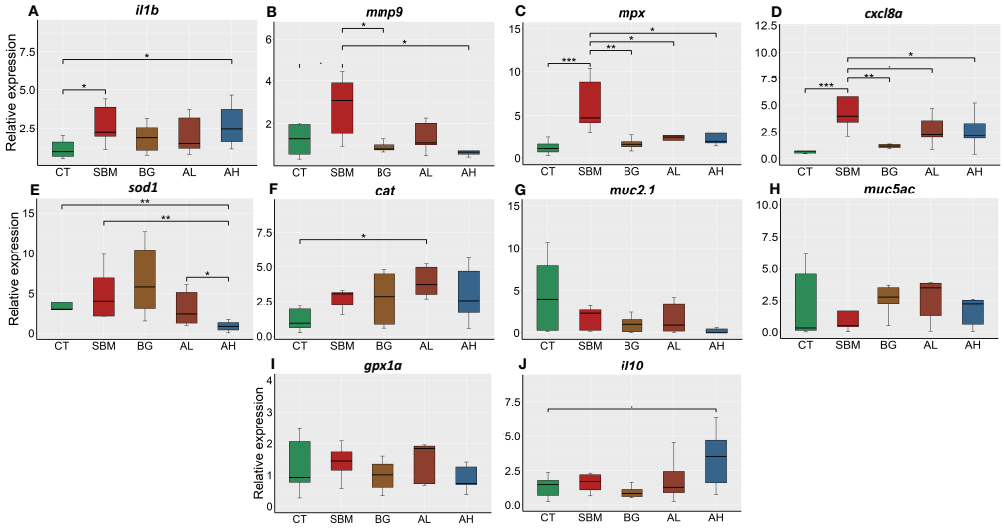

FIGURE 1
Relative expression of immune genes in the intestine of zebrafish fed different diets. **(A)** *interleukin-1b* (*il1b*); **(B)** *matrix metalloproteinase-9* (*mmp9*); **(C)** *myeloid-specific peroxidase* (*mpx*); **(D)** *chemokine (C-X-C motif) ligand 8a (cxcl8a)*; **(E)** *superoxide dismutase 1* (*sod1*); **(F)** *catalase* (*cat*); **(G)** *mucin2.1* (*muc2.1*); **(H)** *mucin5ac* (*muc5ac*); **(I)** *glutathione peroxidase* (*gpx1a*); **(J)** *interleukin-10* (*il10*). CT- control diet; SBM- soybean diet; BG- algal β-glucan; AL- AOS with 31% < 3kDa; AH- AOS with 3% < 3kDa. Asterisk * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, • $p < 0.1$. Each treatment group consisted of five biological replicates.

data (Figure 2B). Hierarchical clustering (Figure 2C) revealed a clear separation of upregulated and downregulated DEGs in the SBM group compared to the CT group.

The GO enrichment analysis based on the upregulated DEGs revealed significant enrichment of several GO terms including those linked to immune system process, endoplasmic reticulum (ER) part, leukocyte chemotaxis, response to stress, response to external stimulus and leukocyte migration (Figure 2D). GO enrichment analysis employing the downregulated DEGs revealed terms like cilium dependent motility, flagellum dependent cell motility, sexual reproduction, alpha-tubulin activity and male gamete generation (Figure 2E).

3.3 Algal β-glucan targets distinct immune-related genes

We performed a comparison of the transcriptome of the BG and SBM groups. Forty-two genes were differentially expressed, of which 16 were upregulated and 26 were downregulated in the BG group (Figure 3A and Supplementary Table 5). The PCA plot shows the differential clustering of the SBM and BG groups along the PC1, which reveals 63% variability in the data (Figure 3B). Hierarchical clustering (Figure 3C) revealed a clear separation of DEGs in the SBM group compared to the BG group. Several downregulated DEGs were immune-related, for example *GTPase IMAP family member-like gimap7* (*LOC799889*), *gimap8* (*LOC103910175*), *lectin, galactoside-binding, soluble, 9 (galectin 9)-like 6* (*lgals9l6*), *matrix metalloproteinase-13a* (*mmp13a*), *chemokine ccl-c17a* (*LOC100002392*), *TIMP metallopeptidase inhibitor 2b* (*timp2b*) and *complement component* (*c7a*). The GO enrichment analysis employing the downregulated DEGs revealed the enrichment of terms like endopeptidase activity, hydrolase activity and proteolysis (Figure 3D). However, the upregulated DEGs did not reveal any significant GO enrichment.

3.4 Specific shift in the expression of immune-related genes caused by AOS

We first compared the intestine transcriptome of the AL group with the SBM group. The analysis revealed 32 DEGs, of which 10 were upregulated and 22 were downregulated in the AL group (Figure 4A). The PCA plot shows the differential clustering of the SBM and AL groups along the PC1, which explains 65% of variability in the data (Figure 4B). Hierarchical clustering (Figure 4C) revealed a clear separation of differentially expressed genes in the SBM group compared to the AL group. Several downregulated DEGs were immune related; *chemokine (C-C motif) ligand 36, duplicate 1* (*ccl36.1*), *intelectin 3* (*itln3*), *CD59A glycoprotein-like* (*LOC103910140*), *aquaporin 1a* (*aqp1a.1*), *NLR family CARD domain-containing protein 3-like* (*LOC101882744*),

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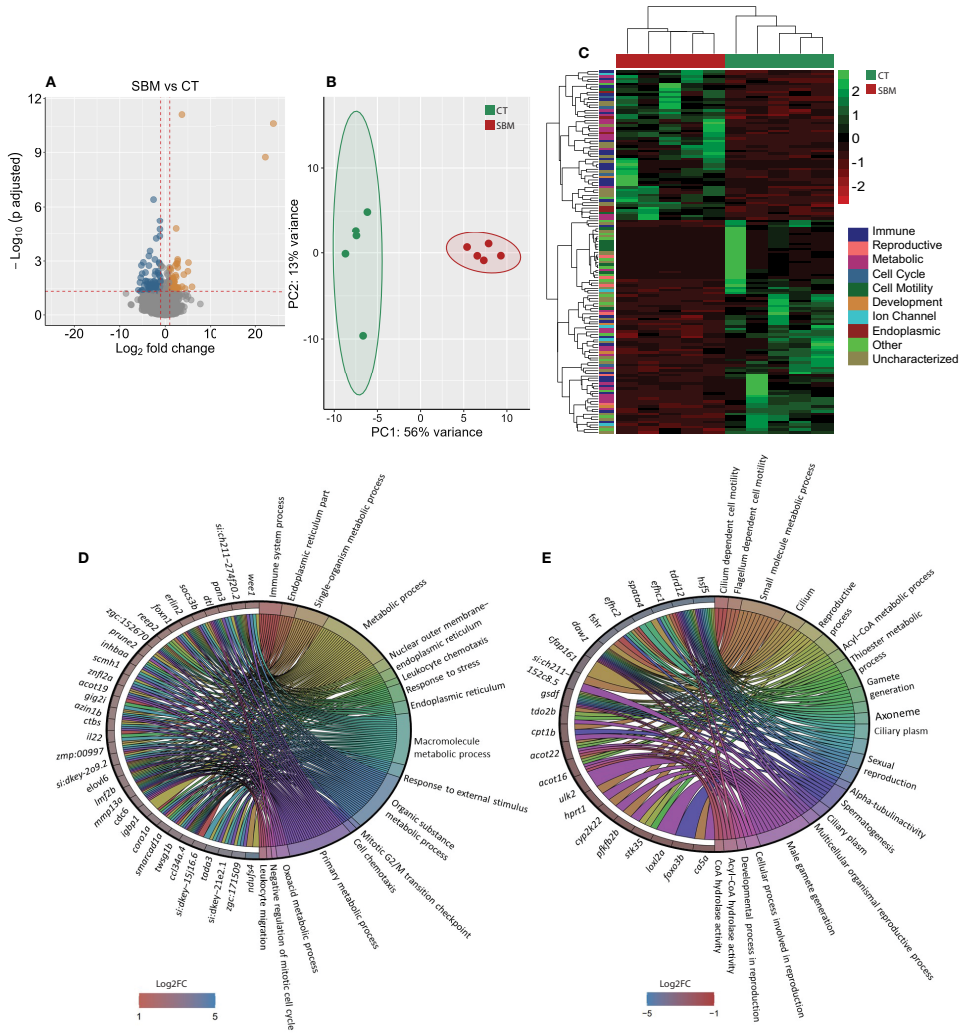

FIGURE 2
Transcriptome-based differences in the intestine of zebrafish from the soybean group compared to the control group. Volcano plot **(A)**, PCA plot **(B)** and heatmap **(C)** of the differentially expressed genes in the soybean (SBM) group compared to the control (CT) group. Chord diagram showing the link between the enriched GO terms in the soybean group and the associated genes that were upregulated **(D)** and downregulated **(E)** in the soybean (SBM) group compared to the control (CT) group. The enriched GO terms are colour-coded and on the right side of the circles one finds the differentially expressed genes contributing to the enriched GO terms that are shown on the left of the circles. The gradient colour bar intensity varies with the Log2 fold change (adjusted p-value < 0.05 and |Log2 fold change| ≥ 1). There are five biological replicates in each study group.

myeloid-specific peroxidase (*mpx*), *c7a*, *G protein-coupled receptor* 142 (*gpr142*) and *matrix metalloproteinase-25b* (*mmp25b*) (Figure 4C and Supplementary Table 6). GO enrichment analysis employing the downregulated DEGs revealed terms like response to stress, inflammatory response, immune response, humoral immune response and complement activation (Figure 4D). The upregulated DEGs based analyses revealed the enrichment of intracellular organelle lumen (Supplementary Table 7).

Comparison of the AH group with the SBM group revealed 20 DEGs of which 10 were upregulated and 10 were downregulated in the AH group (Figure 4E and Supplementary Table 8). The PCA plot shows the clustering of samples into SBM and AH groups. The

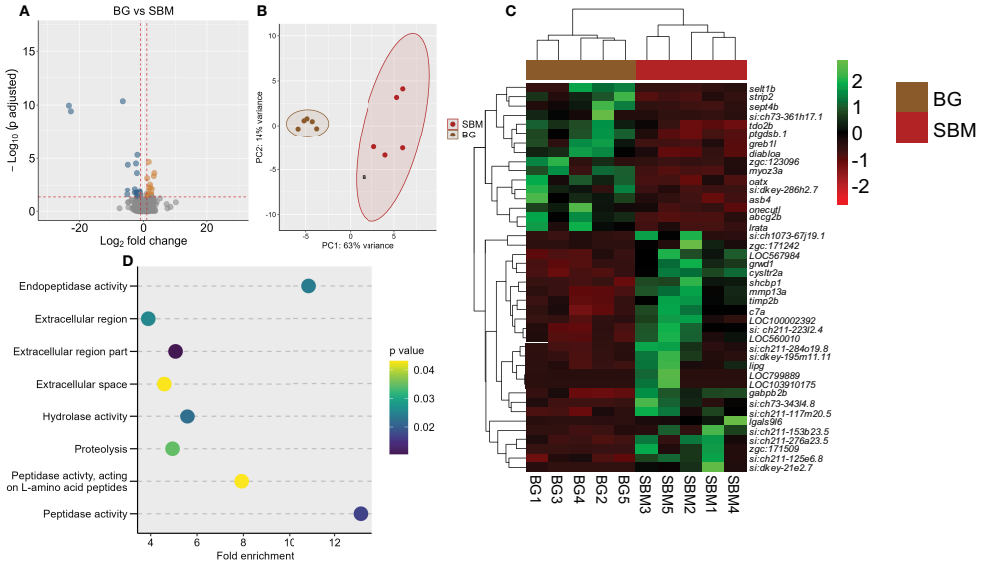

FIGURE 3
Transcriptome-based differences in the intestine of zebrafish from the algal β-glucan group compared to the soybean group. Volcano plot **(A)**, PCA plot **(B)** and heatmap **(C)** of the differentially expressed genes in the algal β-glucan (BG) group compared to the soybean (SBM) group. Transcripts with an adjusted p-value below 0.05 and $|\text{Log}_2 \text{ fold change}| \geq 1$ were considered as significantly differentially expressed. Dot plot **(D)** showing enriched GO terms in the BG group based on the genes that were differentially downregulated compared to the SBM group. The gradient colour bar intensity of GO terms varies with the p value of each GO term. There are five biological replicates in each study group.

clusters are separated from each other along the PC1, which explains 63% of variability in the data (Figure 4F). Hierarchical clustering (Figure 4G) revealed a clear separation of up- and downregulated DEGs in the AH group compared to the SBM group. The downregulated DEGs-based GO analysis revealed enrichment of negative regulation of immune system processes (Supplementary Table 9). Our analysis did not detect a significant GO term enrichment based on the upregulated DEGs. The upregulated DEGs were immune related: *B-cell receptor CD22 (LOC100151328), NLR family CARD domain-containing protein 3-like (LOC108183498), Fc receptor-like protein 5 (LOC101886098)* and *macrophage mannose receptor 1-like (LOC100331140).* The downregulated DEGs were, among others, *interleukin 26* (*il26*), *adhesion G protein-coupled receptor E15* (*adgre15*), *lectin galactoside-binding, soluble, 9 (galectin 9)-like 6 (lgals9l6)* and *CD59 glycoprotein-like (LOC103910140)* (Figure 4H).

To find out if the AOS has the capacity to shift the expression of genes that were altered by SBM, we examined the common DEGs from the transcriptome comparisons, viz. SBM vs. CT as well as AL vs SBM and AH vs SBM (Supplementary Figure 2 and Figures 5A, B). Here we list the DEGs that had contrasting expression patterns in the abovementioned comparisons. The expression of four upregulated DEGs in SBM vs CT comparison, namely *fin TRIM family, member 37* (*LOC567984*), *CD59A glycoprotein-like* (*LOC103910140*), *cell division cycle 6 homolog* (*cdc6*), *alcohol dehydrogenase 5-like* (*zgc:63568*) and four downregulated DEGs in SBM vs CT comparison namely *RAS like family 11 member B* (*rasl11b*), *HECT and RLD domain containing E3 ubiquitin protein ligase 56.3* (*zgc:163136*), *si:cabz01076231.1*, *RNA polymerase II subunit* C (*polr2c*) were shifted in the AL group to a level almost similar to that in the control group (Supplementary Figure 2 and Figures 5C, D). On the other hand, the expression of five upregulated DEGs in SBM vs CT namely *NADH:ubiquinone oxidoreductase subunit S4* (*ndufs4*), *CD59A glycoprotein-like* (*LOC103910140*), *fibroblast growth factor binding protein 1b* (*fgfbp1b*), *alcohol dehydrogenase 5-like* (*zgc:63568*), *acyl-CoA thioesterase 19* (*acot19*) and four downregulated DEGs in SBM vs CT comparison namely *Fc receptor-like protein 5* (*LOC101886098*), *B-cell receptor CD22* (*LOC100151328*), *si:dkey-286h2.7*, *RNA polymerase II subunit* C (*polr2c*) were shifted in the AH group to levels almost similar to those in the control group (Figures 5E, F). Venn diagram reveals the two common downregulated DEGs (*LOC103910140*, *zgc:63568*) (Figures 5C, E) and one common upregulated (*polr2c*) (Figures 5D, F) DEG in the AL and AH diet groups compared to the SBM group, respectively.

A direct comparison of AL versus AH group revealed 14 DEGs, of which 2 were upregulated and 12 were downregulated in the AL group. Among the downregulated DEGs, immune genes like *ccl36.1* had a 12-fold and *C-reactive protein -6* (*crp6*) had a 5-fold downregulation in the AL group compared to the AH group (Supplementary Table 10).

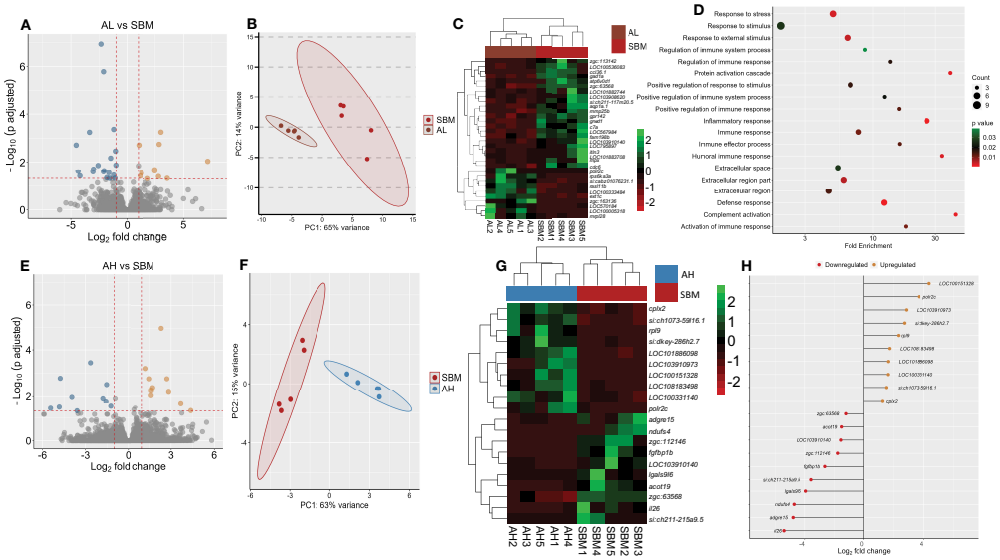

FIGURE 4
Transcriptome-based differences in the intestine of zebrafish fed AOS with 31% <3kDa and AOS with 3% <3kDa compared to the soybean group. Volcano plot **(A)**, PCA plot **(B)** and heatmap **(C)** of the differentially expressed genes in the AL (AOS with 31% <3kDa) group compared to the soybean (SBM) group. Dot plot **(D)** showing the enriched GO terms in the AL group based on the differentially downregulated genes compared to the SBM group. The gradient colour bar intensity of GO terms varies with the p value of each GO term. Volcano plot **(E)**, PCA plot **(F)**, heatmap **(G)** and differentially expressed genes **(H)** obtained by comparing the AH (AOS with 3% < 3kDa) group with the soybean (SBM) group. Transcripts with an adjusted p value below 0.05 and $|\text{Log}_2 \text{ fold change}| \geq 1$ were considered as significantly differentially expressed. There are five biological replicates in each study group.

Furthermore, these 2 DEGs caused the enrichment of the GO term "response to virus" (Supplementary Table 11).

3.5 Soybean-based diets (both with and without glucans or AOS) altered the plasma metabolome

To gain deeper insights into the impact of different dietary treatments, we compared the plasma metabolome of the various treatment groups. We identified a total of 71 metabolites (level 1). Partial least squares discriminant analysis revealed a group-based clustering of the samples (Supplementary Figure 3). Comparison of the SBM group with the CT group revealed aldopentose, ethylmalonic acid, xanthine, itaconic acid, 2-(hydroxymethyl) butanoic acid, citrulline, ornithine, taurochenodeoxycholic acid and trigonelline as the significantly altered metabolites (Figures 6A, B; Supplementary Figure 4 and Supplementary Table 12). The pathway analysis using these nine significantly altered metabolites identified arginine biosynthesis as the significantly enriched pathway (Figure 6C). Comparison of the BG group with the SBM group revealed pantothenic acid and isocitric acid as the significantly altered metabolites (Supplementary Figure 5A and Supplementary Table 13). Furthermore, the AL group versus the SBM group revealed 2-hydroxybutyric acid as the significantly abundant metabolite (Figure 6D; Supplementary Figure 5B and Supplementary Table 14). We did not find any significantly altered metabolite from the AH vs SBM comparison.

3.6 AOS altered the intestinal histomorphology

We investigated histological changes in the intestine of zebrafish to understand the effect of different diets (Figure 7A). We found a significantly higher number of goblet cells per villi ($p < 0.05$) in the AL group compared to the CT and SBM groups (Figure 7B). We also found an increase in the villi length in the AL group compared to the CT and BG groups (Figure 7C). The diets seem to have no effect on goblet cell size, eosinophils and lamina propria width in zebrafish (Figures 7D–F).

4 Discussion

Prebiotics are often administered through diet to obtain a "synergistic or complementary synbiotic" effect, and currently, scientists are gathering evidence on the IBD-alleviating potential of

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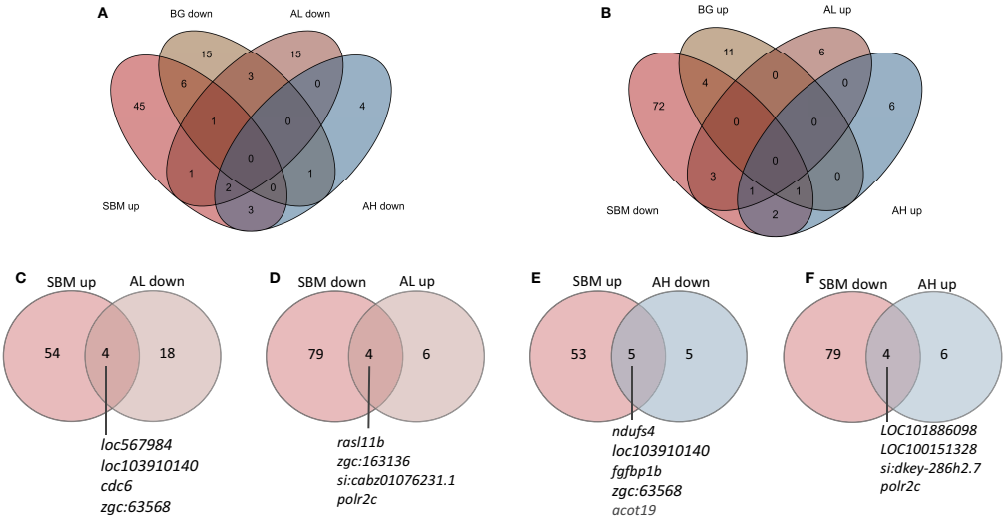

FIGURE 5
Venn diagrams showing the total number of genes that were altered by the experimental diets. The genes that were differentially upregulated in the SBM vs CT comparison **(A)** but downregulated in the BG, AL and AH vs SBM comparisons. The genes that were differentially downregulated in the SBM vs CT comparison **(B)** but upregulated in the BG, AL and AH vs SBM comparisons. Genes that were altered in the SBM group had mRNA levels in the AL **(C, D)** and AH **(E, F)** groups, respectively, similar to that in the control group.

this approach. The belief is that dietary prebiotics change the composition of intestinal microbiota, which influences mucosal as well as systemic immune responses in a host (23). In one of our previous studies (13), we profiled the intestinal bacterial communities in Atlantic salmon fed two levels of AOS (0.5 and 2.5%). We reported the potential ability of 0.5% AOS to stimulate the proliferation of bacteria with SCFA-producing capacity. The same product was added to the AL diet of the current study. For comparative purposes, we formulated the AH diet that contained an AOS with a lower proportion of < 3kDa. The two products were incorporated at 0.962% (AL) and 0.658% (AH) (both w/w) into the diet of zebrafish, taking into consideration the content of the active component. We performed an *in vivo* study to compare the anti-inflammatory effects imparted by two AOS products (with 31% < 3kDa and with 3% < 3kDa), using an intestine inflammation model in adult zebrafish. We targeted the transcriptome and metabolome of the fish to evaluate the anti-inflammatory potential of AOS. We have also studied the transcriptome and metabolome of zebrafish fed an algal β-glucan that we studied previously (16).

The generated transcriptomic and metabolomic profiles revealed the distinct responses evoked by the products (based on the comparison with the SBM group). Downregulated DEGs-based enriched GO terms of the AL group were complement activation, inflammatory response and humoral response, compared to the negative regulation of the immune system in the case of the AH group. The significantly abundant plasma metabolite in the AL group was 2-hydroxybutyric acid. Histological evaluation indicated that the AL group had more goblet cells and longer intestinal villi.

4.1 Dietary soybean meal altered the expression of genes linked to inflammation, endoplasmic reticulum, reproduction and cell motility

Soybean meal contains several anti-nutritional factors (ANFs) including saponins, lectins, isoflavones, and β-conglycinin (24). These ANFs can hamper growth, reduce digestive enzyme activity, and alter the gut mucosal integrity to induce inflammation. Such an inflammatory response could be due to soy saponins as reported in fish studies (25, 26). Increased granulocyte recruitment and higher expression of inflammatory marker genes (*il1b* and *il8*) were the characteristics described in zebrafish larvae fed a diet containing soybean meal and soy saponin (27). In our previous studies, we found that the dietary soybean meal (50% inclusion) affected barrier-related genes in the intestine of juvenile zebrafish (28) and soy saponin developed inflammation features such as increased lamina propria width, infiltration of immune cells, and increased expression of genes related to antimicrobial peptides and ion transport in the intestine of Atlantic salmon (26). In the present study, the expression of inflammatory marker genes (*il1b*, *mpx*, *cxcl8a*) were upregulated in the SBM group. The proinflammatory cytokine IL-1β is secreted by innate immune cells and is an important mediator of inflammatory response (29). The chemokine CXCL8A is a neutrophil chemoattractant that stimulates the migration of neutrophils from blood to the inflamed sites. Granulocytes (mainly neutrophils) are the first responders that migrate to an inflamed site and a high

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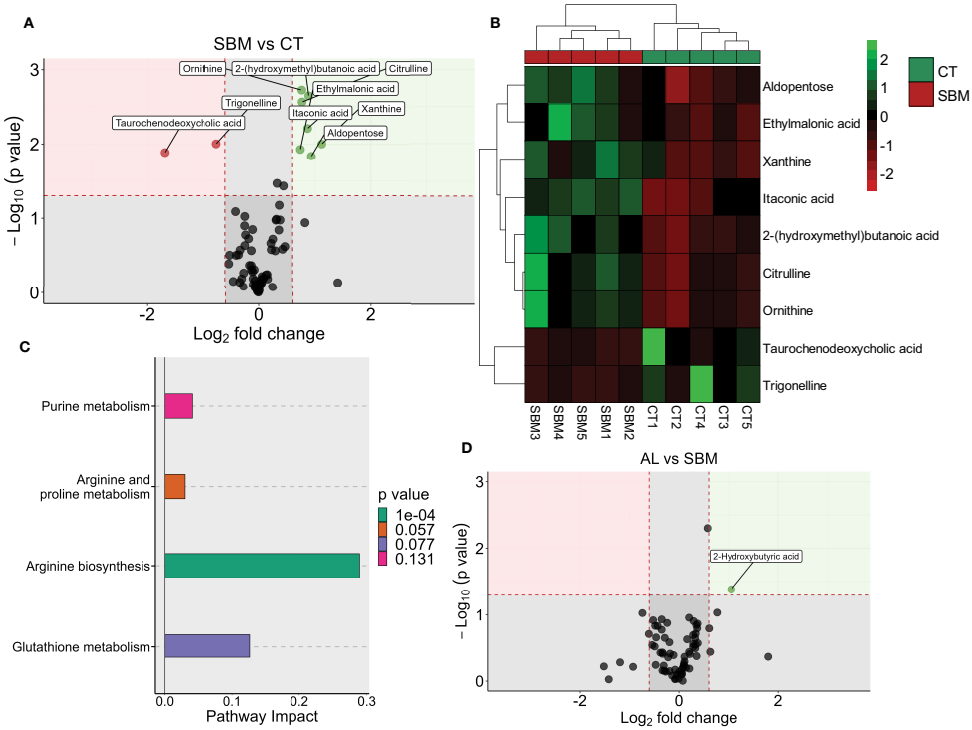

FIGURE 6
Diet-induced changes in plasma metabolites of zebrafish. Volcano plot **(A)**, heatmap **(B)** and enriched KEGG pathways **(C)** by the differentially altered metabolites in the soybean (SBM) group compared to the control (CT) group (|Log_2 fold-change| ≥ 0.6, p value < 0.05). Volcano plot **(D)** showing the differentially abundant metabolites in the AL group (AOS diet with 31% < 3kDa) compared to the soybean (SBM) group. There are five biological replicates in each study group.

concentration of granulocytes represents a transition from an acute phase to a chronic inflammatory state (30). We also gathered evidence on increased presence of neutrophils in the intestine of larval zebrafish (16) and in the present study the expression of the neutrophil marker *mpx* was elevated by the SBM diet. Moreover, MPX was found to be involved in the production of ROS in the mucosa of patients suffering from intestinal inflammation (31). The MMPs secreted by neutrophils degrade the extracellular matrix, facilitating the transendothelial migration of neutrophils to the inflamed sites (32). In the present study, several inflammation-related GO terms like leukocyte chemotaxis, leukocyte migration were enriched in the SBM group with significantly upregulated immune genes (*mmp13a*, *coro1a*, *il22*, *ccl34a.4*, *cd59*, *foxn*, *gig2i*). The metalloprotease gene *mmp13* codes for an endopeptidase that plays a critical role in intestinal epithelial barrier disruption and is therefore considered a potential therapeutic agent for treating IBD (33). LPS-induced goblet cell depletion, ER stress, permeability and tight junction alterations were reduced in the gut of *Mmp13* knockout mice (33). The gene *il22* codes for a cytokine that regulates the intestinal barrier integrity and its expression was altered during inflammation (34). A previous study on juvenile Jian carp (*Cyprinus carpio* var. Jian) reported that soybean β-conglycinin can also cause intestinal damage and induce inflammation and oxidative stress as a result of the elevated expression of inflammatory cytokine *il-8*, *tumor necrosis factor-α* (*tnf-α*), and *transforming growth factor-β* (*tgf-β*) genes and reduction of the anti-oxidant enzymes SOD and CAT (35). Hence, the negative effects of soybean meal can be compounded by the actions of all the antinutritional factors. For instance, soybean lectins can potentiate the detrimental effects of saponin on epithelial barrier function (36). Furthermore, dietary soybean meal can also have other metabolic effects like altering the cholesterol metabolism and hampering reproductive development (37, 38). In the present study, several downregulated DEGs in the SBM group significantly enriched the GO term sexual reproduction. These results corroborated those reported in our previous article; 50% soybean meal feeding altered genes related to reproduction and cholesterol metabolism in zebrafish (16, 28). This could be attributed to isoflavones present in soybean meal, which can bind to oestrogen receptors (39). As reported in previous studies, alteration in the membrane cholesterol by soy saponin might have affected cell motility and lipid metabolism by influencing the functioning of ER (40, 41). Furthermore, dietary soybean meal can

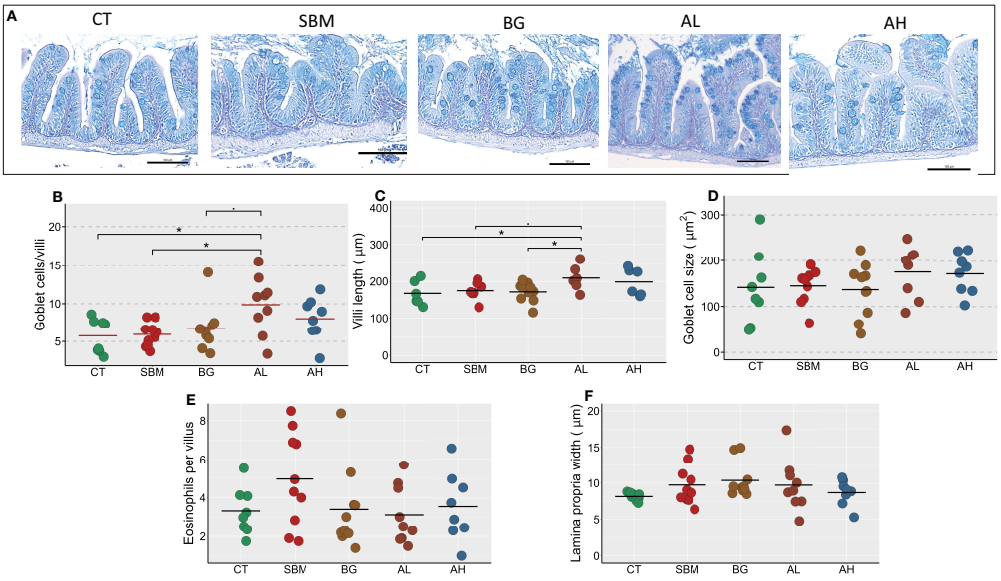

FIGURE 7
Diet-induced histomorphological changes in the intestine of zebrafish. Representative histological images **(A)** showing the changes in tissue architecture of the intestine of zebrafish stained with AB-PAS. Scale bar = 100 µm. The measured parameters include **(B)** Goblet cells per villus **(C)** Villi length **(D)** Goblet cell size **(E)** Eosinophils per villus **(F)** Lamina propria width. Horizontal bars indicate mean values. * indicates $p < 0.05$ and $p < 0.1$ (n = 6-9 per group). CT- control diet; SBM- soybean diet; BG- algal β-glucan diet; AL- AOS diet with 31% < 3kDa; AH- AOS diet with 3% < 3kDa.

increase the rate of respiration (16), thereby increasing the production of reactive oxygen species (42) and aggravating the inflammatory response (43). Soybean meal diet increased the oxygen consumption (16) and altered the genes related to oxidoreductase activity in zebrafish (28). Thus, the intestinal inflammatory response to soybean meal can be a direct effect of anti-nutritional factors or due to cumulative metabolic changes caused by multiple factors in the soybean diet.

4.2 Distinct changes in the intestine of adult zebrafish fed soybean meal and algal β-glucan or AOS

Defects in the barrier function caused by intestinal structural changes can increase luminal antigen penetration and the associated chemokine-induced recruitment of neutrophils. We found that the expression of genes associated with neutrophil recruitment (*mpx* and *cxcl8a*) and barrier disruption (*mmp9*) was downregulated in the AOS and BG fed groups. The expression of proinflammatory cytokine *il1b* was upregulated in the SBM and AH group. Conversely, the expression of *il1b* was not altered in the AL group compared to the control group suggesting an immune modulation in the zebrafish intestine by the AL diet. Furthermore, in the AL group the downregulated DEGs (*cd59, c7a, mpx, ccl36.1, itln3, aqp1a.1, nlrc3, gpr142 and mmp25b*) enriched the GO terms inflammatory response, complement activation and humoral immune response. Note that *mpx* and *mmp* (*mmp25b*) were downregulated in the AL group. Furthermore, the expression of the gene, *cat* was upregulated in the AL group, and this antioxidant is a key regulator of ROS generated during inflammatory conditions (44). It should be noted that catalase activity was lower in patients suffering from intestinal inflammation (45) and catalase administration can reduce ROS levels and ameliorate inflammation, as shown in colitis mice models (44). Intestinal epithelial cells are sources of complement components and appropriate regulation of complement activation is essential to prevent intestinal epithelial cell damage. Increased complement activation has been associated with the pathogenesis of IBD (46). The C7A protein is part of the membrane attack complex (MAC), and the downregulation of gene expression of this component points to the prevention of complement activation. Therefore, the suppression of several processes related to inflammation by the downregulated DEGs and the increase in the antioxidant gene *cat* in the AL group suggest the ability of AOS (AL) to reduce the intestinal inflammation induced by the dietary soybean.

Conversely, in the AH group, we found one GO term, viz. negative regulation of immune system process, enriched by the downregulated DEGs (*lgals9l6*, *CD59 glycoprotein-like*). The downregulated DEG in the AH group, *lgals9l6* that codes for protein galactoside-binding, soluble, 9 (galectin 9)-like 6, is an ortholog of human *LGALS9* (galectin 9/Gal-9). Gal-9, a β-

galactoside binding lectin with a carbohydrate recognition domain, is expressed in human crypt cells and its expression is lowered in IBD patients (47). Furthermore, mice lacking *gal-9* were reported to have impaired intestinal mucosal antigen-specific IgA response and were more susceptible to developing watery diarrhoea (47). Because CD59 prevents the activation of the complement system and the associated assembly of MAC, the decrease in epithelial expression of CD59 in IBD patients renders the epithelial cells prone to complement lysis and may lead to destruction of gut epithelium (48). Furthermore, the comparison of AH group with the SBM group revealed the upregulation of several immune genes (*il26*, *cd22*, *nlrc3*, *cd206*). The gene *il26* is a mediator of inflammation and is overexpressed in activated or transformed T cells (49). The protein CD22 is abundantly expressed on the cell surface of activated B-lymphocytes and it can negatively regulate lamina propria eosinophil levels, as in the case of mice (50). Based on these facts, we speculate that the AL group is effective in reducing inflammation.

Venn diagrams created to understand the differential effects of AL and AH on the expression of genes in zebrafish intestine revealed that the results of the AL vs SBM comparison was distinct from those of AH vs SBM comparison. We found only three common DEGs (*loc103910140*, *zgc:63568*, *polr2c*) in the two comparisons; two (zgc:63568 and *loc103910140*/*CD59*) of these were downregulated DEGs and one was an upregulated (*polr2c*) DEG. As mentioned before, the protein CD59 prevents the complement activation and MAC formation. We performed a direct transcriptomic comparison of the AL group with AH group to delineate further the specific effects of the two products. This comparison revealed the downregulation of *ccl36.1* and *crp6* in the AL group. The zebrafish gene *crp6* is an ortholog of human *CRP*, which is used as a biomarker of systemic inflammation and has been reported as a valuable marker of IBD (51). In our previous study, we have reported that the positive effects of yeast-derived β-glucan on soybean meal-induced inflammation could also be due to a downregulation in the expression of *ccl36.1* (28). In the present study, dietary algal β-glucan downregulated the DEGs (*zgc:171509, timp2b, lipg, mmp13a, c7a*) linked to the GO terms like endopeptidase activity and proteolysis. We found that the immunostimulant can suppress the expression of *mmp13a* while the expression of *mmp13* was upregulated in Atlantic salmon infested with sea lice in response to chronic tissue damage (52). The genes *mmp13* and *timp2* have an essential role during tissue remodelling because the expression of the molecules determine the intricate extracellular matrix turnover (53, 54). Therefore, these genes could be markers of tissue damage caused by the dietary soybean. Furthermore, as noted in this study on adult fish, in larval zebrafish also algal β-glucan reduced endopeptidase and proteolytic activity (16).

4.3 AOS diet altered the histological architecture of the intestine

We studied five histomorphological parameters of the intestine and found that the AL group had longer villi with significantly higher number of goblet cells. Goblet cells are responsible for the synthesis, storage, and release of intestinal mucin proteins. Mucus production is an indication of a healthy barrier function as it restricts the entry of pathogens and unwanted luminal antigens into the intestine. It is reported that oligosaccharides can support the mucosal barrier function by stimulating intestinal goblet cells to produce more mucus (55). More mucus cells per villi is an indication that more intestinal cells differentiate into goblet cells to reinforce the barrier. We noted changes specific to the AL group. AL diet fed fish had longer villi and more goblet cells. More goblet cells, longer villi and an increase in the villus height-to-crypt depth (V:C) ratio were reported in a study on AOS diet fed pigs that had better growth (11). β-glucans increased the V:C ratio as well as the average body weight of broiler chicken (56). Mannan oligosaccharide enhanced the growth, increased the villi height and decreased the intestine crypt depth of the juvenile striped catfish (*Pangasianodon hypophthalmus*) (57). There are not many reports on AOS induced alteration in V:C ratio and its correlation with the growth of fishes. Since zebrafish lacks intestinal crypts (58), we cannot relate the growth to the V:C ratio. Nevertheless, increased villus height has been associated with increased nutrient absorption, higher transport of nutrients and improved growth in mammals and fish (59, 60). However, the AL diet did not stimulate the growth of zebrafish. Our previous study on the larval zebrafish model also did not reveal any effect of the SBM and β-glucan diets (also used in the present study) on the standard length (16). Conversely, SBM diet caused several developmental defects like impairment of eye, swim bladder and skeletal deformities in the larval zebrafish (16). Zebrafish is known to have a determinate growth (46) and therefore fish of age 20–40 dpf is considered suitable for a reliable growth study. During this period, energy is predominantly allocated for rapid growth and this time window permits a 40-fold increase in body weight (61). However, in our study we did not find any changes in the growth of the AL group as the feeding experiment was conducted using adult zebrafish. A previous study has also reported that inclusion of soybean meal can stimulate an inflammatory response in the intestine without any effect on the growth of zebrafish (62).

4.4 Plasma metabolites indicate soybean meal-induced inflammation, and AOS and algal β-glucan induced SCFA and vitamin production

To our knowledge, this is the first study on the metabolites of zebrafish plasma. Plasma metabolomics can give indications of the systemic perturbations caused by intestinal inflammation (39). Only few metabolites have been detected in plasma from zebrafish due to the small sample amount that can be retrieved from the fish. A comparison of the SBM group with the control group yielded 9 differentially abundant metabolites, out of the 71 detected metabolites. Among the altered metabolites, itaconic acid was significantly abundant in the SBM group compared to the control group. Itaconic acid is considered a biomarker of inflammation, and M1 macrophages are known to produce

substantial amounts of itaconate (63). Furthermore, itaconate concentration was markedly increased during lipopolysaccharide- and interferon-γ-induced activation of mammalian macrophages (64), probably due to polarization of macrophages to their M1 phenotype. We also found a decrease in the metabolite taurochenodeoxycholic acid (TCDCA) in the SBM fed group. TCDCA, the secondary bile acid that is conjugated with taurine, is a derivative of the primary bile acid chenodeoxycholic acid. Secondary bile acids are microbiota-associated metabolites and studies have reported an increase in primary bile acids and a reduction in secondary bile acids in IBD patients (65). Furthermore, the amino acid residues in soybean protein have a high bile acid-binding ability and can suppress enterohepatic circulation, even in fishes (66). Therefore, the decreased concentration of TCDCA in plasma is also likely due to soybean feeding. On the other hand, arginine nitric oxide and arginine urea pathways are implicated in the pathogenesis of IBD; in the former case NOS2 (the inducible form of nitric oxide synthase [iNOS]) metabolizes L-arginine to NO and L-citrulline and in the latter arginases (ARG1 and ARG2) catalyse the conversion of arginine to urea and ornithine. The abundance of ornithine and citrulline was higher in the plasma of zebrafish fed the inflammation-inducing diet, as reported for IBD patients (67). However, pathway analysis using metabolites with significantly higher abundance in SBM group detected arginine biosynthesis as the significantly enriched pathway. Our results point to the enrichment of the arginine biosynthesis pathway and upregulation of ornithine and citrulline. Increase of arginine biosynthesis in the SBM group could be a compensatory response in the body due to decreased arginine availability associated with the inflammatory response (68).

SCFAs are produced by microbial fermentation of non-digestible carbohydrates in the posterior segment of the intestine of mammals (69). We found significantly higher levels of 2-hydroxybutyric acid (2-HB) in the plasma of the AL group. While a high fat diet decreased the abundance of 2-HB in the serum of mice, dietary polysaccharide increased the metabolite (70). The same study reported that pre-treatment of macrophages with 2-HB can significantly decrease LPS-induced up-regulation of TNF-α. In the present study, proinflammatory genes were downregulated in the AL group with a high abundance of 2-HB. Interestingly, we found that AOS (AL) can elevate 2-HB, which was positively correlated with *Alloprevotella* in another study (71). Furthermore, dietary AOS could increase the abundance of *Alloprevotella* and butyric acid which are positively correlated (72). The AL and AH groups exhibited distinct transcriptomic and metabolomic responses although the two diets differed only in terms of the percentage of the low molecular weight fraction (AL, with 31% < 3kDa and AH, with 3% < 3kDa). Our results indicate that this difference can affect the immune modulatory and prebiotic potential of the diet; the AL diet was more effective in reducing the intestinal inflammation compared to the AH diet. Low molecular weight polysaccharides are more soluble and have greater fermentability (73). Low and high molecular polysaccharides are utilized by different intestine bacteria (74), and the former type is fermented faster to produce SCFAs and have better prebiotic potential (75). This could be the reason for the detection of a SCFA in the plasma of the AL group.

A comparison of the BG group with the SBM group indicated an increase in the abundance of pantothenic acid also known as vitamin B5 (VB5). A previous study has found an inverse correlation between dietary VB5 intake and serum CRP concentration (marker of inflammation) in humans (76). Although we observed a downregulation of *crp6* in the AL group compared to the AH group, such changes were not noted for the BG group. A study on mice revealed that VB5 could enhance the phagocytosis of macrophages to reduce the pathogen load in macrophages (77). In addition, *in vitro* studies have shown that VB5 can increase glutathione levels in cells, suggesting a role of VB5 as an antioxidant to reduce cell damage (78). Although previous studies have not indicated a connection between pantothenic acid and dietary β-glucan, it is possible that algal β-glucan might have stimulated the proliferation of gut microbes such as *Bacteroides fragilis*, *Prevotella copri* and *Ruminococcu*s spp. that possess the genes to synthesize vitamin B5 (79).

5 Conclusion

Dietary soybean affected both the expression of inflammatory marker genes (*il1b, mpx, cxcl8a*) and metabolites like itaconic acid and taurochenodeoxycholic acid in the intestine of zebrafish. Conversely dietary AOS with a higher percentage of low molecular weight reduced the expression of several inflammatory marker genes, increased goblet cell number, villi height and a SCFA in the plasma. The BG diet suppressed several immune genes linked to the endopeptidase activity and proteolysis, suggesting a possible role of algal β-glucan in controlling the tissue damage caused by dietary soybean. In the future, it would be interesting to study the impact of structurally different AOS on the microbiota composition and SCFAs in zebrafish and explore the synergetic effect of AOS and algal β-glucan in reducing soybean induced intestinal inflammation.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found here: SRA Bioproject, PRJNA896758.

Ethics statement

The approval for the conduct of this study was obtained from the Norwegian Animal Research Authority, FDU (Forsøksdyrutvalget ID-22992). We adhered to the rules and regulations regarding the research on experimental animals; FOR-2015-06-18-761. Also, the biosafety rules and regulations stipulated by the Health, Safety and Environment system of the Faculty of Biosciences and Aquaculture, Nord University, were followed during the experiment.

Author contributions

SR, VK, and JD designed the study. RP provided AOS products, KM supplied the algal β-glucan and helped to plan the study. JD formulated and prepared the experimental feeds. SR and AG performed the feeding experiment. AG, SR, and YA did the sampling. SR did the molecular analyses. AG and SR performed the bioinformatic analysis. SR and VK wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Author JD was employed by company SPAROS Lda., RP by CEVA and KM by Kemin Aquascience.

The remaining 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/fimmu.2023.1183701/full#supplementary-material

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It is widely recognized that intestinal inflammation is a major health problem in humans. Certain dietary components can evoke an inflammatory status in the intestine due to their ability to disrupt the intestinal barrier. On the other hand, some natural bioactive compounds can prevent the development of intestinal inflammation. This PhD project elucidated the molecular characteristics and extraintestinal effects of soybean meal-induced intestinal inflammation in zebrafish. The ability of dietary ß-glucans (yeast and alga-derived) and alginate oligosaccharides to counter soybean meal-induced inflammation in zebrafish was also studied to reveal the efficacy of the bioactive compounds. Transcriptome and metabolome-based studies were employed to delineate the markers of inflammation. Furthermore, soybean meal-induced inflammation was associated with defects in development and locomotor activity of zebrafish. The interesting finding is that algal ß-glucans and alginate oligosaccharides restored the expression of inflammation marker genes altered by soybean meal diet. The algal ß-glucan prevented the developmental defects and normalized the soybean meal diet-induced changes in the locomotor behavior of zebrafish. The alginate oligosaccharides and algal beta-glucans could elevate the abundance of a short chain fatty acid and vitamin in the plasma of zebrafish, respectively. The PhD project gave insights into diet-induced inflammatory features and distinct modes of action of ß-glucan and alginate oligosaccharides to counteract inflammation and associated extraintestinal manifestations.

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