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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$|$ ≥ 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

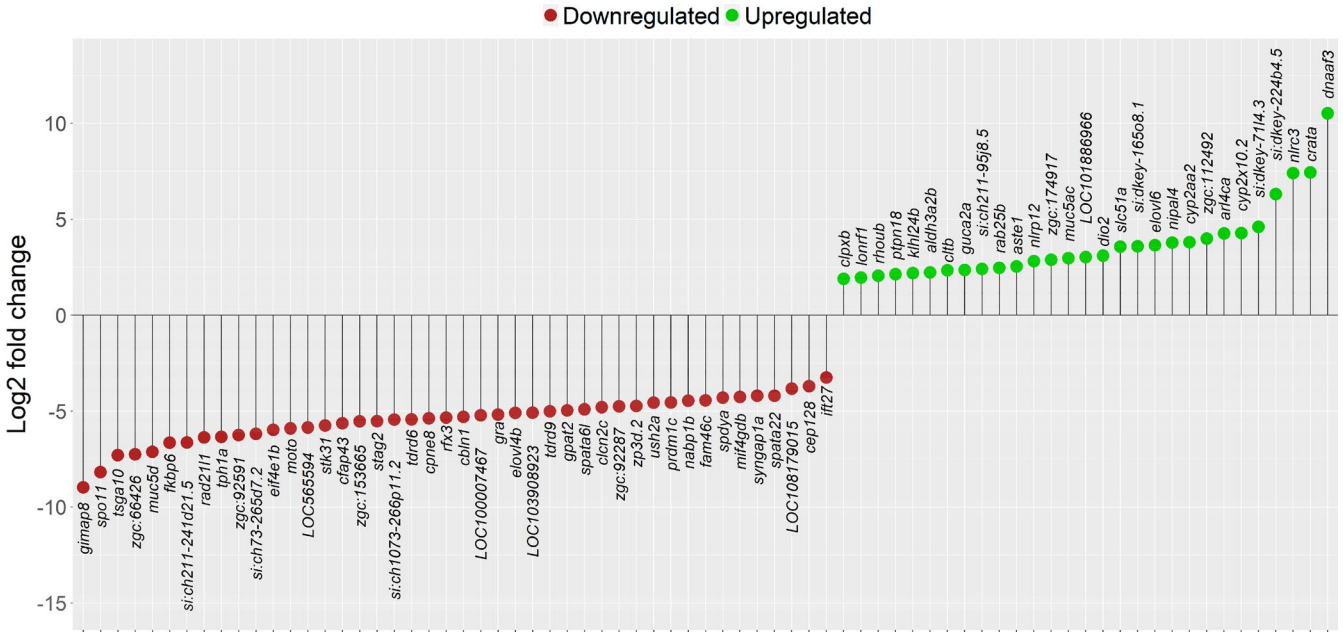

FIGURE 1 Differentially expressed genes in the intestine of the soya bean group (CP) compared to the control (CZ) group. An adjusted *p*-value below 0.05 and |Log2 fold change| ≥1 were employed in the function of DESeq2 (dots are plotted from left to right in the increasing order of Log2 fold change).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

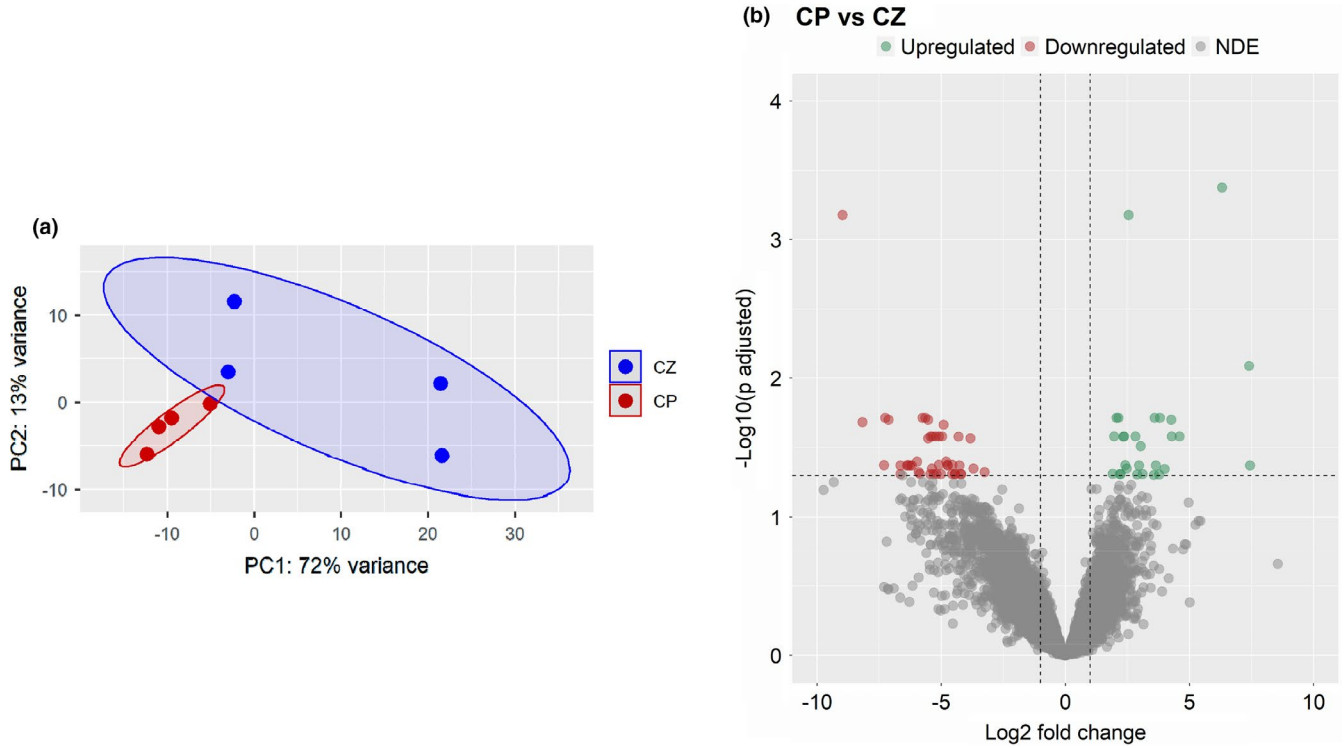

FIGURE 2 Intestinal transcriptome-based differences in the soya bean group (CP) compared to the control (CZ) group. Principal component analyses (a) and Volcano plot (b) of the differentially expressed genes in the CP group compared to the CZ group. Volcano plot shows the differentially expressed genes (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

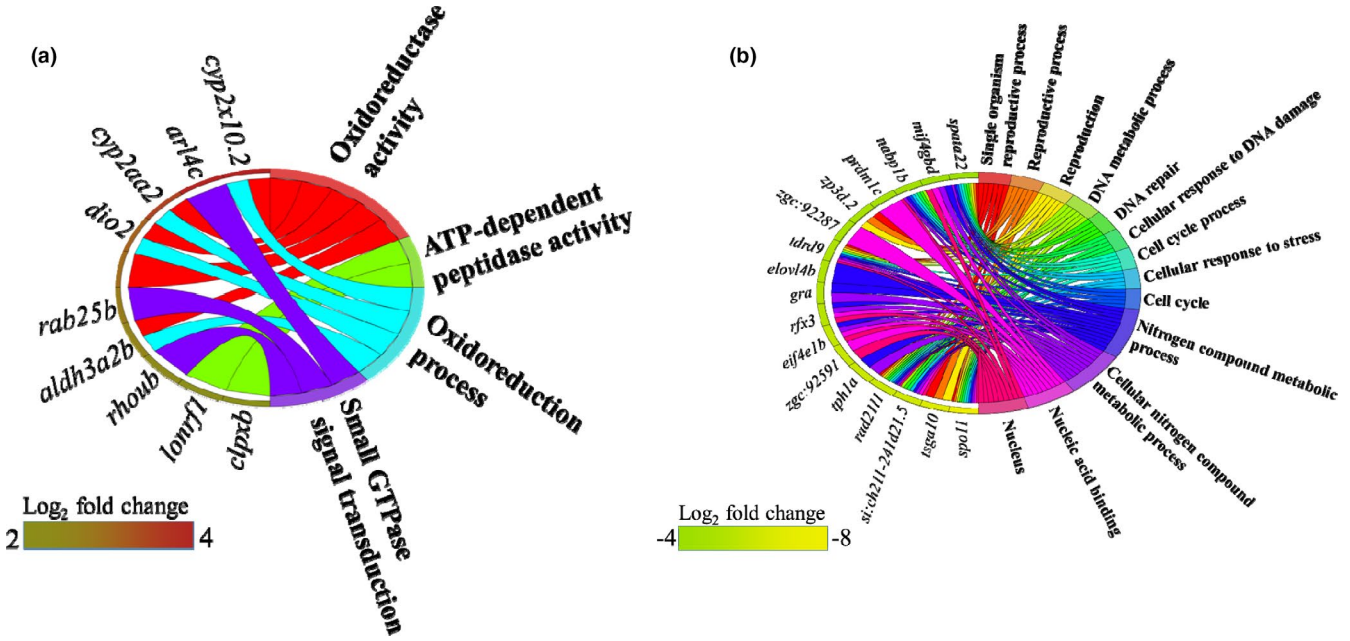

FIGURE 3 Chord diagram showing the link between the enriched GO terms in the soya bean (CP) group and the associated genes. The genes were upregulated (a) and downregulated (b) in the CP group compared to the control (CZ) group. The enriched GO terms are colour-coded, and differentially expressed genes 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)

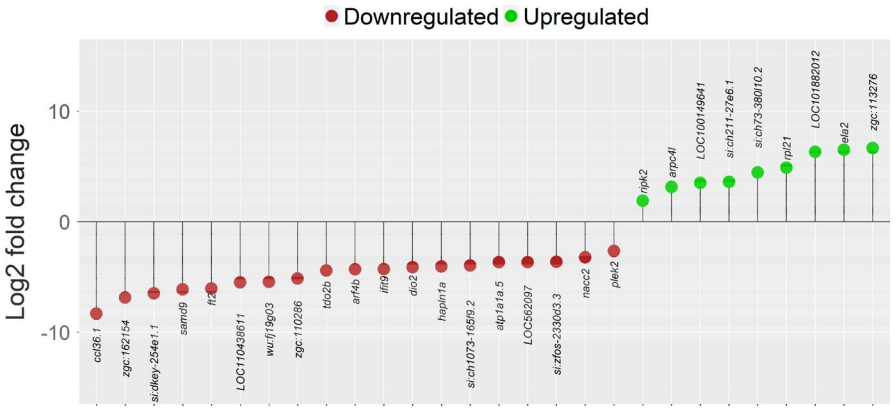

FIGURE 4 Differentially expressed genes in the intestine of the MacroGard® group (PM) compared to the soya bean group (CP). An adjusted p-value below 0.05 and |Log2 fold change| ≥1 were employed to generate the results (dots are plotted from left to right in the increasing order of Log2 fold change).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

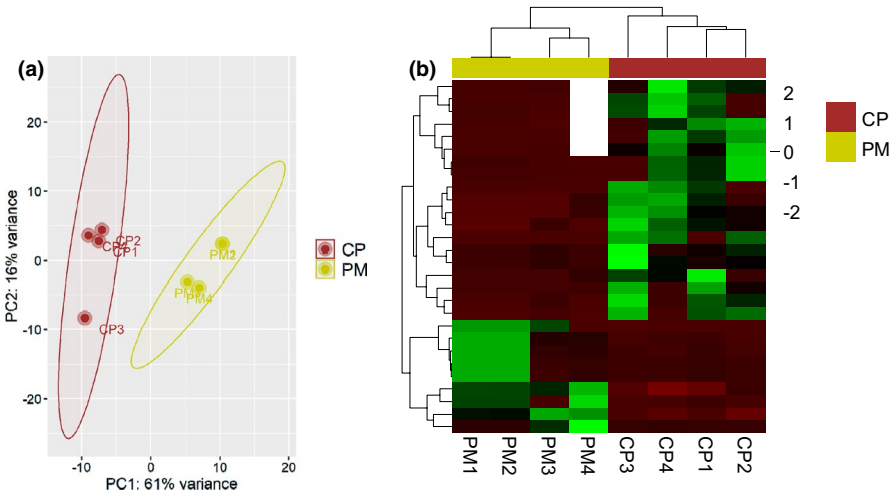

FIGURE 5 Intestinal transcriptome-based differences in the MacroGard® group (PM) compared to the soya bean group (CP). Principal component analyses (a) and heatmap (b) of differentially expressed genes in the PM group compared to the CP group. Each treatment group consisted of four biological replicates

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.

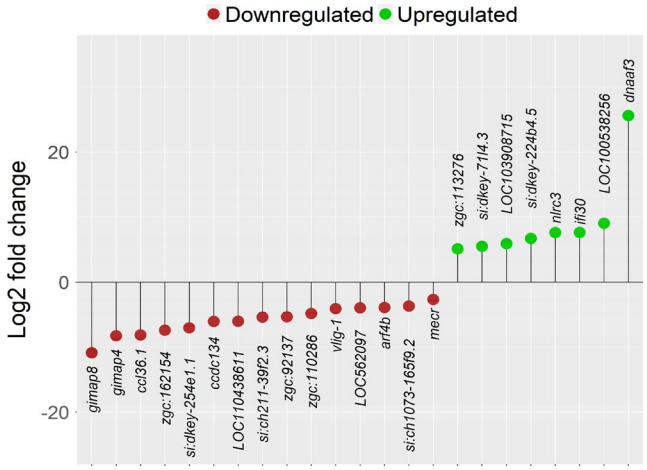

FIGURE 6 Differentially expressed genes in the intestine of the MacroGard® group (PM) compared to the control group (CZ). An adjusted *p*-value below 0.05 and |Log2 fold change| ≥ 1 were employed to generate the results (dots are plotted from left to right in the increasing order of Log2 fold change).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

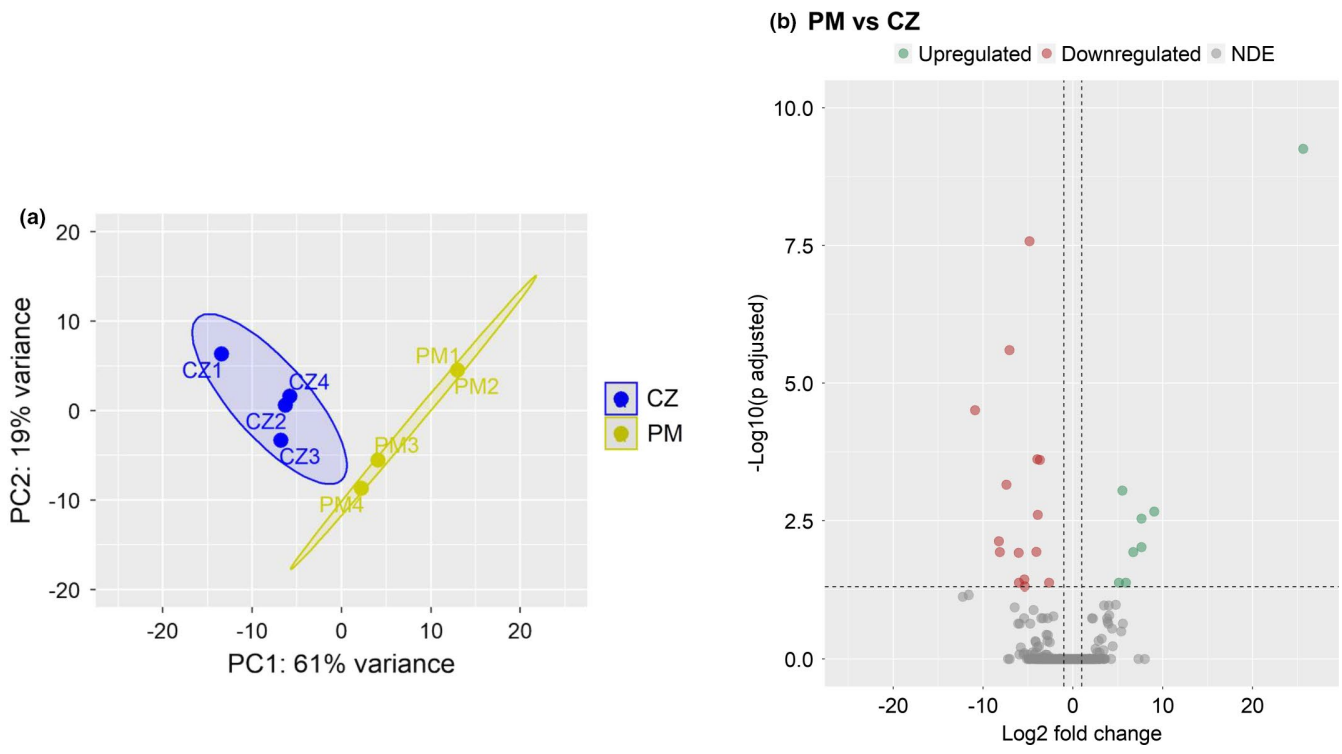

FIGURE 7 Intestinal transcriptome-based differences in the MacroGard® group (PM) compared to the control (CZ) group. Principal component analyses (a) and Volcano plot (b) of the differentially expressed genes in the PM group compared to the CZ group. Volcano plot shows the differentially expressed genes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

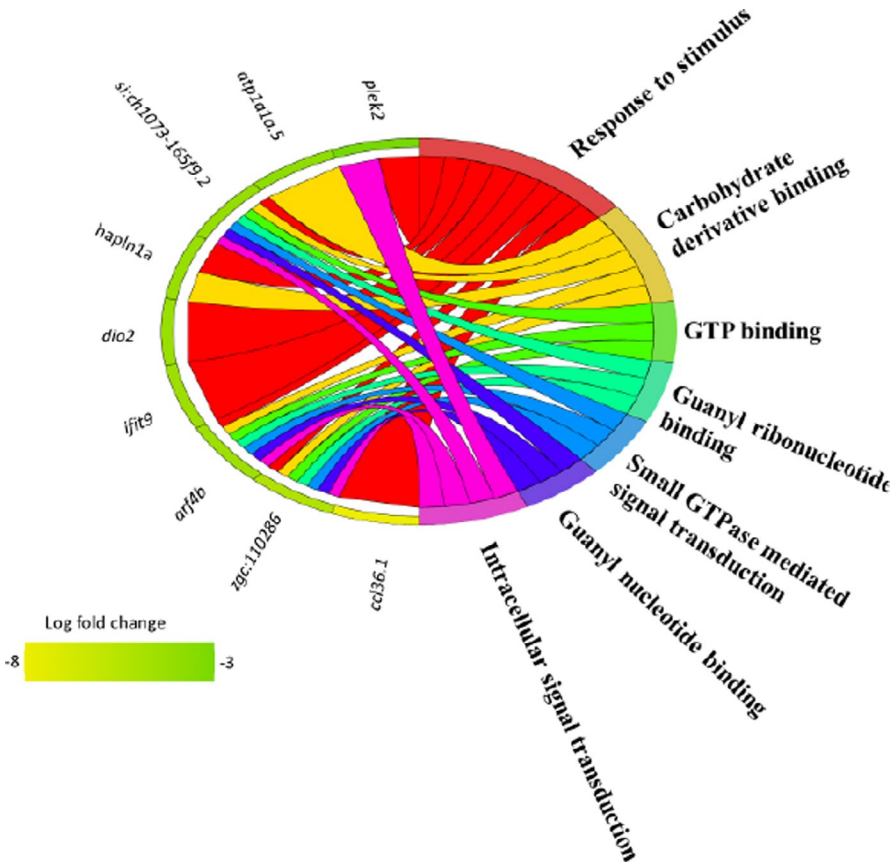

FIGURE 8 Chord diagram showing the link between the enriched GO terms in the MacroGard® group and the associated genes. The genes were downregulated in the PM group compared to the soya bean (CP) group. The enriched GO terms are colour-coded, and differentially expressed genes contributing to this enrichment 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*

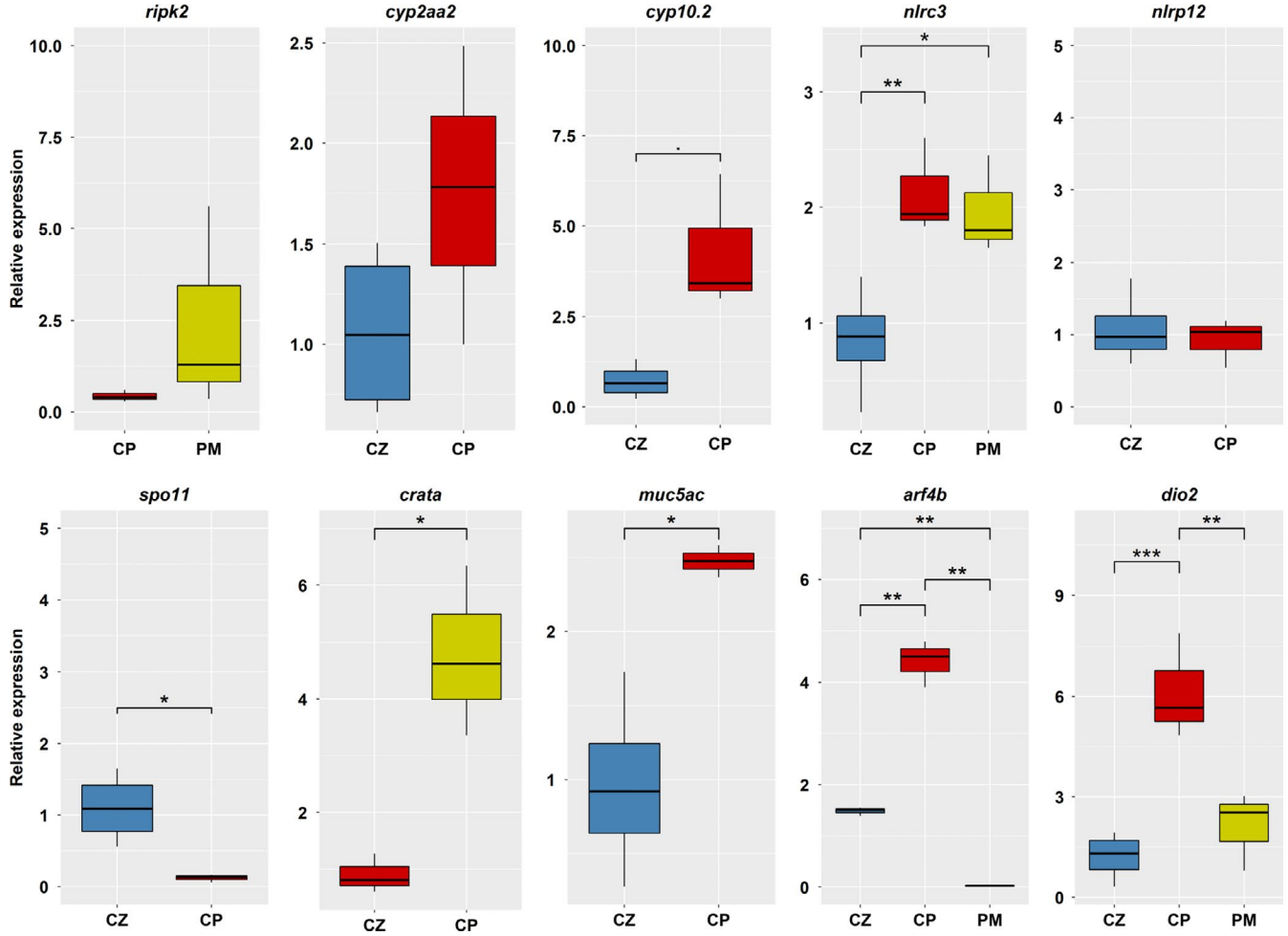

FIGURE 9 Relative gene expression of 10 selected genes in the intestine of the zebrafish fed control (CZ), soya bean (CP) and MacroGard® (PM) diets ($n = 4$). Asterisks *** indicate $p < 0.001$, ** indicate $p < 0.01$, * indicates $p < 0.05$, and • indicates $p < 0.1$

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

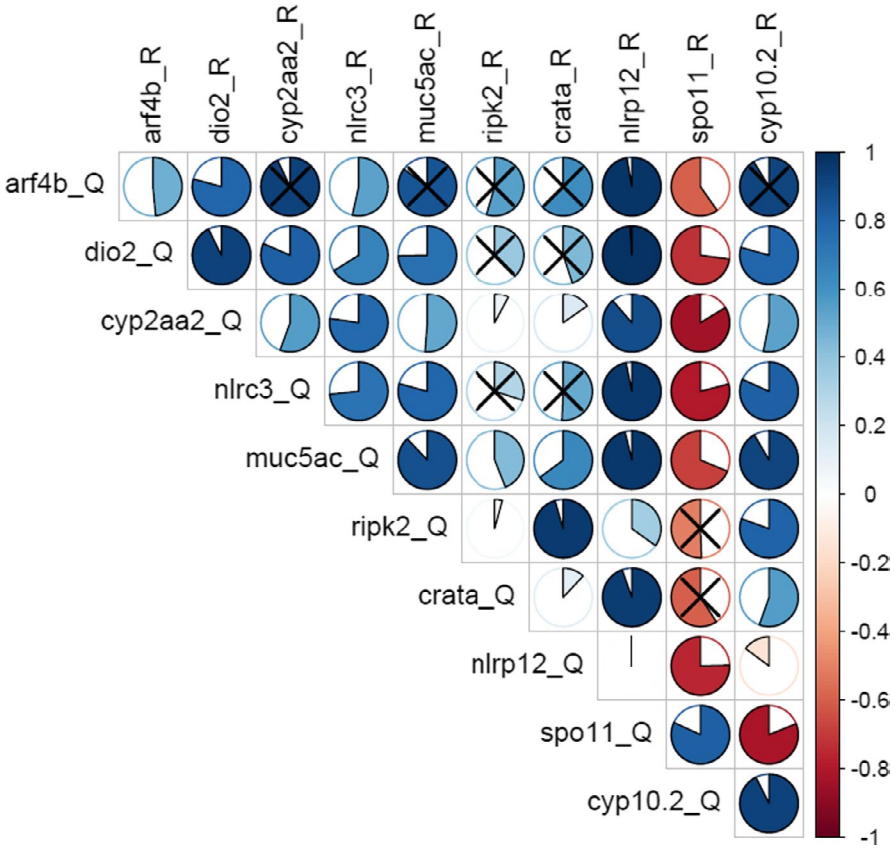

FIGURE 10 Correlation between the normalized counts from the RNA-Seq data and gene expression values from the qPCR data

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

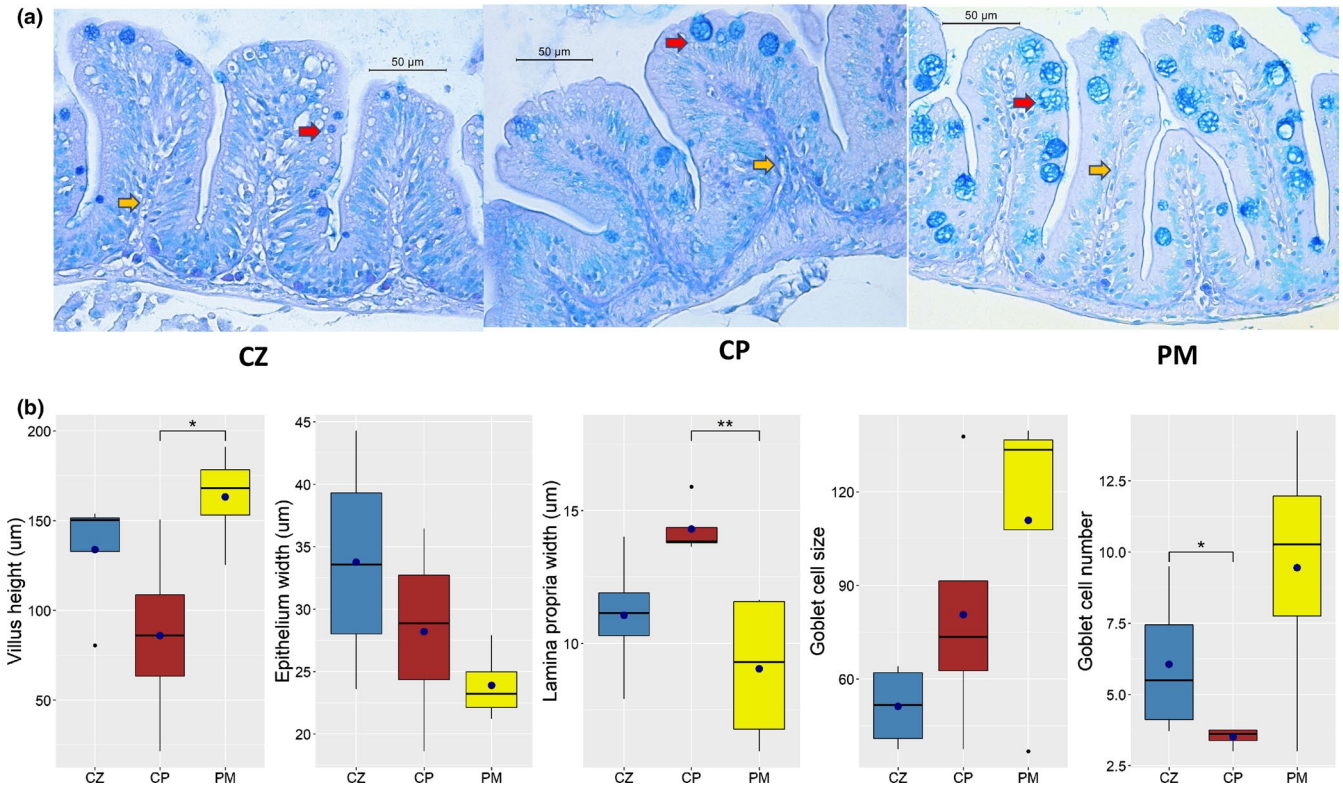

FIGURE 11 Differences in micromorphology of the distal intestine of zebrafish fed different feeds. Representative histological images (a) and box plots (b) showing the changes in the tissue architecture of the distal intestine of zebrafish stained with AB-PAS. The control group (CZ) has more absorptive 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

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