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Soy diet induces intestinal inflammation in adult Zebrafish: Role of OTX and P53 family

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Abstract

Inflammatory bowel diseases (IBDs) are a group of inflammatory conditions of the colon and small intestine, including Crohn's disease and ulcerative colitis. Since *Danio rerio* is a promising animal model to study gut function, we developed a soy-dependent model of intestinal inflammation in adult zebrafish. The soya bean meal diet was given for 4 weeks and induced an inflammatory process, as demonstrated by morphological changes together with an increased percentage of neutrophils infiltrating the intestinal wall, which developed between the second and fourth week of treatment. Pro-inflammatory genes such as interleukin-1beta, interleukin-8 and tumour necrosis factor alpha were upregulated in the second week and anti-inflammatory genes such as transforming growth factor beta and interleukin-10. Interestingly, an additional expression peak was found for interleukin-8 at the fourth week. Neuronal genes, OTX1 and OTX2, were significantly upregulated in the first two weeks, compatible with the development of the changes in the gut wall. As for the genes of the p53 family such as p53, DNp63 and p73, a statistically significant increase was observed after two weeks of treatment compared with controls. Interestingly, DNp63 and p73 were shown an additional peak after four weeks. Our data demonstrate that soya bean meal diet negatively influences intestinal morphology and immunological function in adult zebrafish showing the features of acute inflammation. Data observed at the fourth week of treatment may suggest initiation of chronic inflammation. Adult zebrafish may represent a promising model to better understand the mechanisms of food-dependent intestinal inflammation.

KEYWORDS

gut, inflammation, otx, p53, p63, p73, soy, zebrafish

1 | INTRODUCTION

Inflammatory bowel diseases (IBDs) are a group of inflammatory conditions of the colon and small intestine, such as Crohn's disease and ulcerative colitis, with increasing incidence worldwide.[1] Oxidative stress has an essential role both in the pathogenesis and in the progression of IBD.[2] Intestinal epithelial damage is thought to be important for the development and maintenance of the inflammatory condition both in preclinical animal models and in IBD patients.[3] So far, the most well-established animal model to study gut inflammation is the murine model.[4]

In the last decades, zebrafish (*Danio rerio*) has been used mainly in developmental biology and embryology research, but, more recently, it has been introduced as a model for food-induced intestinal inflammation for aquaculture applications and for gastrointestinal studies.[5–10]

Despite identical temporal sequence and functional homology with mammalian gut, zebrafish gut has a simpler structure with some differences that must be considered when making comparisons. The zebrafish lacks a proper stomach and gastric glands, but the intestinal bulb shares motility and goblet cells characteristics with mammalian stomach.[11,12] The intestinal epithelium does not have crypts and villi resembling those seen in mammals - they are substituted by finger-like protrusions called folds,[8,12] Some cell types or structures are lacking, such as Paneth cells and microfolds.[5] From an architectural point of view, zebrafish gut wall lacks the submucosa layer and the associated neuronal plexus[8] and the enteric nervous system is organized in scattered neurons instead of ganglions.[13]

Taking these characteristics into account, there are nonethless sufficent similarities to justfy the use of the zebrafish as a useful model system so several studies on intestinal inflammation have been performed already. Both larval and adult zebrafish models of enterocolitis have been developed, taking advantage of studies performed in mice.[5] By now, intestinal inflammation has been induced by an administration of 2,4,6-trinitrobenzenesulfonic acid (TNBS) in larvae[14–16] and adults,[17] dextran sulphate sodium (DSS)[18,19] and 0.2% oxazolone intrarectal injection.[20]

In addition zebrafish have aquired importance as a laboratory animal model in aquaculture and in the study of food-induced inflammation.[5]

Soya bean meal (SBM) was proposed initially as a substitute food for industrial fish aquaculture instead of fish-derived meal. However, recent studies showed that SBM negatively influences food ingestion, intestinal morphology and immunological function in several fish species, such as Atlantic salmon,[21–26] rainbow trout,[24] common carp,[25] juvenile turbot[27] and zebrafish larvae.[6]

Saponin contained in SBM triggers an inflammatory process in the distal intestine, which is characterized by shorter mucosal folds, an increased number of goblet cells[12,15] and infiltration of macrophages, neutrophils, lymphocytes, eosinophils, immunoglobulin M (IgM) and T cells into the lamina propria, reducing the capacity of the distal intestine to adsorb nutrients.[23,26,28]. These studies add further support to the suggestion that zebrafish could be used successfully to study food-associated effects on intestine, both in terms of inflammation and microbiota alterations.[6,7]

OTXs (orthodenticle homeobox) are transcription factors, homologues to *Drosophila orthodenticle* (*odt*) gene, deeply involved in embryonic development and morphogenesis.[29] The physiological expression of OTX1 and OTX2 in adult tissues is limited to specific tissues, such as the choroid plexus, some areas of the nervous system and sensory organs.[30] OTX1 expression has also been observed in the erythroid lineage of haematopoietic cells.[31] OTX gene mutations are often associated with congenital, somatic and metabolic human disorders associated with growth and cellular differentiation alterations.[32,33] Furthermore, the importance of OTX gene expression in the gut has also studied in two preclinical models of gastrointestinal disease, that is ischaemia/reperfusion[34] and DNBS-induced colitis.[35] In both these conditions, OTX1 was upregulated mainly in enteric glial cells and colocalized with inducible nitric oxide synthase (iNOS), suggesting a neurodamaging effect. OTX2 was mainly upregulated in neurons in association with neuronal nitric oxide synthase (nNOS).

Another important gene linked to inflammation and injury is the tumour suppressor p53.[36] It is known as the 'guardian of the genome' because of its ability to arrest proliferation and prevent the generation of altered cells in response to several stress signals. Also, p63 and p73, relatives of p53, can activate pro-apoptotic pathways and stimulate cell differentiation.[37]

Taking these data into account, therefore, we decided to test the involvement of OTX and p53 genes in the SBM zebrafish model of intestinal inflammation.

2 | METHODS AND MATERIALS

2.1 | Zebrafish maintenance, strain and diets

This experimentation has been performed in the Zebrafish Laboratory (IRCCS R. Galeazzi, GSD Foundation) according to the Italian and European guidelines on research practice (EU Directive 2010/63/EU). Zebrafish experimentation and all protocols of this study were approved

by the Italian Ministry of Health (authorization No. 742/2019-PR).

AB wild-type strain of zebrafish (*Danio rerio*) was maintained and raised in the ZEBTEC© Bench Top system (Tecniplast Spa, Italy) according to standard protocols.[38]

As control diet (D1), we used a commercial pellet that does not contain soya bean flour (Vipagran, Sera GmbH) with the following composition: corn starch, wheat gluten, whole egg powder, fish meal, calcium caseinate, wheat flour, fish oil, bean yeast, Spirulina algae, wheat germ, aromatic herbs, alpha-alpha, nettle, gammarus, seaweed, parsley, peppers, spinach, mannan-oligosaccharides (MOS 0.4%), carrots, mollusc flour Perna canaliculus and garlic.

Coloured with CE additives.

The experimental soy-based diet (D2) consisted of 50% w/w commercial pellet (Vipagran Sera GmbH) + 50% w/w soya bean defatted flour (Sigma-Aldrich). Food was produced as extruded 2-mm sinking pellets and crushed in a mortar and sieved with a mesh of 75 microns to obtain a particle size that was suitable for zebrafish consumption.

Adult zebrafish were maintained at 28°C in fish water from the aquarium system to have a density of 8 fish per litre. Fishes were fed three times a day, with an interval of at least 3 h between feedings, for 4 weeks (T1–T4), with an automated system.

Each fish was fed 10 mg/kg/day of meal, resulting in 33 g/kg/day. For D2, each fish received 16.5 g/kg/day of SBM. Fish were euthanized by rapid cooling.

2.2 | Histological analysis and morphometric measures

To evaluate morphological changes due to inflammation, microscopic analyses were conducted in collaboration with Dr Anna Maria Chiaravalli, UO di Istologia e Anatomia Patologica, Ospedale di Circolo, ASST-Sette Laghi, Viale L. Borri 57, 21100 Varese, Italy. Fresh samples were fixed in a 10% formalin buffer for 12 h in a w/w 20:1 ratio. For perfect dehydration and fixing processes, we used the Donatello Fast (DIAPATH) automatic processor. Samples were then embedded in paraffin with the Leica EG1150H automated system. Transversal sections of 8–10 μm thickness for optic microscopy were obtained with the Leica biosystem RM2235 microtome. Sections were then stained with haematoxylin-eosin and Alcian blue/PAS and observed on an optic microscope (Leica DMRA Nussloch). Imagines were acquired with a Nikon D5-5M camera.

Thickness of circular and longitudinal muscle and height and density of folds were evaluated using cross sections of distal intestine.

2.3 | Myeloperoxidase staining and cell count

Deparaffinization of 8-μm-thick formalin-fixed, paraffin-embedded (FFPE) sections was performed following standard protocols. Slides were mounted on poly-L-lysine-coated slides, heated in an oven (60°C, 30 min) and re-hydrated through an alcohol series to water. Endogenous activity was blocked with 3% hydrogen peroxide solution for 12 min, and antigen retrieval was obtained by 10 mM Citrate Buffer (pH 6) treatment.

Slides were incubated overnight at 4°C with a rabbit polyclonal anti-myeloperoxidase (MPO) primary antibody (ab210563, Abcam; 1:100 dilution) followed by goat anti-rabbit IgG H&L (HRP) (ab6721, Abcam; 1:200 dilution) secondary antibody for 1 h and ABC peroxidase complex. To localize MPO, 3,3′-diaminobenzidine tetrahydrochloride (DAB) was added. The immunohistochemical reaction was developed with diaminobenzidine-hydrogen peroxide reaction, and sections were counterstained with haematoxylin.

The immune cell evaluation was performed by counting the MPO-positive cells, corresponding to neutrophils, present in a defined area of each histological section respect to total cells. The zebrafish neutrophils, in fact, are very similar to human neutrophils and express myeloperoxidase.[39]

2.4 | RNA extraction and qRT-PCR

Total intestines were sampled every week for total RNA extraction. Total RNA extraction was performed using the EuroGold Total RNA Mini kit (Euroclone) according to manufacturer's instructions. RNA was quantified with the Nanodrop® ND 100 instrument, and its integrity was verified on a 2% agarose gel. cDNA was synthesized from RNA samples with the High Capacity cDNA Synthesis Kit (Applied Biosystems, Life Technologies, Grand Island) according to manufacturer's instructions.

Real-time quantitative PCR was performed on a Bio-Rad CFX Maestro™ (Bio-Rad Laboratories) instrument with 30 ng of cDNA per well with the SYBR Green (iTac Universal SYBR green Supermix, Bio-Rad) method. The thermal profile was 50°C for 2 min, 95°C for 10 s and 40 cycles of 95°C for 15 s, and 60°C for 1 min. Each reaction was carried out in triplicate. Analysis was performed with the Ct method with β-actin as the endogenous control. The average ΔCt value was calculated by subtracting the control ΔCt from the treated ΔCt. The relative quantity of mRNA was calculated as $2^{-\Delta\Delta Ct}$.

Primers were designed with the Primer3 free online software, and primers used are summarized in Table 1:

TABLE 1 Zebrafish gene primers for real-time PCR

GENE	Forward primer	Reverse primer
B-actin	5′ ACGGTCAGGTCATCACCATC 3′	5′ TGGATACCGCAAGATTCCAT 3′
il-1β	5′ CAGATCCGCTTGCAATGA 3′	5′ TTGTGCTGCGAAGTCCAC 3′
cxcl8a	5′ GCAAAATCATTTCAGTGTGTGTT 3′	5′ CAGACCTCTCAAGCTCATTCC 3′
il10	5′ AACTCAAGCGorGGATATGGTG 3′	5′ GACCCCCTTTTCCTTCATCT 3′
Tgfb	5′ AATGGCTGCAGGGTTCAG 3′	5′ GGTTTGCTTTACAGTCGCAGT 3′
Tnfa	5′ AGGCAATTTCACTTCCAAGG 3′	5′ AGGTCTTTGATTCAGAGTTGTATCC 3′
nos1	5′ GAGGTCAGAATCCATTGCGTA 3′	5′ TGATGACTATGAGCAAAACACCTC 3′
otx1	5′ CTTCCCGAGTCCCGAGTT 3′	5′ CGAGTTTTCGTGCTGCTG 3′
otx2	5′ ACCCCTCCGTTGGATACC 3′	5′ ATCCGGGTAGCGTGTTTTC 3′
tp53	5′ GGAGCAAAAAGGCCAAGG 3′	5′ TCCAGACTGTCGTTCAATTTCTT 3′
tp63	5′ GTGGGCACAGAGTTCACAAC 3′	5′ CGACCCAAAACCTGACCAT 3′
tp73	5′ CTTTCCAGCAGTCCAGCAC 3′	5′ TTCTTGAGCAATGGAGAGTACG 3′

2.5 | Statistical analysis

At the beginning of the experiment, 5 fish were analysed as initial controls for the population, and then, every week, 5 controls and 5 treated fish have been used to a total of 45 animals for each experiment. The entire experimentation has been repeated 3 times, with comparable results, using a total of 135 fish. After each week of treatment, 2 controls (D1) and 2 treated fish (D2) intestines were sampled for morphological analysis and 3 intestines were used for qRT-PCR.

Data are expressed as mean ± SEM (mean standard error) of the three biological tests. We used a non-parametric test, the Kruskal-Wallis one-way ANOVA test followed by Dunnett's post hoc test using the GraphPad Prism Software (INC). Significance values have been set at less than $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

3 | RESULTS

3.1 | Intestine morphology in SBM fish

After each week of treatment, 2 control (D1) and 2 treated fish (D2) intestines were sampled for morphological analysis. All optical microscope sections were stained with haematoxylin-eosin (H&E) and analysed at a 40X magnitude to perform anatomical morphometric measurements of the gut wall (Figure 1A). The total wall thickness significantly increased after 2 weeks of treatment (Figure 1B), as a consequence of an increase in the thickness of both the circular (Figure 1C) and longitudinal smooth muscle layers (Figure 1D).

The height of the folds (Figure 1E) and the number of folds per area (Figure 1F) were not significantly different between soy-treated samples and relative. To evaluate the number of goblet cells, we performed Alcian blue/PAS staining on medial intestine sections. An increased number of goblet cells were observed after exposure to SBM for 2 weeks (Figure 1G).

3.2 | Inflammation was increased in SBM fish intestine

We evaluated the immune cell infiltrate in the intestine by myeloperoxidase (MPO) staining (Figure 2A). The SBM diet (D2) induces an inflammatory process, as demonstrated by the increasing percentage of neutrophils at two, three and for weeks in the intestinal mucosa (Figure 2B).

Tissue morphology and neutrophil infiltrate indicate an inflammatory condition in the intestine after 2 weeks of SBM treatment. Gene expression analysis has been performed by real-time PCR on pro-inflammatory cytokine, anti-inflammatory genes and other regulatory genes. In particular, pro-inflammatory genes such as interleukin-1b (*il-1β*), interleukin-8 (*cxcl8a*) and tumour necrosis factor alpha (*tnfa*) were found upregulated in the second week as well as anti-inflammatory genes such as transforming growth factor beta (*tgfb*) and interleukin-10 (*il10*). Moreover, the expression levels of nNOS, another regulatory gene that plays an essential role in mucosal homeostasis and neuronal protection,[40] were found downregulated at T1, T2 and T4 but unchanged at T3 (Figure 3). Interestingly, an additional expression peak was found in *cxcl8α* at the 4th week and *tnfa* at the 3rd week.

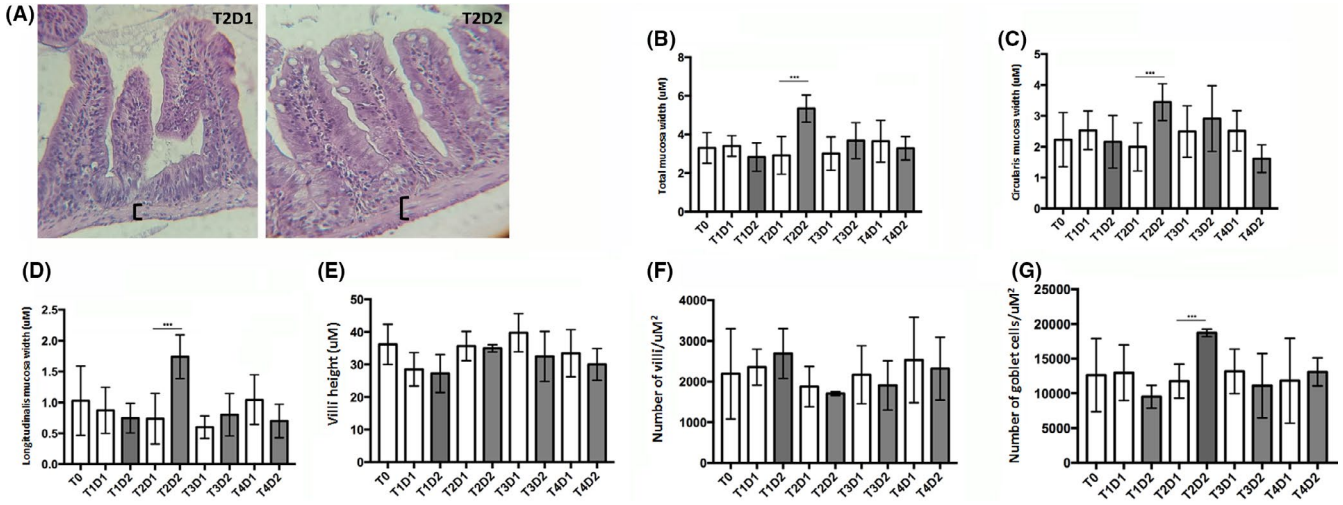
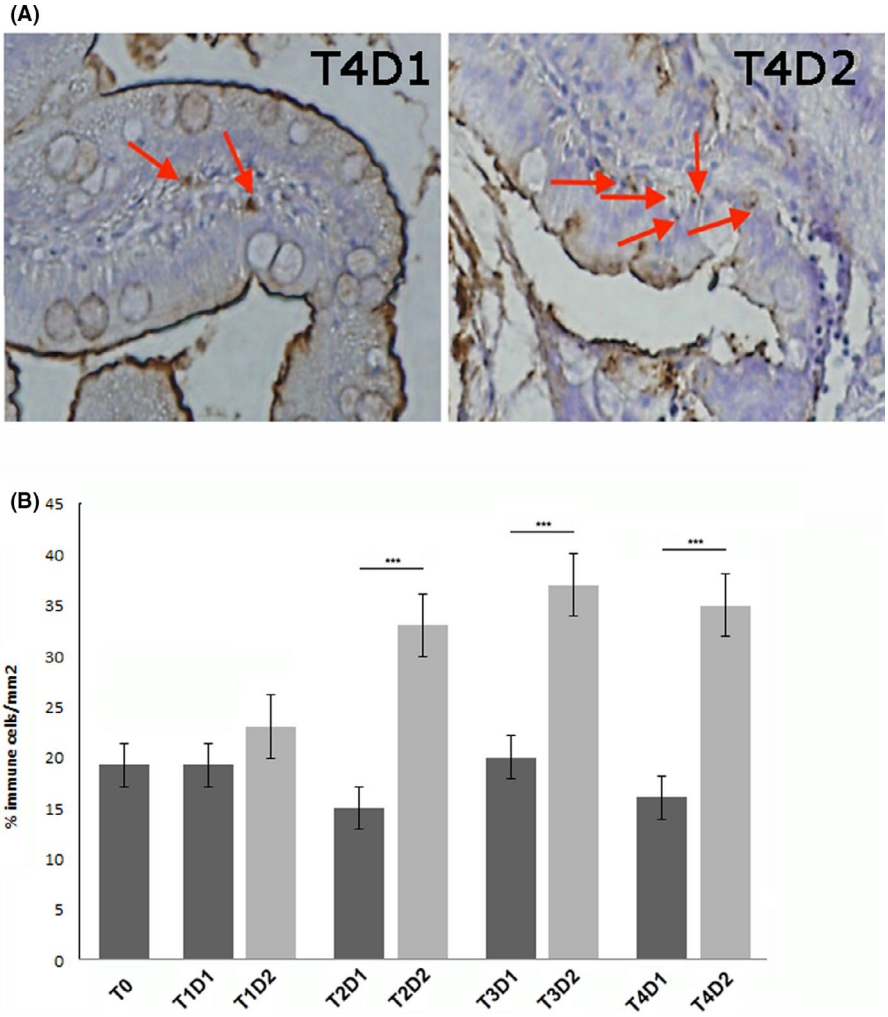


FIGURE 1 Morphometric analysis of the gut wall. Fish have been fed for one (T1), two (T2), three (T3) and four (T4) weeks with two different diet: normal (D1) or soy (D2). (A) Morphological representation of intestinal sections stained with H&E. Images represent wall thickness in control (T2D1) and treated fish (T2D2) at 2 weeks of treatment. As we can see from square brackets, treated sample shows a higher wall thickness. (B) The total wall thickness was increased after 2 weeks of treatment (T2D1 vs T2D2, $p < 0.001$). (C–D) The alteration can be detected in the circular smooth muscle layer (T2D1 vs T2D2, $p < 0.001$) as well as in the longitudinal smooth muscle layer in T2D2 (T2D1 vs T2D2, $p < 0.001$). (E) Morphometric measure of fold height and (F) number of folds per area indicated no significant morphological changes with soy diet. (G) Number of goblet cells per area was found increased after 2 weeks of treatment (T2D1 vs T2D2, $p < 0.001$). *=p<0,05 ; **=p<0,01 ; ***=p<0,001

FIGURE 2 Immune cell analysis in the intestinal mucosa in fish fed with D1 and D2 at different weeks (T1–T4). (A) Myeloperoxidase (MPO) staining of neutrophils (red arrows) on intestine sections counterstained with haematoxylin. (B) An increased percentage of immune cell can be detected in the submucosal layer of SBM-treated fish from T2 to T4 (T2D1 vs T2D2, $p < 0.001$; T3D1 vs T3D2, $p < 0.001$; T4D1 vs T4D2, $p < 0.001$). *=p<0,05 ; **=p<0,01 ; ***=p<0,001



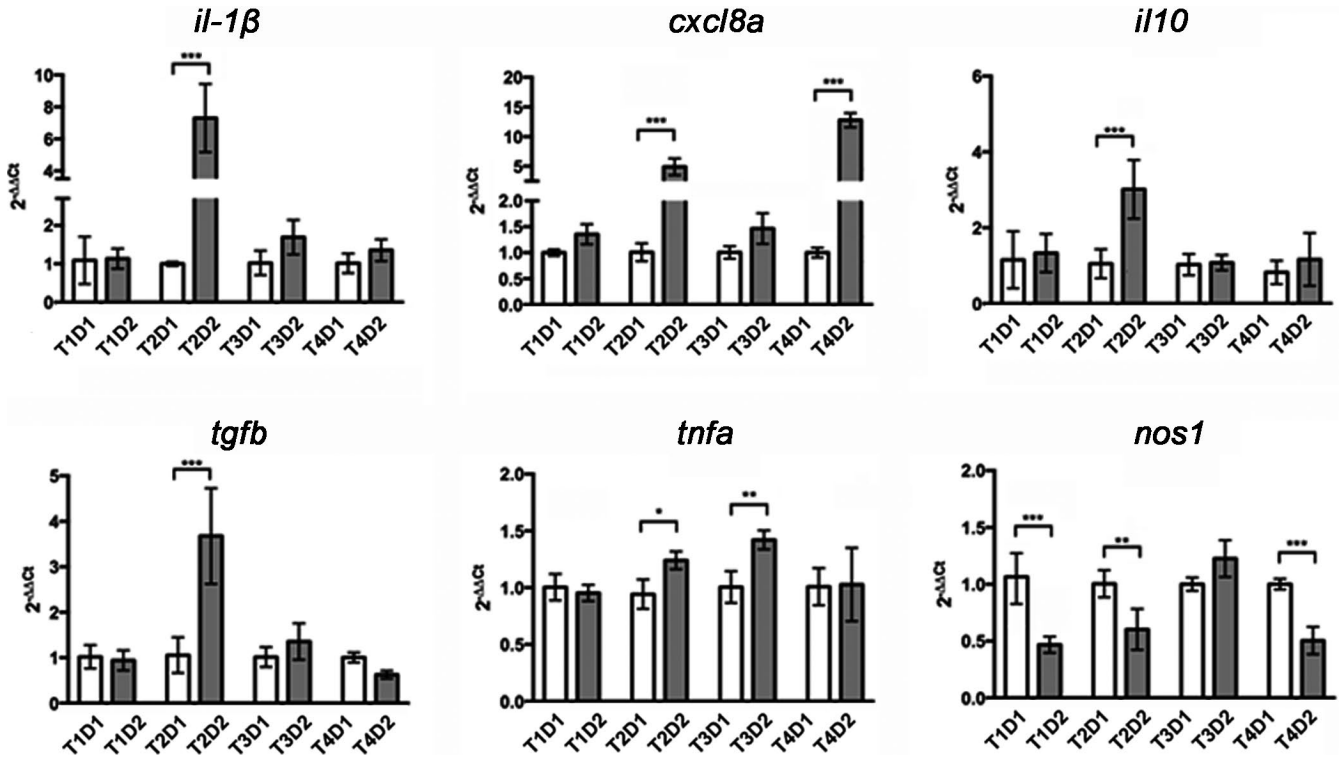


FIGURE 3 Gene expression levels of pro-inflammatory (*il-1β*, *cxcl8a*, *tnfa*), anti-inflammatory (*tgfb* and *il10*) and *nos* genes obtained by RT-PCR from intestinal tissue of fish fed with D1 and D2 at different treatment times (from T1 to T4). *il-1β*: T2D1 vs T2D2, $p < 0.001$; *cxcl8a*: T2D1 vs T2D2, $p < 0.001$; T4D1 vs T4D2, $p < 0.001$; *il10*: T2D1 vs T2D2, $p < 0.001$; *tgfb*: T2D1 vs T2D2, $p < 0.001$; *tnfa*: T2D1 vs T2D2, $p < 0.05$, T3D1 vs T3D2, $p < 0.01$; *nos1*: T1D1 vs T1D2, $p < 0.001$; T2D1 vs T2D2, $p < 0.01$; T4D1 vs T4D2, $p < 0.001$. *=p<0,05 ; **=p<0,01 ; ***=p<0,001

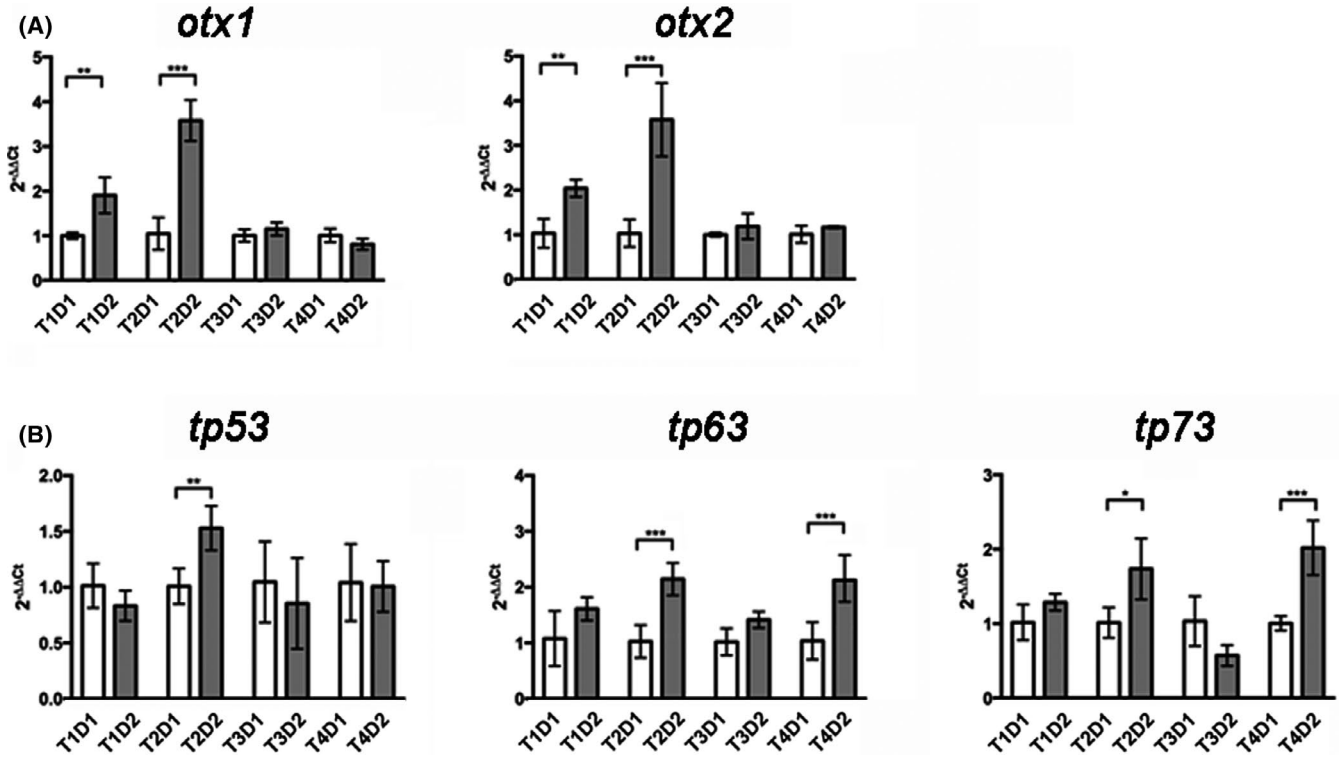


FIGURE 4 Expression analysis by RT-PCR of OTX and p53 family genes. (A) *otx1* and *otx2* were found upregulated in the first 2 weeks of treatment (*otx1*: T1D1 vs T1D2, $p < 0.01$; T2D1 vs T2D2, $p < 0.001$; *otx2*: T1D1 vs T1D2, $p < 0.01$; T2D1 vs T2D2, $p < 0.001$). (B) *tp53* was found upregulated in treated fish at T2 only (*tp53*: T2D1 vs T2D2, $p < 0.01$), but *tp63* and *tp73* at T2 and T4 (*tp63*: T2D1 vs T2D2, $p < 0.001$; T4D1 vs T4D2, $p < 0.001$; *tp73*: T2D1 vs T2D2, $p < 0.05$; T4D1 vs T4D2, $p < 0.001$). *=p<0,05 ; **=p<0,01 ; ***=p<0,001

3.3 | otx family genes were modulated in SBM fish intestine

To investigate the role of the p53-OTX axis in our model, gene expression analysis of the OTX1, OTX2 genes and the p53 family was conducted by real-time PCR on intestines explanted from 3 SBM-treated fish (D2) and 3 controls (D1). As shown in Figure 4A, both *otx1* and *otx2* increased from Week 1 and were significantly upregulated in the second week after the beginning of SBM treatment, compatibly with the wall derangement and immune response activation, whereas the expression levels at three and 4 weeks return to the levels observed in the controls. As for the genes of the p53 family such as *tp53*, *tp63* and *tp73*, a statistically significant increase was observed after 2 weeks of treatment with respect to the controls (Figure 4B). Interestingly, as for *tp63* and *tp73* an additional peak was observed after 4 weeks.

4 | DISCUSSION

A 50% SBM defatted diet was administered to adult zebrafish for 4 weeks in order to generate a model of intestinal inflammation.

As reported in the literature, SBM is able to trigger an inflammatory process in the intestine of rainbow trout, carp and Atlantic salmon.[21–28,41] The effects proved to be dose-dependent[26]; several studies indicated the soy protein is not the cause of inflammation, which, instead, seems to be caused by its saponin component.[27,28]

Hedrera et al demonstrated that zebrafish larvae fed with a 50% SBM for 4 days showed an inflammatory process. The authors reported no changes in gut morphology but a marked neutrophil migration into the intestine from the second day of treatment. This work also confirmed that a 43% soy protein diet per se is not able to induce intestinal inflammation, but a 50% soy saponin meal induces an inflammatory process in the gut.[6]

Our results are in agreement with those reported by Hedrera et al, since we showed a statistically significant overexpression of pro-inflammatory cytokines such as *cxcl8a*, *il-1β* and *tnfa*. We also evaluated the expression of anti-inflammatory genes such as *tgfb* and *il10*, which increase after 2 weeks of SBM diet presumably in order to negatively regulate the inflammatory stimulus.

As reported by Hedrera in larval studies, no structural changes were observed in the intestinal wall except an increase of adult intestinal wall thickness probably due to oedema at T2.[6]

Our studies were performed in adult fish and show very similar results to those found by Hedrera et al in zebrafish larvae, suggesting that similar pathological mechanisms underlie both larval and adult soya bean-induced inflammation. Moreover, the adult model allows the analysis of soya bean-induced inflammation in a fully matured fish, with full-developed organs and metabolic regulations, which are not present in embryos.

NOS1, in the zebrafish intestine, is known to be specifically expressed in the enteric neuronal system.[42] Our results show a significant change in the expression of NOS1 mRNA at T1, demonstrating that saponin exposure caused a significant increase in oxidative stress on intestinal wall and in particular on resident neurons. Interestingly, a downregulation of nNOS expression has been found in neurons damaged by free radicals and anti-oxidant imbalance is a typical IBD feature.[43]

In this new dietary-dependent zebrafish model of intestinal inflammation, our results suggest the development of an acute-like inflammatory process at 2 weeks (T2), associated with increase in muscular layer and immune cell infiltrate. Muscle thickness increase is strictly associated with inflammatory damage, as already demonstrated in vivo,[44] and gene expression analysis performed by real-time PCR on pro-inflammatory genes revealed an overexpression of *otx1* and *otx2*, *tp53*, *il-1β*, *cxcl8a*, *il10*and *tgfb*.

The acute inflammatory stimulus returned to baseline in 3 weeks (T3), and this could be due to the plasticity of fish intestine and its ability to adapt to the inflammation.

Nevertheless, in SBM-treated intestines the late expression of *cxcl8a* and *tp73* and the persistence of immune infiltrate suggest that a chronic inflammation may superced the acute phase by the fourth week.

It has been demonstrated that IL-8 can lead to chronic inflammatory conditions such as rheumatoid arthritis,[45] IBD[46] and psoriasis.[47] In patients with IBD, both neutrophils and recruited macrophages are responsible for the production of IL-8,[46] but recent human studies have demonstrated that early recurrent ileal lesions in Crohn's patients were characterized by IL-8 production from neutrophils, whereas, in chronic lesions, IL-8 was produced by macrophages and T cells.[48]

In our model, *tp63*and *tp73* (but not *tp53*) share the same expression profile as *cxcl8a*, suggesting that a link exists between the p53 family and IL-8 in intestinal inflammation.

DNp63 is involved in the stress response development of chronic inflammatory diseases; for example, its overexpression in skin keratinocytes that are induced in murine atopic dermatitis through upregulation of IL-33 and IL-31.[49] Some in vitro studies reported a direct link between DNp63 and IL-8 expression in osteosarcoma cell lines[50] and squamous epithelial cells.[51]

P73 has been found to be involved in colonic cell protection after DSS-dependent inflammation injury, through

the activation of apoptosis.[52] Mice functionally deficient for all p73 isoforms exhibit profound defects, including hippocampal dysgenesis, hydrocephalus, chronic infections and inflammation.[53] These data suggested a wide range of mechanisms supported by p73, from immune cell regulation,[54] epithelial damage repair[55] to neuronal protection.[56]

We hypothesize that the saponin content of soy causes a low-grade plasma membrane injury that stimulates inflammatory processes and cellular defence responses, in particular, in the early phase (T2), consisting of *tp53*, *tp63* and *tp73* overexpression, to protect enterocytes and muscle thickening. In the third week (T3), acute inflammation is switched off due to the adaptive capacity of zebrafish intestine versus food change. In the late phase (T4), *tp63* and *tp73* are upregulated in a possible *cxcl8*-dependent inflammation, which persists in zebrafish intestine with infiltrate after the acute phenomena. Since *tp53* expression is quickly activated after DNA damage,[57] we can hypothesize that only in T2, the inflammation-dependent injury is so severe to affect the genome integrity of intestinal cells. Moreover, *nos1* downregulation indicates that an oxidative stress is always present at T1 (saponin injury), T2 (acute inflammation) and T4 (late inflammation) on intestine wall.

Recent studies demonstrated the involvement of homeobox genes (OTX1 and OTX2) and p53-related genes in rat ischaemia/reperfusion model,[34] proliferative vitreoretinopathy[58] and cancer.[32,59,60] Inflammation could be a common link to these phenomena, and OTX1 and OTX2 could play an important role in the damage response-inflammation axis.

A recent study on a DNBS-induced rat model of colitis highlighted a peculiar role for OTX genes during inflammation. Both these genes are upregulated in longitudinal muscle myenteric plexus (LMMP) whole-mount preparations following inflammation, and their expression is observed also distally from the site of DNBS administration.[37]

The upregulation of *otx1* and *otx2* in the first 2 weeks of treatment suggests an involvement of these genes in response to inflammation. In this context, probably, *otx* genes interact with tp53 family at T2 to protect gut from acute inflammation-dependent damage.

Further experiments will be focused on localization of *otx1* and *otx2* in the intestinal wall, in order to verify that these two genes are strictly associated with neuronal cells, as observed in the DNBS-induced rat colitis model, and thus may be involved into the neuronal adaptation system.

Taken together, these data suggest that otx1 and otx2 could be considered early markers of intestinal injury and acute inflammation.

In summary, our data confirm that SBM, one of the best candidates to replace fishmeal protein in aquaculture, negatively influences intestine triggering an acute-like inflammation process in adult zebrafish. Further experiments are needed to characterize how this inflammatory process develops after 4 weeks of treatment and whether a chronic inflammation is established, as we can hypothesize from increased expression of *cxcl8a*, *tp63* and *tp73* at fourth week.

These data also support the use of this adult zebrafish model to study the mechanisms of dietary-induced intestinal inflammation.

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CONFLICT OF INTEREST

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

MM and GP designed the study and the experimental procedure and finalized the manuscript for submission. GB received the fund. MR, GMill and MC did all the experiments, analysed the data and prepared the initial version of the manuscript. GMich, VM, FF and LC analysed the data and prepared the second version of the manuscript. CG, FA, GMont, RV, FF and LC revised the manuscript.

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