Fish Physiology and Biochemistry

# Butyrate and taurine exert a mitigating effect on the inflamed distal intestine of European seabass fed with a high percentage of soybean meal

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| Abstract: | Due to the paucity of oceanic resources utilized in the preparation of diets for cultured fish, commercial feed producers have been trying to replace fishmeal (FM) using alternative protein sources such as vegetable proteins meals (VMs). One of the main drawbacks of using plant-derived proteins in fish feed is related to the presence of a variety of anti-nutritional factors, which could trigger an inflammation process in the distal intestine commonly referred to as VM-induced enteropathy. This reduces the capacity of the enterocytes to absorb nutrients leading to reduced fish growth performances. The typical signs of intestinal inflammation are a shortening of primary and secondary intestinal mucosal folds, an increase in the number of goblet cells, and the infiltration of inflammatory cells, particularly macrophages and eosinophilic granulocytes, into the lamina propria. Once an inflammatory immune response is initiated, proinflammatory cytokines and a panel of chemokines (secreted proteins that play a major role in the inflammatory response) instigate the coordinated expression of downstream genes.<br>Butyrate may promote the healing of inflamed intestine through its major role in enhancing epithelial cell proliferation and differentiation and in improving the intestinal absorptive function. Another organic acid that has been shown to have a restorative effect on VM-induced enteritis in fish is the organic acid taurine. It represents an essential nutrient with powerful antioxidant and anti-inflammatory properties that is required as a supplement in the feed when a relevant percentage of vegetable protein sources are utilized.<br>In view of these considerations, the present research evaluated the mitigating effects of |

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# Butyrate and taurine exert a mitigating effect on the inflamed distal intestine of European seabass fed with a high percentage of soybean meal

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**Keywords:** Aquaculture; intestinal inflammation; TEM; light microscopy; gene expression; cytokines; interleukins, soybean meal, butyrate, taurine, enteritis.

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**Abstract**

Due to the paucity of oceanic resources utilized in the preparation of diets for cultured fish, commercial feed producers have been trying to replace fishmeal (FM) using alternative protein sources such as vegetable proteins meals (VMs). One of the main drawbacks of using plant-derived proteins in fish feed is related to the presence of a variety of anti-nutritional factors, which could trigger an inflammation process in the distal intestine commonly referred to as VM-induced enteropathy. This reduces the capacity of the enterocytes to absorb nutrients leading to reduced fish growth performances. The typical signs of intestinal inflammation are a shortening of primary and secondary intestinal mucosal folds, an increase in the number of goblet cells, and the infiltration of inflammatory cells, particularly macrophages and eosinophilic granulocytes, into the lamina propria. Once an inflammatory immune response is initiated, proinflammatory cytokines and a panel of chemokines (secreted proteins that play a major role in the inflammatory response) instigate the coordinated expression of downstream genes.

Butyrate may promote the healing of inflamed intestine through its major role in enhancing epithelial cell proliferation and differentiation and in improving the intestinal absorptive function. Another organic acid that has been shown to have a restorative effect on VM-induced enteritis in fish is the organic acid taurine. It represents an essential nutrient with powerful antioxidant and anti-inflammatory properties that is required as a supplement in the feed when a relevant percentage of vegetable protein sources are utilized.

In view of these considerations, the present research evaluated the mitigating effects of butyrate and taurine used as feed additives on the morphological abnormalities caused by a soybean meal (SBM) based diet in the distal intestine of European sea bass. We used three experimental diets, containing the same low percentage of FM and high percentage of SBM; two diets were supplemented with either 2% sodium butyrate or taurine. Light and transmission electron microscopy (TEM) were used to determine histological changes in the intestine of fish. We also quantified the intestinal mRNA abundance of several genes involved in the mucosal inflammatory response such as TNF$\alpha$, (tumor necrosis factor alpha) that makes up the inflammatory acute phase reaction, and interleukins such as IL-8, IL-1β, IL-10, and IL-6, which are involved in the regualtion of immune responses, inflammatory reactions, and hematopoiesis.

Fish that received for two months the diet with a concentration of a soy protein source close to 30% (16.7% as SBM and 12.8 as full-fat soy) developed a severe inflammation in the distal intestine. However, histological and gene expression data obtained in fish fed the other two diets, suggest that butyrate and taurine used as a feed additive could have a role in normalizing the intestinal

abnormalities caused by the SBM, albeit at different success rates. The meccanisms underlying these effects of butyrate and taurine should be further investigated along with any possible synergistic effect of the two substances in the feed in normalizing the intestinal abnormalities caused by soyabean meal.

## Introduction

Due to the paucity of oceanic resources utilized in the preparation of diets for cultured fish, commercial feed producers have been trying to replace fishmeal (FM) by using alternative nitrogen and protein sources such as vegetable proteins meals (VMs) (Tacon and Metian, 2008). Vegetable products include soy, canola (rape), corn, wheat, cottonseed, lupin, sunflower, flax linseed, and peas. Of these, soybeans are promising because of their higher protein content, higher digestibility, and better amino acid profile in comparison to other grains and oilseeds (Hardy, 1999; Gatlin et al, 2007; Zhang et al, 2014).

The main drawbacks of using plant-derived proteins in fish feed are related to the low level of indispensable amino acids (in particular, methionine and lysine), and to the presence of a wide variety of anti-nutritional factors, such as saponins, lectins, phytate, trypsin inhibitors, phenols, and tannins (Francis et al., 2001), which could damage the intestinal tract thus reducing nutrient absorption and fish growth (van den Ingh, 1991; Bonaldo et al., 2008; Knudsen, 2008; Uran et al., 2006a,b).

Indeed, studies in different fish species, such as Atlantic salmon, rainbow trout, European sea bass, gilthead sea bream, and common carp have indicated that the inclusion of soybean meal (SBM) in the diet triggers an inflammation process in the distal intestine, commonly referred to as SBM-induced enteropathy (Olli and Krogdahl, 1994; Baeverfjord and Krogdahl, 1996; van den Ingh et al., 1991; Knudsen et al., 2008; Bonaldo et al., 2008; Urán, et al., 2008a,b). Although the provoking mechanism remains to be established, the SBM-induced enteritis is believed to be caused by a disruption of the intestinal barrier, with subsequent exposure of otherwise shielded layers of the mucosa to luminal ingredients, including food-derived and microbial antigens (Romarheim et al., 2011). The typical signs of such inflammation are a shortening of the primary and secondary intestinal mucosal folds, an increase in the number of goblet cells and the infiltration of inflammatory cells, particularly macrophages and eosinophilic granulocytes, into the lamina propria. This situation reduces the capacity of the enterocytes lining the epithelium to absorb nutrients (Baeverfjord and Krogdahl, 1996; van den Ingh et al., 1991; Buttle et al., 2001). These effects proved to be dose–dependent; the worst symptoms were observed at the highest inclusion level (30%), but even diets containing as low as 7.6% SBM produced morphological changes at the intestinal level (Krogdahl,

2003). SBM inclusion in fish feed has also been associated with impaired cholesterol and bile acid reabsorption, which probably occurs as a direct result of distal intestine inflammation (Kaushik et al., 1995; Regost et al.,1999; Refstie et al., 1999; Kortner et al., 2013).

It has been well documented that the responsible for the off-target effects of SBM it is not the soy protein but other components present in the SBM, such as the anti-nutritional factor saponin, in combination with at least one more unidentified components (van den Ingh et al., 1991; Baeverfjord and Krogdahl 1996; Bakke-Mckellep et al., 2000; Krogdahl et al., 2003; Knudsen et al., 2007).

Once an inflammatory immune response is initiated, proinflammatory cytokines and a panel of chemokines (secreted proteins that play a major role in the inflammatory response) instigate the coordinated expression of downstream genes (Martin, et al., 2006). The expression of inflammatory and anti-inflammatory cytokine genes was quantified in the isolated intraepithelial lymphocytes of common carp (*Cyprinus carpio* L.), in which an intestinal inflamation was observed (Urán et al., 2008a). The enteropathy was developed when carp continuously fed on animal protein, were transferred to a diet in which 20% of the protein was replaced by SBM. After 3 weeks of feeding, the pro-inflammatory interleukin 1β (IL-1β) and tumour necrosis factor α1 (TNF-α1) genes were up-regulated whereas the anti-inflammatory interleukin 10 (IL-10) was down-regulated after an initial up-regulation at the first week of feeding.

The effects of replacement of FM with VM in fish intestine have been described also in several carnivorous species cultured in Mediterranean area, reporting similar inflamation symptomps. Nevertheless, the reported data are sometimes conflicting. Bonaldo et al. (2008) did not find any histological differences in the intestine of European seabass or Gilthead sea bream when fed for 80 and 89 days, respectively, with up to 300 mg/kg SBM. Similarly, a certain ability of Gilthead sea bream to cope with saponins and phytosterol components of SBM was reported by Couto et al. (2014), whereas other authors reported that no more than 50% of SBM can replace FM in methionine supplemented diets for both European sea bass (Tibaldi et al., 2006) and Gilthead seabream (Venou et al., 2006).

Different studies on the replacement of FM with high percentages of soybean meal (SBM) have reported a reduction of fish growth (Boonyaratpalin et al., 1998; Wang et al, 2006; Kikuchi and Furuta, 2009). However, investigations in trout, *Oncorhynchus mykiss* (Kaushik et al., 1995), and cobia, *Rachycentron canadum* (Salze et al., 2010), indicated that more than 90% of FM can be replaced with soy protein concentrate without affecting fish growth performance or nutrient utilization, whereas replacement of FM with soyflour (up to 50%) reduced the growth rate in trout (Kaushik et al., 1995).

The effects of replacement of FM with VM, often accompanied by reduced fish performance, are not restricted to SBM inclusion solely, but have been observed after inclusion of many plant protein sources in several teleost species such as gilthead sea bream, turbot, Atlantic cod, and parrot fish (Gomez-Requeni et al., 2004; Sitja-Bobadilla et al., 2005; Regost et al., 1999; Yun et al., 2011; Hansen et al. 2007; Lim and Lee 2009). Baeza-Ariño et al. (2014) described liver and gut alterations of gilthead sea bream, *Sparus aurata* L., fed diets in which FM was replaced by a mixture of rice and pea protein concentrates. The results of the histological analysis showed significant changes in the case of the 90% substitution in parameters such as thickness of the gut layers, number of goblet cells and villi's length and thickness, whereas the integrity of the gut structure was not significantly affected by a diet with up to 60% of replacement. In some cases, severe vacuolization was encountered, which consequently deformed hepatocytes and displaced the nucleus.

The short chain fatty acid butyrate, may promote the healing of inflamed intestine through its major role in enhancing epithelial cell proliferation and differentiation and in improving the intestinal absorptive function (Canani et al., 2012; Gálfi and Neogrády 2002; Wong et al., 2006). Like other short chain or volatile fatty acids (acetic, propionic, valeric, and caproic), butyric acid is produced during the fermentation of dietary fibers by the anaerobic microbiota associated with the epithelium of the animals' digestive tract. In addition to being the main respiratory fuel source of the intestinal bacteria, and preferred to glucose or glutamine, this four-carbon chain organic acid molecule has potential immunomodulatory and anti-inflammatory properties (Vinolo et al., 2009; Toden et al., 2007), and exert multiple other beneficial effects on host energy metabolism (Hamer et al., 2008; Den Besten et al., 2013). Although the mechanisms underlying these effects are still enigmatic and the subject of intense scrutiny, it is believed that they encompass the complex interplay between diet, gut microbiota, and host energy metabolism.

However, much of the research on butyrate has been focused on terrestrial vertebrates, including humans whilst very few studies have been conducted in fish. In particular, little is known about the effects of butyrate used as a feed additive on fish intestinal integrity. In terrestrial farmed animals such as pig and chicken, butyrate included in the diet has had a positive influence on body weight gain, feed utilization, and composition of intestinal microflora. It exerted trophic effects on the intestinal epithelium through an increase in the villi length and crypt depth, too (Gálfi and Bokori, 1990; Kotunia et al., 2004; Hu et al., 2007). In fish, Robles et al. (2013) reported an effect of butyrate used as a feed additive in increasing the availability of several essential amino acids and nucleotide derivatives, which have been demonstrated to increase fish growth when they are added individually to the diet. An effect of butyrate supplemented to the diet in enhancing the expression of the intestinal oligopeptide transporter PepT1 was observed both *in vitro* and *in vivo* in carp (*Ctenopharyngodon*

*idella*) intestinal cells (Liu et al., 2013). PepT1 is an integral plasma membrane protein located on the brush-border membrane of the intestinal epithelium and plays an important role in the absorption of the end products of intestinal protein digestion (Terova et al., 2009; 2013; Rimoldi et al., 2015; Kwasek et al., 2012). In the study of Liu et al., (2013) PepT1 was increased in a dose-dependent manner and dietary protein and butyrate had a significant effect on its gene expression in grass carp. Among the effects of butyrate in fish, Pérez-Sànchez et al. (2013), quoting Gaudier et al. (2004), and Burger-van Paassen et al. (2009) reported that in Gilthead sea bream, butyrate can stimulate the gene expression of several mucins, which are a major component of intestinal mucus gel secretions that serve as a barrier to protect the intestinal epithelium against aggressions arising from the luminal content.

Another feed additive that has been shown to have a restorative effect on soya saponin- and soya lectin-induced enteritis in fish (Iwashita et al., 2008; 2009) is the organic acid taurine. A number of recent studies reviewed by El-Sayed, (2014) and Salze and Davis, (2015) have demonstrated the essentiality of dietary taurine for many commercially relevant cultured species, including marine teleosts. According to these studies, taurine is involved in many physiological functions in fish and represents an essential nutrient, which exert powerful antioxidant and anti-inflammatory properties and is required as a supplement in the feed when a relevant percentage of vegetable protein sources are utilized. Taurine is a neutral β-amino acid found in high concentrations in animal tissues whereas plants contain less than 1% of the taurine levels found in animals. It is a simple molecule, containing an acidic sulfonate group, a basic amino group, and two carbons in between. This is in contrast to α-amino acids where the amino group is bound to the same carbon holding the acidic group (i.e., first carbon, α) (Salze and Davis, 2015). Although there are naturally occurring taurine-containing peptides, taurine cannot be part of translated peptide chains as there is no transfer RNA that carry it to the ribosome. In mammals, taurine is mainly synthesized in liver and brain through enzymatic oxidation and direct conversion of cysteine (derived from methionine) to cysteine sulfinic acid and hypotaurine. In this pathway the flux of cysteine to cysteine sulfinate is mainly determined by the highly regulated enzyme cysteine dioxygenase (CDO), whereas the decarboxylation of cysteine sulfinic acid into hypotaurine, which is then oxidized to taurine, is regulated by cysteinsulfinate decarboxylase (CDS) (Griffith, 1987; El-Sayed, 2014). In animal taxa with limited or no activity of the CDS, such as marine fish species, a continuous supply of taurine should be provided with the diet (El-Sayed, 2014; Battim and Brumaghim, 2009). Indeed, Yokoyama et al. (2001), by comparing the CSD activity and hypotaurine production in several teleost and mammalian species, found that the only fish species with a high CSD activity were rainbow trout and tilapia. However, their enzyme activity was an order of magnitude lower than that found in mammalian species and other data

indicate that both species benefit from dietary taurine supplementation (see Salze and Davis, 2015 for a review), thus suggesting that levels of CSD activity are insufficient to provide the necessary amounts of taurine for maximum growth of rainbow trout and tilapia.

Accordingly, the objective of the present research was to evaluate the mitigating effects of butyrate and taurine used as feed additives on the morphological abnormalities caused by a SBM based diet in the intestine of European sea bass. In order to attain this aim, we utilized both light and transmission electron microscopy (TEM) to determine intestinal changes in fish. We also quantified the intestinal mRNA abundance of several genes involved in the mucosal inflammatory response such as TNF$\alpha$, which is a cell-signaling protein (cytokine) that makes up the inflammatory acute phase reaction, and interleukins such as IL-8, IL-1β, IL-10, and IL-6, which are well-known cytokines that regulate immune responses, inflammatory reactions, and hematopoiesis.

## Materials and methods

The feeding experiments were conducted at the water recirculating system facility situated at the Department of Biotechnology and Life Sciences, University of Insubria, Varese, Italy. After being individually weighted and tagged, 30 European seabass (*Dicentrarchus labrax*) of an average weight of 514 ± 67.4g were transferred from 2500-L acclimation tanks into six circular 750-L tanks with 5 fish/tank. After being transferred into the respective tanks, fish were allowed to acclimatize for one week before the start of the feeding trial. Fish were fed two times a day *ad libitum*, in duplicate (two tanks per diet) with 3 different diets (Tab. 1) and sampled at the end of the feeding experiment which lasted 60 days. The experimental tanks were connected to a 20 $m^3$ water recirculation system. The water parameters such as pH, temperature, and dissolved oxygen (DO) were strictly controlled during all the experiment. Temperature was maintained at 20 ± 0.5°C; salinity at 22 g $L^{-1}$; pH 7.2; total N-$NH_3$ ≤0.1 mg $L^{-1}$; N-$NO_2^-$ ≤0.02 mg $L^{-1}$; N-$NO_3^-$ ≤ 5 mg $L^{-1}$, DO 8-8.5 mg $L^{-1}$, and DO saturation, over 97%.

Fish were euthanised by anaesthetic overdose (tricaine-methasulfonate, Finquel MS-222 ®, Agent Chemical Laboratories, USA) and then weighed individually. The specific growth rate (SGR) was calculated using the following formula: SGR= 100 x [ln (final BW) – ln (initial BW) ]/days.

The distal intestine of all sampled fish was dissected out and half of it was fixed in 4% buffered formalin, pH 7.2, for either histological or immunohistochemical examination, whereas the other part was conserved at -80°C for gene expression analyses.

The experimental feed

We used 3 experimental diets, named C, B, and T, which contained the same low percentage of fish meal and high percentage of soybean meal, as shown in Table 1. Diets B and T were supplemented with 2% of sodium butyrate or taurine, respectively, whereas Diet C, which represented the control diet was not supplemented.

Light microscopy

Sampled intestine was fixed by immersion in 4% buffered formalin, dehydrated with a graded ethanol series (20%, 30%, 50%, 70%, 95%), embedded in paraffin, and sectioned at 4 μm. After dewaxing and rehydrating the sections, they were consecutively stained with hematoxylyn-eosin. Periodic Acidic Schiff (PAS) and Alcian blue stain at pH 2.5 were used to reveal neutral and acidic mucosubstances, respectively.

Immunohistochemistry for leukocyte assessment

The indirect immunohistochemical methodology protocol included a primary antibody specific for the leukocyte antigen CD45 and a secondary antibody conjugated with alkaline phosphatase, substrate, and chromogen. The sections (4 μm) of distal intestine were dewaxed and rehydrated according to normal procedures and incubated for 18 hrs at 37°C. To avoid false positives due to the chromogen reaction with endogenous alkaline phosphatase, the latter were deactivated with $CH_3COOH$ 20% applied for 15 min at 4°C. To avoid other kinds of false positives due to the antibody reaction with aspecific antigens, the samples were treated with PBS/BSA 2% Tween20 0.1% at environmental temperature for 30 min. Then, the samples were washed 5 x 1 min in phosphate buffer (PBS 1x) before applying the primary antibody (CD45 Antibody, pAb, Rabbit GenScript NJ,USA) at a 1:100 dilution overnight at 4°C. After another 5 x 1-min washing cycles in phosphate buffer, the secondary antibody directed to immunoglobulins belonging to the same species was incubated for 1h and utilized to produce the primary antibody, anti-rabbit IgG (whole molecule)-alkaline phosphatase antibody produced in goat (SIGMA-ALDRICH). The substrate system (SIGMAFAST BCIP/NBT tablet) was used to develop the reaction, which was monitored at the light microscope. After 15-20 min, a final washing with phosphate buffer was performed to stop the reaction; the glass was mounted with PBS/glycerol 1:1. Negative controls were prepared with samples without applying the primary antibody.

Electron microscopy

Specimens already fixed in formalin were transferred to a 2% Karnovsky solution at 4°C for 2 hours and then embedded in Epoxy resins. Ultra-thin sections were stained with uranyl acetate and lead citrate and examined with a TEM Morgagni Philips/FEI electron microscope.

Semi-quantitative PCR: expression levels of cytokines

Total RNA was extracted from 125 mg of distal intestine (5 fish/group). Tissue lysis and homogenization were performed in special disposable sterile tubes (GentleMACS M tubes™, MiltenyiBiotec), in order to minimize the possibility of cross contamination between samples, and using the gentleMACSDissociator (MiltenyiBiotec). After an automated purification process by using the Maxwell® 16 Instrument and Maxwell® 16 Tissue LEV total RNA purification Kit (Promega, Italy), the RNA was isolated. The concentration and purity of total RNA were determined by a spectrophotometer NanoDrop ™ (Thermo Scientific), measuring the absorbance at 260 nm and the absorbance ratio 260/280, respectively. The integrity of RNA was verified by electrophoresis on 1% agarose gel stained with ethidium bromide.

Reverse transcription of 1 μg total RNA from each fish intestine tissue was performed with random decamers in a volume of 20 μl using the High-Capacity cDNA Archive Kit (Life Technologies, Italy) following the manufacturer's instructions.

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis was performed to quantify the transcript levels of *interleukin-1β* (*IL-1β*) (GenBank Acc. No. **AJ269472**), *interleukin-6* (*IL-6*) (GenBank Acc. No. **AM490062**), *interleukin-8* (*IL-8*) (GenBank Acc. No. **AM490063**), *interleukin-10* (*IL-10*) (GenBank Acc. No. **AM268529**), and *tumor necrosis factor α* (*TNF-α*) (GenBank Acc. No. **DQ070246**) genes. Each PCR reaction was carried out in triplicate using forward and reverse primers selected for target genes and β-actin (GenBank Acc. No. **AY148350**), which represented the internal normalizer (Tab. 2). The reaction mixtures included 1 μl of cDNA, 0.5 μL of each specific primer (50 μM), and 12.5 μL of GoTaq 2X Master Mix (Promega) in a final volume of 25 μL. The PCR reactions were incubated at 95 °C for 4 min, followed by 30 cycles of thermal cycling (30 s at 95 °C, 30 s at 55 °C and 35 s at 72 °C). The final cycle was followed by a 5-min extension step at 72 °C.

PCR products were subsequently analyzed on 2% agarose gel, and bands were quantified using GelDoc acquisition system (Bio-Rad) and QuantityOne software (Bio-Rad). The intensity of each band obtained (INT/ $mm^2$) was normalized on the basis of intensity levels of *β-actin*. The expression

levels of each target gene were documented with reference to expression in control group (C), for which levels were arbitrarily set as equal to 1.

Statistical analyses

The quantitative data were submitted to ANOVA variance analyses and the Duncan's test used for post hoc analysis, applying the software IBM SPSS Statistics 21. The value $p<0.05$ was considered significant for differences.

## Results

Fish growth

Growth performance of fish fed diet T was significantly different ($P<0.05$) from fish fed diets C and B (Tab. 3).

Light microscopy and Immunohistochemistry

Histologically, the intestinal wall surrounding the lumen of seabass intestinal tract is made up of four layers of specialised tissue – from the lumen outwards: mucosa, submucosa, muscle layer and serosa. By light microscopy, we evaluated different aspects of the various anatomical portions of the distal intestine. The thickness of the muscle and serous layers were evaluated. The shape and length of the folds of the intestinal mucosa and the thickness of the lamina propria of the folds were observed. In addition, we paid attention to the presence and distribution of mucous cells and to the presence, size, and content of supranuclear vacuoles of enterocytes. The presence and extent of any inflammatory infiltrates was also examined.

The distal intestines of sea bass fed with diet C had very developed serous and muscular layers. The average length of villi were characterized by simple morphology with areas of flaking and discontinuity of the mucosa (Fig. 1a). The lamina propria was thickened with respect to the physiological condition. In enterocytes, we observed abundant vacuoles of various sizes not regularly aligned, eosinophils, and PAS-positive areas occupying much of the cytoplasm and forcing the nucleus at the base of the cell (Fig. 1b, 1c). We observed a few muciparous cells that were uniformly distributed and examined them by using a specific staining procedure for mucins, Alcian Blue PAS (Fig. 1c). An important leukocyte infiltrate was found between the mucosal epithelial cells (Fig. 1b,

1c yellow arrows) at the level of the submucosa and also at the level of the serous and muscle layer, as further shown by immunohistochemistry (Fig.1d).

In the intestines of sea bass receiving the diet B in which 2% of butyrate was added, the thickness of the serous and muscular layers was normal (Fig.2a); the intestinal plicae, long, were characterized by a complex structure and the lamina propria appeared slightly thickened, particularly in those areas that corresponded to the branching points of the structure of the fold (Fig. 2c). Supranuclear vacuoles were plentiful and diverse in form, size, and content (Fig. 2b, red arrow). Numerous muciparous cells were found that were well distributed along the folds (Fig. 2b, the green arrow), which was demonstrated specifically by Alcian blue PAS staining (Fig. 2c). A hyperproliferative epithelium was observed in areas in which the typical single layer showed an abnormal pluristratification (blue arrow Fig. 2c). The inflammatory infiltrate was identified between the epithelial cells in the lamina propria and in the submucosa (yellow arrow in Fig. 2b), and corroborated by immunohistochemistry (Fig. 2d yellow arrows).

The seabass fed with diet T which was supplemented with 2% of taurine, presented a well-developed muscle layer (Fig. 3a). The long folds tended to be simple and showed the lamina propria to be slightly thickened in some places, but generally thin. Muciparous cells were abundant, preferentially concentrated in the medium-apical fold (Fig. 3c). The supranuclear vacuoles were characterized by a heterogeneous content (Fig. 3b red arrow). A mild overgrowth of epithelium was observed (blue arrow Fig. 3c). The inflammatory infiltrate was detected in the submucosa and in the mucosa, both in the lamina propria and between epithelial cells, which was confirmed by immunohistochemistry (Fig. 3d yellow arrows).

Electron Microscopy

Further investigation by using transmission electron microscopy allowed us to highlight the cell ultrastructure in the three types of samples. As in most cells with absorbing properties, enterocytes are characterized by the presence of microvilli and numerous invaginations in the apical plasmamembrane, many pinocytotic vesicles and tubules (tubulo-vescicular system) in apical cytoplasm, and vacuoles in the supranuclear region. Different aspects of the enterocytes were investigated. From the lumen to the lamina propria of the fold we considered: the brush border, the tubulovesicular system occupying the apical area of enterocytes, the supranuclear vacuoles, the nuclei, and infiltrating inflammatory cells.

The distal intestines of seabass receiving feed C showed brush border areas with microvilli that appeared irregular and damaged (Fig. 4a). The content of the numerous large supranuclear vacuoles

was different, from homogeneous electrondense to granular (Fig. 4a, 4b). In some cases some vacuoles were fused into a single structure (Fig. 4b). Numerous leukocytes infiltrating the epithelial cells could be observed (Fig. 4c) and, in some cases, the nuclei of enterocytes appeared pyknotic.

In the intestines of fish fed with feed B, the microvilli were often interrupted for alteration of the apical plasma membrane (Fig. 5a). In the apical cytoplasm, a developed tubulo-vescicular system was observed, and in the supranuclear cytoplasm were evident many characteristic vacuoles that had an irregular shape and heterogeneous content, with clear areas mixed with dense material (Fig. 5b, 5c). Some enterocytes showed marked cytoplasmic degeneration (Fig. 5a), and others had infiltrated the mucosa.

Sections of distal intestines from fish fed with diet T presented long and well-shaped microvilli, sometimes topped by a layer of mucus (Fig.6c). A tubulo-vesicular system was observed in the apical cytoplasm, and in the supranuclear cytoplasm, there were many vacuoles with a heterogeneous content showing lamellar profiles (Fig.6b). Numerous muciparous Goblet cells (Fig. 6a) and leukocytes infiltrating the mucosa were detected, too (Fig. 6a).

Gene Expression Analysis

The expression levels of the proinflammatory interleukins *IL-1β*, *IL-6*, *IL-8*, and *TNF* and the anti-inflammatory interleukin *IL-10* genes were evaluated in the distal portion of sea bass intestine by semi-quantitative RT-PCR. The results of agarose gel electrophoresis of amplified PCR products are shown in Figure 7. The semi-quantitative PCR analysis, based on the densitometric analysis of band intensity, showed that the expression of target genes in the intestine was deeply influenced by the type of fish diet. Although *IL-1β* was expressed at low levels in all analyzed fish, sea bass fed diet C displayed the highest expression level of *IL-1β* gene in comparison to fish fed on other two diets. Indeed, only one out of five analysed fish of group B and T expressed quantifiable levels of *IL-1β* transcript (fig. 7). In fish fed the T diet, which was supplemented with taurine, the expression of both *IL-8* and *IL-10* genes was significantly lower ($P$ <0.05) than in fish which received diet C or B (Fig. 8A, D). Dietary butyrate caused an upregulation of *TNFα* gene transcription; indeed, fish fed on diet B showed higher levels of TNFα mRNAs than fish fed on other diets ($P$<0.05) (Fig. 8B). Finally, among the interleukins measured in this study, *IL-6* was the only one, to be not influenced by diet (Fig. 8E).

**Discussion**

The feeding experiment conducted in this study lasted 60 days which is a short period for SGR and FCR (feed conversion rate) reliable evaluation. Nevertheless, SGR of fish fed diet T was 2.7 times higher ($P<0.05$) than that of fish fed diet C or B. This result is in line with findings of Brotons-Martinez et al. (2004) on sea bass fingerlings fed a diet containing SBM and supplemented with taurine for 30 days.

With regard to the histological data, we observed a reduction in the number of intestinal goblet cells, in fish fed diet C with SBM and this result is in agreement with other studies conducted in Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) (Baeverfjord and Krogdal, 1996; Iwashita et al., 2008). However, we found also high concentrations of absorptive vacuoles containing PAS-positive substances (probably glicoproteins, considering their acidophilic properties) in the supranuclear region of the enterocytes from fish fed diet C. This could indicate an interruption of the digestive processes occuring within the enterocytes, since these cells are involved not only in the uptake of different nutrients but also in the digestion. For example, once inside the enterocyte, the absorbed di- and tripeptides are digested into amino acids by cytoplasmic peptidases and exported from the cell into blood; only a very small number of these small peptides enter blood intact. In contrast, other authors have reported a reduction of the same supranuclear vesicles in salmonids and in carps. In fish fed diet C, lamina propria and epithelial lining were heavily infiltrated with inflammatory cells, including macrophages and polymorphonuclear leukocytes. We also observed shortening of the mucosal folding height, and widening of the lamina propria, which are all effects similar to those reported in literature in other fish fed SBM meal based diets (Baeverfjord and Krogdal, 1996; Urán et al., 2008 a,b). The important signs of inflammation in our samples were confirmed by immunohistochemistry staining. This analysis showed a strong positivity for anti-CD45 antibody indicating a relevant diffusion of leukocytes in all the distal intestinal tissue. The TEM observations confirmed the presence of leukocytes and showed an apical tubulovescicular system, large vacuoles occupying the supranuclear cytoplasm and containing a homogeneous electrodense substance, and local intracellular degeneration, which may be related to the fragility of the vacuolar membrane leading to the release of lytic enzymes and the recall of leukocytes. According to Noaillac-Depeyre and Gas, (1973), the supranuclear body is of complex origin. Indeed, in the adult carp, the apical cytoplasm of the enterocytes of the medium intestine shows the presence of a dense tubolovesicular network that form a voluminous supranuclear body. However, the supranuclear bodies are moreover independent of the presence of food material in the intestinal lumen since they are also present in fasting animals. Therefore, it seems that they do not result only from the running together of the food vacuoles but probably arise from the fusion of the Golgi vesicles. The supranuclear bodies would thus have a dual origin, endogenous for the hydrolytic enzymes that

contain, and exogenous for the di- and tripeptides supplied by the food which are then further digested inside them (Noaillac-Depejre and Gas, 1973).

Histological samples from distal intestine of fish fed diet B supplemented with sodium butyrate, showed that the *muscularis externa* was thinner than that of fish fed diets C or T. This suggests that butyrate facilitated the formation of softer fecal pellets in fish of this group. The complex structure of the intestinal folds was suboptimal, not perfectly organized and included a high number of goblet cells, and a thick lamina propria. Although the inflamatory cells were infiltrated less than in the distal intestine of fish fed diet C and were more concentrated at the submucosa level, we found a significant number of leukocytes as confirmed by the positive staining with the CD45-antibody. The number of supranuclear vacuoles was less than in fish fed diet C and they were often heterogeneous. TEM analysis of enterocytes showed an alteration at the microvilli level, with cells at a certain state of degradation. However, other areas appeared much less compromised and microvilli were regularly displayed. The cells showed an intense vesicular system and supranuclear vacuoles with dishomogeneous content: partly dense, like that found in fish of group C, and partly clear. Furthermore, the aforementioned acidophilic and PAS-positive substance that we found in the vacuoles of fish fed diet C was reduced in the distal intestine of fish fed diet B, suggest that butyrate supplementation of the diet, have had a mitigation effect in the intestine of fish.

In fish fed diet T, the *muscularis externa* was developed as much as in fish fed diet C. The folds were long and regular, with enterocytes showing a limited number of vacuoles and an increased number of goblet cells, which were larger in size and mainly concentrated in the medioapical part of the fold. Leukocytes, immunoreactive to the CD45 antibody, were still present in both, the mucosa and the submucosa, similar to those observed in fish fed diet B. The TEM images of enterocytes were very different from those in fish fed diets C and B, with cells showing well-displayed microvilli frequently covered by a protective mucus layer, no alterations in the structure, and supranuclear vacuoles filled with a heterogeneous substance, including membranous bodies suggesting that an autophagic process was in progress. To our knowledge, no other example of taurine used as a feed additive causing autophagic processes has been reported in the literature, and thus this finding should be further investigated. However, this process seems to enable enterocytes of fish fed with diet T to cope with the negative effects induced by the SBM diet, and contyributes to the protection of the intestinal mucosa.

The expression pattern of molecular markers of inflammatory processes such as the inflammatory cytokines supported the light and TEM microscopy observations. The highest transcription level of the IL-1β gene was found in the distal intestine of sea bass fed diet C, indicating a chronic inflammation, which was less evident in fish fed diets B ot T. Cytokine IL-8 was less expressed in

fish fed diet T than in those fed diet C or B, suggesting a protective action of taurine against oxidative stress through the reduction of the inflammatory cascade induced by oxidative stress. The transcription activity of IL-10 gene, which is known as anti-inflammatory cytokine, was strongly reduced in fish fed diet T, remaining however relatively high in fish fed diets C and B. Among the target cytokines, only IL-6 gene expression was not affected by dietary butyrate or taurine supplementation. Proinflammatory IL-6 is of particular interest since it is expressed to a small extent in normal colonic epithelium but to a much greater degree in colonic carcinomas (Shirota et al., 1990). In human colonic cells, increased expression of both IL-6 and its receptor IL-6R, have mainly an anti-apoptotic effect (Yuan et al., 2004). IL-6, indeed, upregulates the expression of anti-apoptotic protein Bcl-xl (Fujio et al., 1997; Scwarze and Hawly, 1995). Yuan and colleagues (2004), clearly demonstrated that butyrate downregulates IL-6 signaling in human colonocytes by inhibiting IL-6 receptor rather than IL-6 expression. Similarly, in our study, butyrate did not influence IL-6 expression, but we cannot exclude that, even in fish, butyrate could act principally on IL-6 receptor rather than in IL-6 expression. So, this is an issue that require further investigation.

It is well documented that taurine inhibits the overproduction of inflammatory mediators such as TNF-α (Kim and Cha, 2014; Locksley et al., 2001). TNF-α produced is known to stimulate further production of itself and to induce the expression of other pro-inflammatory mediators, such as IL-6, IL-8. TNFα was expressed at higher levels in the intestine of sea bass fed diet B, which induced an upregulation also of IL-8 but not of IL-6. Moreover, higher expression of TNF-α in fish receiving B diet supported the TEM results indicating a promotive effect of butyrate in increasing cellular turnover.

Our findings on cytokine activity are mainly in agreement with the literature. Indeed, in the intestine of carps receiving soy as protein source (Urán et al., 2008a), a peak of IL-1β was observed after the first week of feeding, and the expression of this gene as well as that of TNF-α1 remained high over the control levels for the entire experiment. In the same study, a strong upregulation of IL-10 was observed after one week of SBM feeding, but at weeks 3 and 5 expression levels were downregulated at values either lower or similar to the control (Urán et al., 2008a). In zebrafish fed diets containing different amounts of SBM, Hedrera et al. (2013) reported a signficant increase in the intestinal transcription of the mRNAs coding for proinflammatory cytokines, such as IL-1β and IL-8. The transpcriptional activity of IL-10, known to have an anti-inflammatory action, was increased in the group receiving feed with soy protein, too.

In conclusion, sea bass fed a diet containing a concentration of a soy protein source close to 30% (16.7% as SBM and 12.8 as full-fat soy) for two months developed a severe inflammatory status in

the distal intestine. The symptoms of inflammation seem to be mitigated, albeit at different success rates by adding sodium butyrate or taurine at concentrations as low as 2%. However, the meccanisms underlying these effects should be further investigated along with any possible synergistic effect of the two substances combined in the feed in normalizing the intestinal abnormalities caused by soyabean meal.

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## Tables

**Table 1** Diet composition

| **Raw material** | **C (%)** | **B (%)** | **T (%)** |
|---|---|---|---|
| Full fat soy | 12.8 | 12.8 | 12.8 |
| SPC [a] | 13.6 | 13.6 | 13.6 |
| Wheat | 8.0 | 8.0 | 8.0 |
| Wheat gluten meal | 8.19 | 8.19 | 8.19 |
| DCP [b] | 1.72 | 1.72 | 1.72 |
| Mixed oil [c] | 12 | 10 | 12 |
| Lysine (98%) | 0.29 | 0.29 | 0.29 |
| Vitamins and mineral premix [d] | 0.4 | 0.4 | 0.4 |
| Corn gluten | 16.0 | 16.0 | 16.0 |
| Soybean meal (48%) | 16.7 | 16.7 | 16.7 |
| Fish meal (65%) | 10.0 | 10.0 | 10.0 |
| Anti molds | 0.1 | 0.1 | 0.1 |
| Protein | 45.0 | 45.0 | 45.0 |
| Fat | 16.0 | 16.0 | 16.0 |
| Fiber | 2.3 | 2.3 | 2.3 |
| Ash | 6.4 | 6.4 | 6.4 |
| Calcium | 1.0 | 1.0 | 1.0 |
| Total phosphorus | 0.95 | 0.95 | 0.95 |
| Mathionine | 0.9 | 0.9 | 0.9 |
| Methionine + cysteine | 1.6 | 1.6 | 1.6 |
| Lysine | 2.3 | 2.3 | 2.3 |
| Taurine | - | - | 2 |
| Sodium butyrate | - | 2 | - |

[a] SPC - soy protein concentrate
[b] DCP - Dicalcium Phosphate
[c] Mixed oil: 40% corn 60% soy
[d] Vitamin and Mineral premix (quantities in 1 kg of mix): Vitamin A, 4,000,000 IU; Vitamin D3, 800,000 IU; Vitamin C, 25,000 mg; Vitamin E, 15,000 mg; Inositol, 15,000 mg; Niacin, 12,000 mg; Choline chloride, 6,000 mg; Calcium Pantothenate, 3,000 mg; Vitamin B1, 2,000mg; Vitamin B3, 2,000mg; Vitamin B6, 1,800 mg; Biotin, 100 mg; Manganese, 9,000 mg; Zinc, 8,000 mg; Iron, 7,000 mg; Copper, 1,400 mg; Cobalt, 160 mg; Iodine 120 mg; Anticaking & Antioxidant + carrier, making up to 1000 g.

**Table 2** Nucleotide sequencies of the primers used for the semi-quantitative PCR

| GENE | GenBank Acc. n° | Nucleotide sequence 5'-3' |
|---|---|---|
| IL-1β | AJ269472 | F: GCTCGGACAGATGCAAA |
| | | R: CTTTGTCCACCACCTCCAGA |
| IL-6 | AM490062 | F: ACATGCCCTGAGAAGTCCAG |
| | | R: CCGCTGGTCAGTCTAAGGAG |
| IL-8 | AM490063 | F:GTCTGAGAAGCCTGGGAGTG |
| | | R: CTCGGGGTCCAGGCAAAC |
| IL-10 | AM268529 | F: CGACCAGCTCAAGAGTGATG |
| | | R: AGAGGCTGCATGGTTTCTGT |
| TNF-α | DQ070246 | F: CTCAACACAGCGGATATGGA |
| | | R: TGGGGATCTGTTTTCTCAGC |
| β-ACT | AY148350 | F: GAGCGTGGCTACTCCTTCAC |
| | | R: GGTCCTTACGGATGTCAACG |

**Table 3** Growth performances of European seabass

| | $T_0$ | $T_{60}$ | | |
|---|---|---|---|---|
| | | Diet C | Diet B | Diet T |
| **Biomass (g) ± S.D.** | 514 ± 67.40 | 546.8 ± 61.14 | 540.2 ± 60.95 | 641.2 ± 60.95 |
| **SGR (%)** | - | $0.12^{a}$ | $0.12^{a}$ | $0.32^{b}$ |

Fish were fed *ad libitum* for 60 days (T60) with a 45% protein diet, containing 10% FM and 35% VM. Different superscript letters mean significant differences in SGR ($P<0.05$).

**Figure captions**

**Figure 1.** Light microscope images obtained from distal intestine of sea bass fed diet C. (**a**) Morphology of a complete section at 1x, bar = 100 µm, EE stain, (*) indicates a discontinuous area, with folding exfoliation; (**b**) visible at higher magnification are vacuoles in eosinophils (indicated by the red arrow) and leukocyte infiltrate in the intraepithelial skinfold (indicated by the yellow arrow), 40x magnification, bar 25 µm, stain EE; (**c**) Evaluation of mucus cells or goblet cells (GC), Alcian Blue staining and PAS in mucus cells (green arrow), the vacuoles in the eosinophils (red arrow), also show PAS Positive staining Alcian Blue P.A.S. and leukocytes (yellow arrow), 20x magnification, bar 50 µm; (d) Observation of leukocytes highlighted anti-CD45 immunohistochemical analysis, image magnification 10x, bar 100 µm;

**Figure 2.** Light microscope images obtained from distal intestine of sea bass fed diet B. (**a**) Complete morphology of the section, observed at a magnification 1x, bar 100 µm, stain EE, (**b**) at higher magnification intraepithelial lymphocytes (yellow arrow), the vacuoles (red arrow) and mucus cells (green arrow)are visible by EE staining, magnification 20x, bar 50µm; (**c**) Alcian Blue staining for PAS evaluation of mucus cells, visible also a hyperproliferative epithelium (blue arrow), 20x magnification, bar 50µm; (d) anti-CD45 immunohistochemistry, magnification 10x, bar 100 µm.

**Figure 3** Light microscope images obtained from distal intestine of sea bass fed diet T. (**a**) Full morphology observed at a magnification 1x, bar 100 µm, stain EE; (**b**) at higher magnification 20x, bar 50µm, stain EE (red arrow indicating the vacuoles); (**c**) staining Alcian Blue P.A.S., which highlights the many muciparous cells (green arrow); the blue arrow indicates an area where it was possible to detect an overgrowth of enterocytes, 20x magnification, bar 50 µm; (**d**) Immunohistochemistry anti-CD45, (yellow arrows indicate the signal of the inflammatory infiltrate).

**Figure 4.** Electron microscope images obtained from distal intestine of sea bass fed diet C. (**a**) a portion of epithelium, with an advanced degeneration and enterocytes with incipient leukocyte infiltration (→), a focal alteration of the microvilli, and vacuoles with an electrondense content very close to bigger vacuoles with a granular content, magnification 2800x. (**b**) at higher magnification (5600x) is visible a vacuole (*) that pours its contents into a larger one; (**c**) pyknotic nuclei of enterocytes (indicated by arrow) magnification 4400x. V = vacuoles; , M= microvilli

**Figure 5.** Electron microscope images obtained from distal intestine of sea bass fed diet B. (**a**) Epithelium of two adjacent folds. At the base of the fold, nuclei of enterocytes are visible; in the median portion, intracellular spaces are present that originate from degenerated large vacuoles; in the apex portion partially damaged microvilli are present; the cytoplasm of a Goblet cell is observed , 1800x magnification; (**b**) vacuoles at higher magnification of 8900x, showing a heterogeneous content; (**c**) apical portion of a cytoplasm in which there are tubulo-vesicular system and vacuoles with heterogeneous content, 8900x magnification. N =enterocytes nucleus; V = vacuoles; TVS tubulo-vesicular system; GC =goblet cell, M=microvilli.

**Figure 6.** Electron microscope images obtained from distal intestine of sea bass diet T. (**a**) the epithelium of a fold with rather well-preserved cells including a muciparous cell is observed. Enterocytes show numerous vacuoles, 2200x magnification; (**b**) a vacuole containing lamellar body (indicated by arrow) is visible 7100x magnification; (**c**) epithelium with well developed microvilli dominated by mucus, 5600x magnification; V= vacuoles, M= microvilli, GC =goblet cell; Mu = mucus.

**Figure 7.** Agarose gel electrophoresis of the PCR products corrisponding to genes IL-8 (A), TNF (B), IL-1β, IL-10, IL-6 and β-actin.

**Figure 8.** Relative expression levels of IL-8 (A), TNFα (B), IL-1β (C), IL-10 (D) and IL-6 (E) genes. Values are reported as average values (n = 3) ± SD. The group C represents the reference sample whose values of expression for each target genes were placed by convention as equal to 1. Differences between the letters indicate significant differences between groups ($p < 0.05$), One-way ANOVA followed by Duncan's multiple range test.

**Fig. 1**

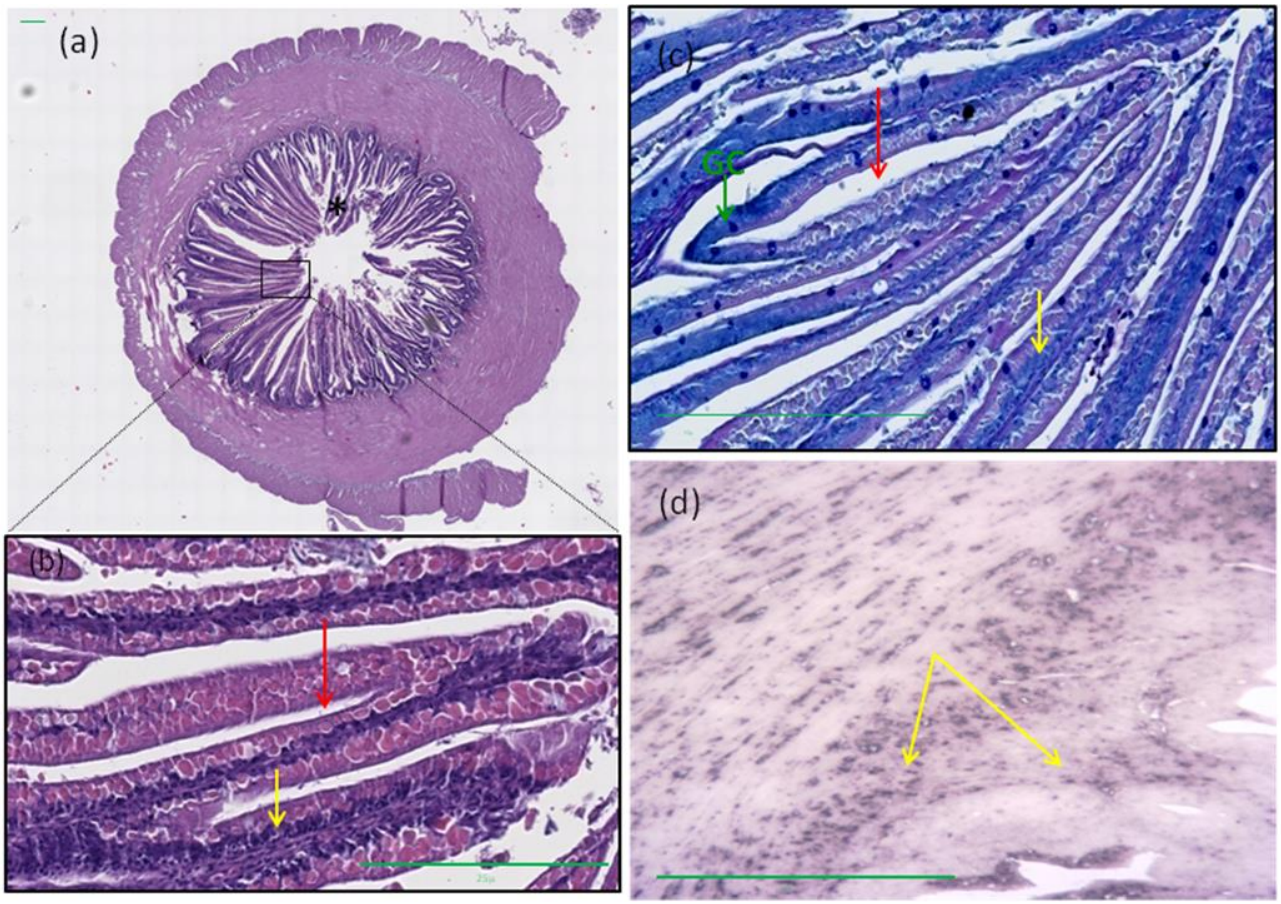


**Fig. 2**

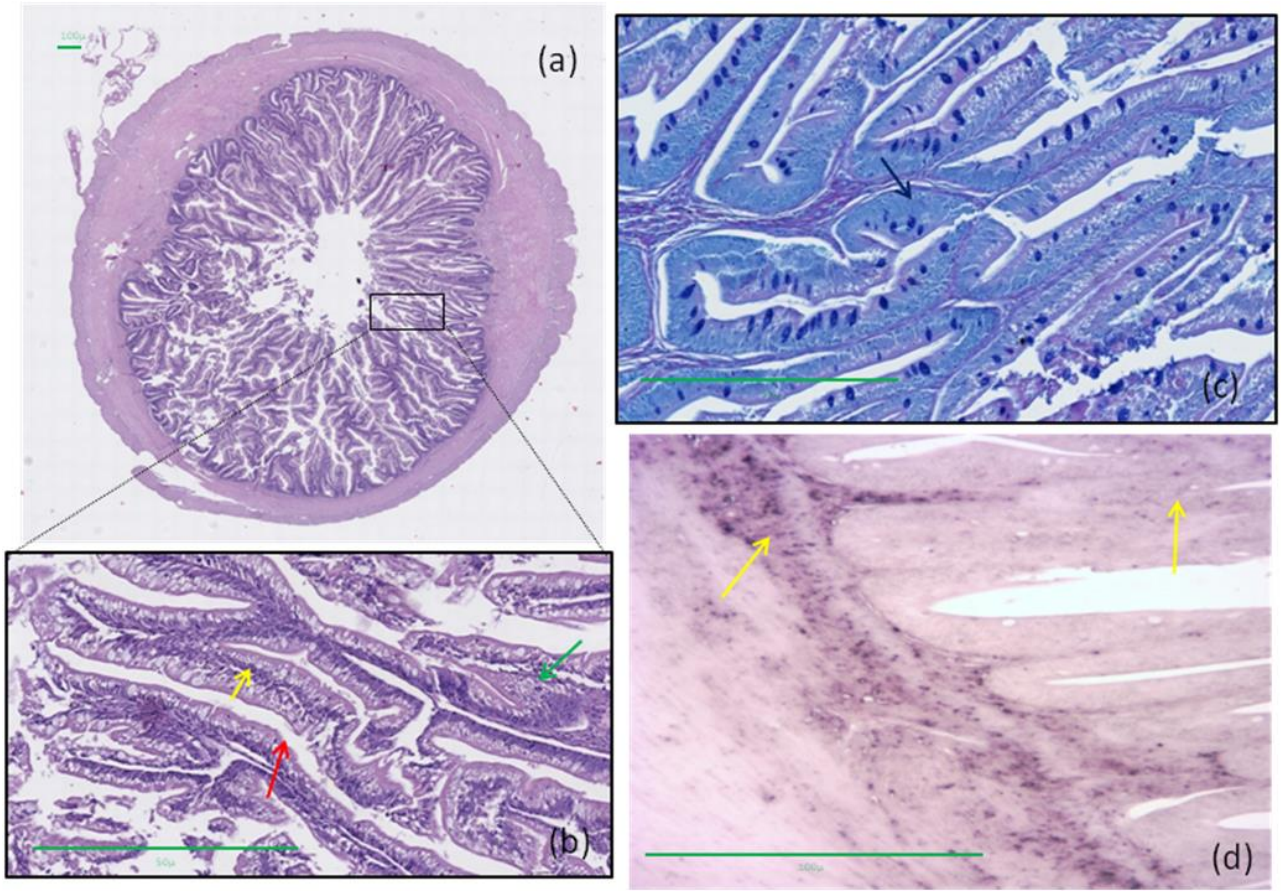



**Fig. 3**

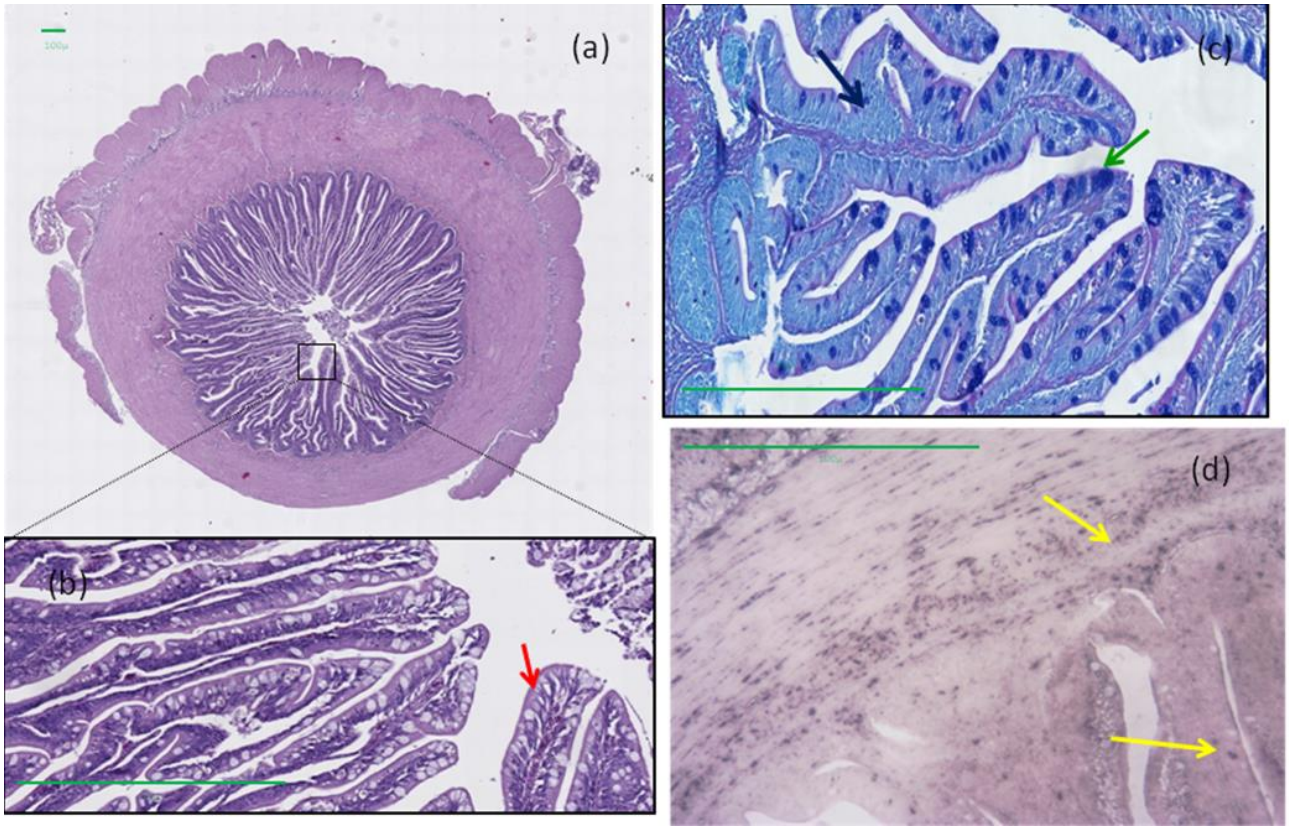
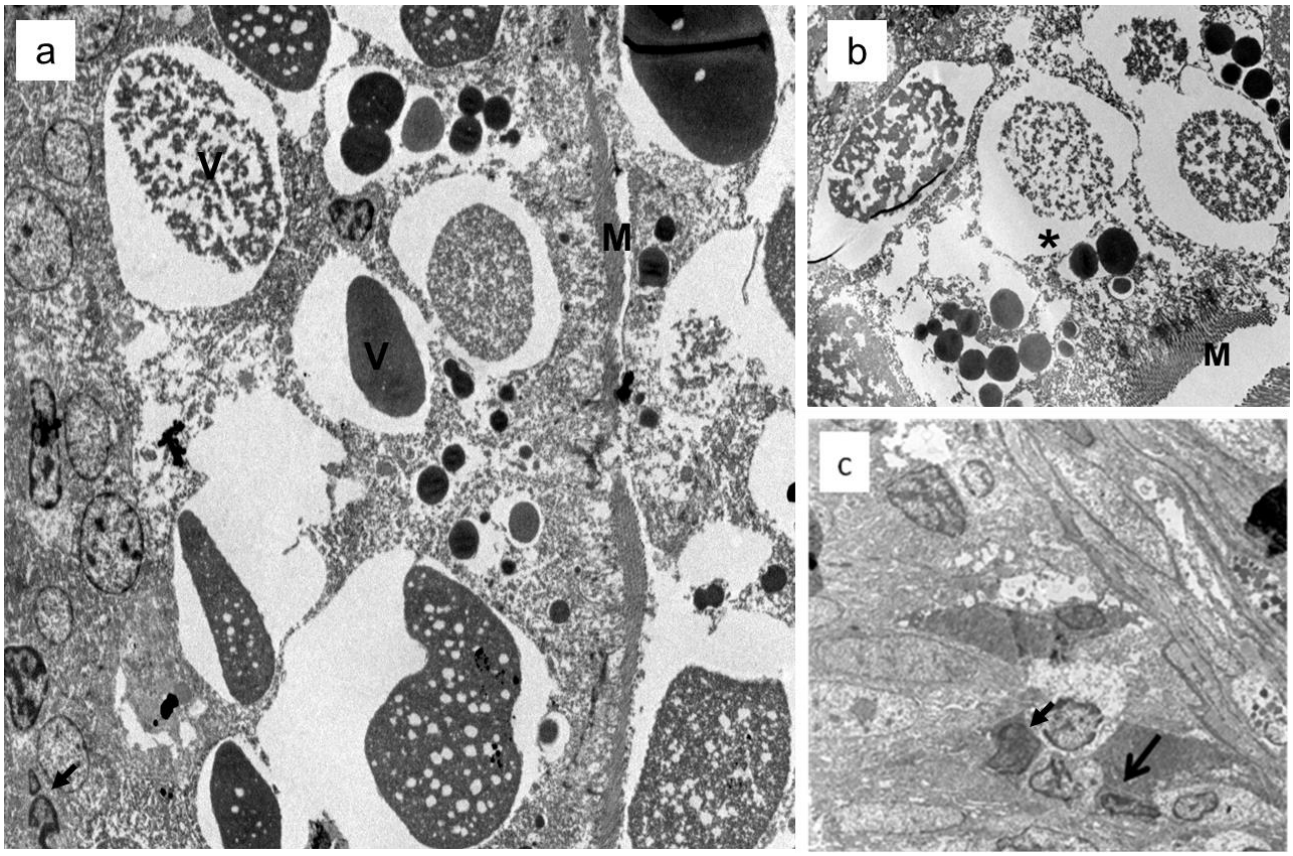


**Fig. 4**

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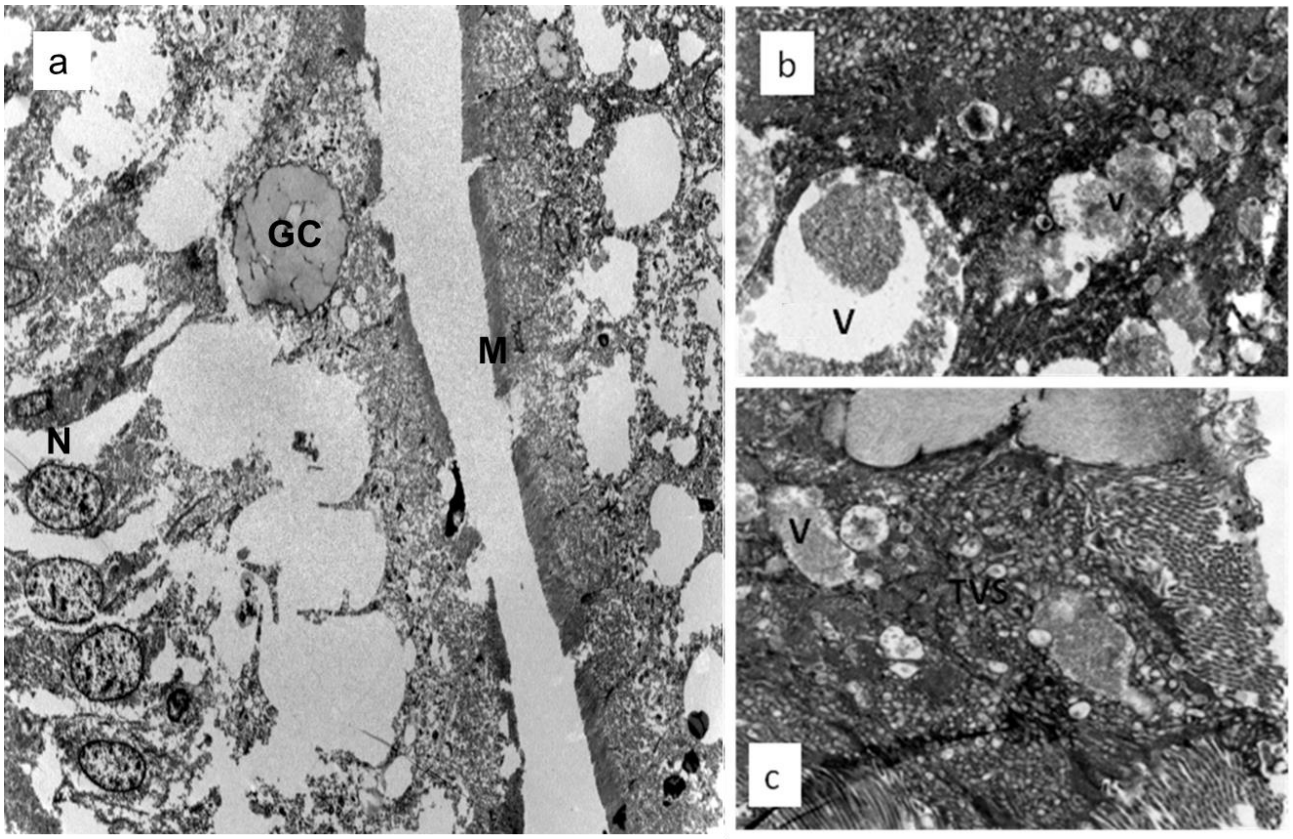


**Fig.6**

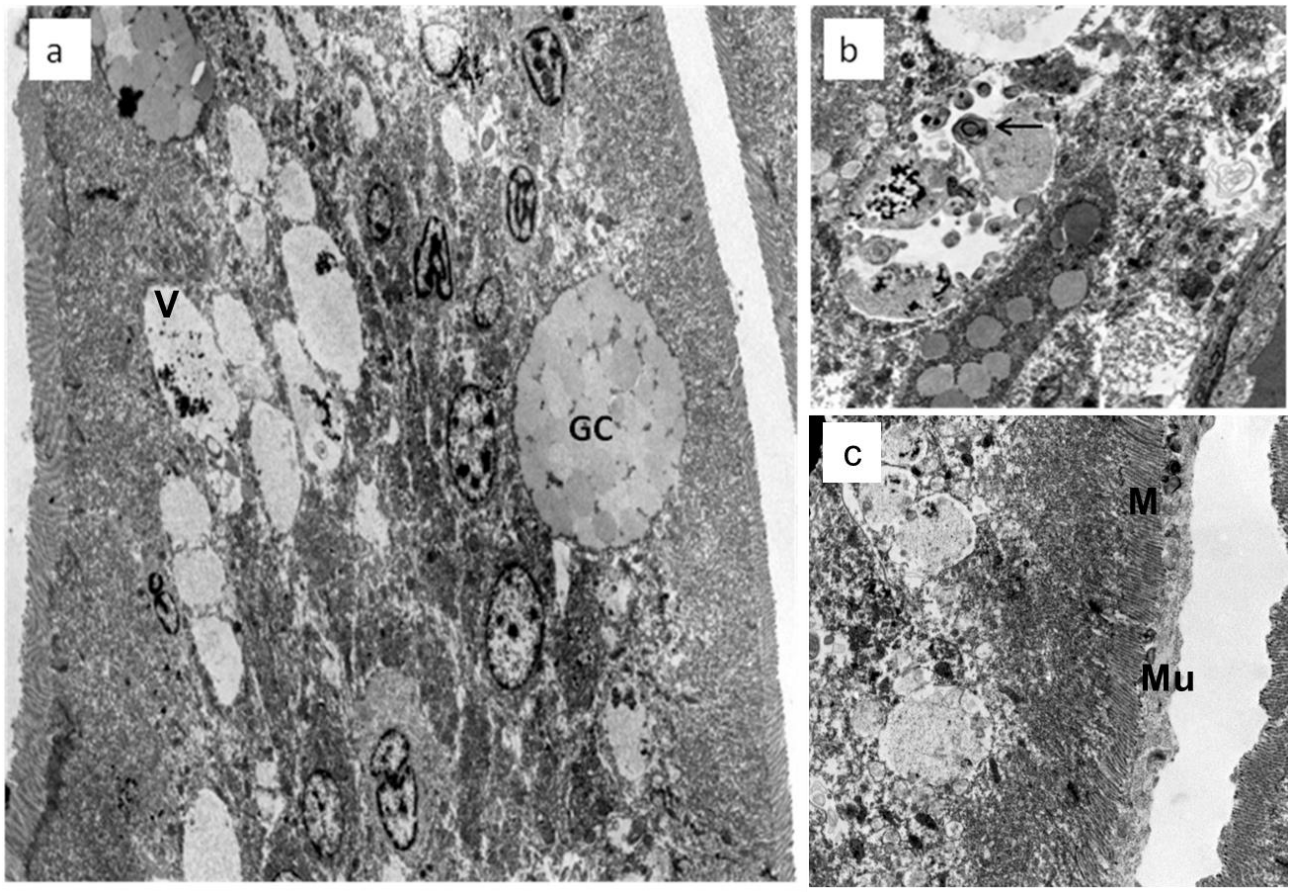

a
V
GC
b
c
M
Mu

**Fig. 7**

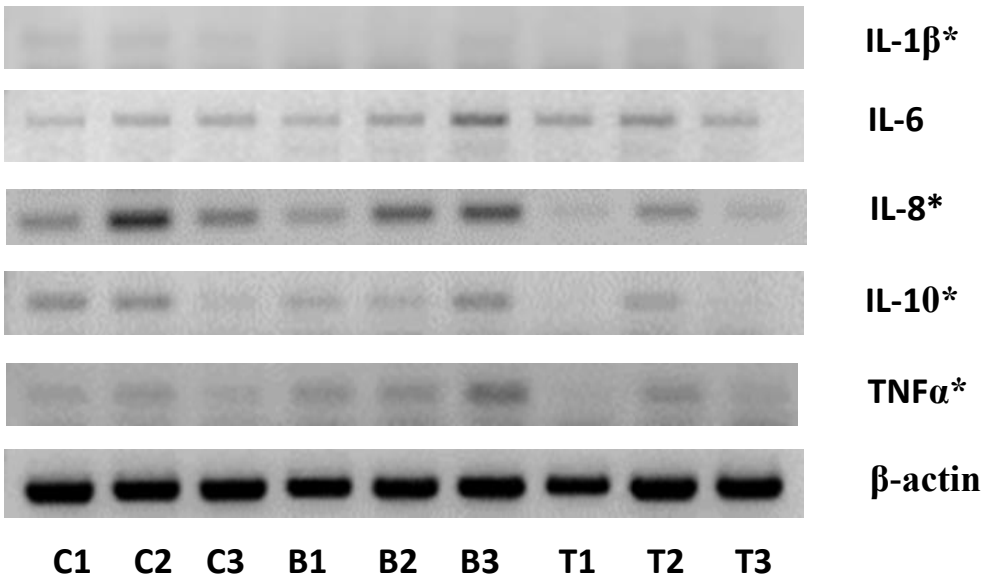
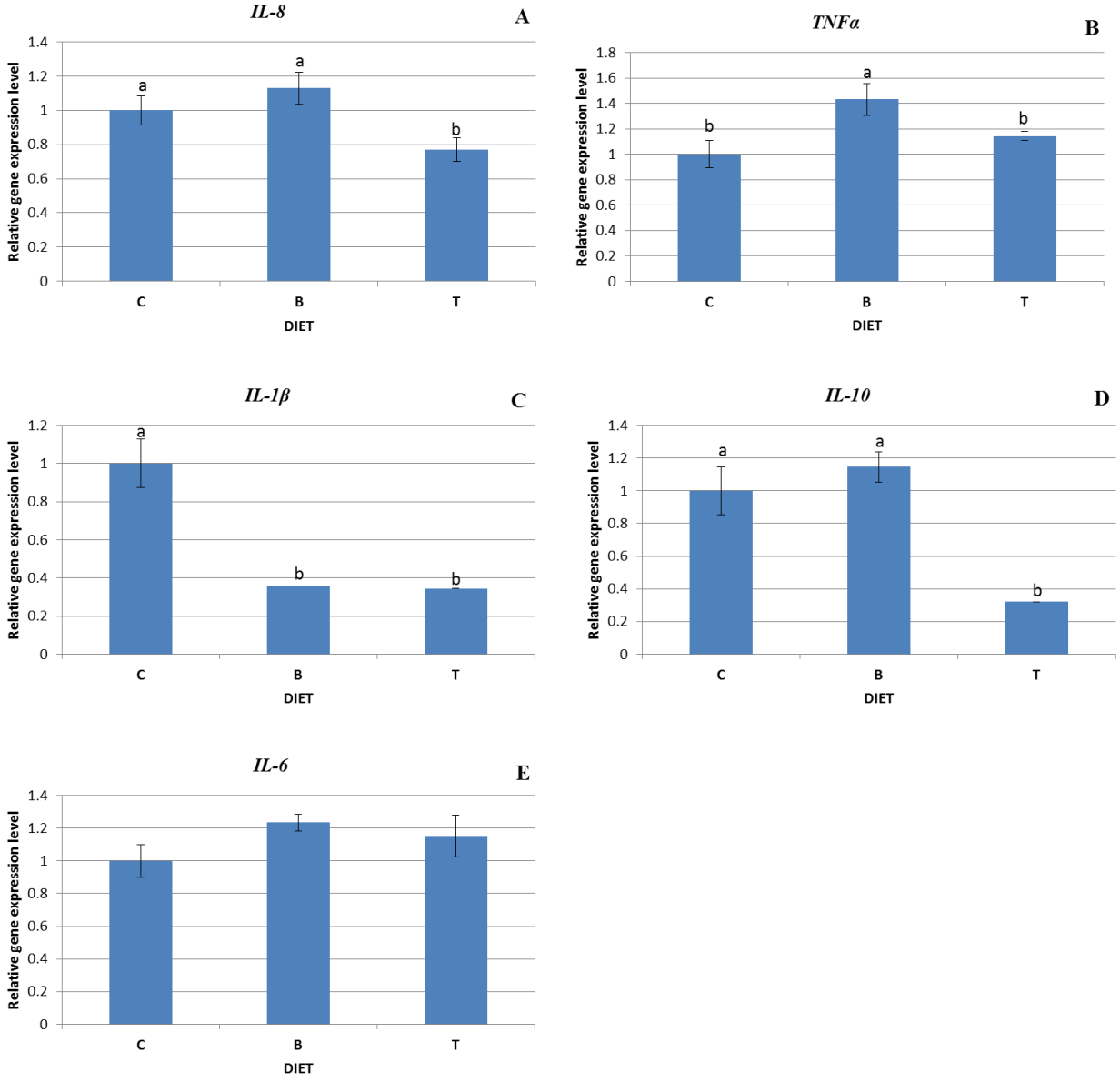

IL-1β*
IL-6
IL-8*
IL-10*
TNFα*
β-actin
C1
C2
C3
B1
B2
B3
T1
T2
T3

**Fig. 8**


IL-8
A
Relative gene expression level
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a
a
b
C
B
T
DIET
TNFα
B
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
b
a
b
C
B
T
DIET
IL-1β
C
1.2
1
0.8
0.6
0.4
0.2
0
a
b
b
C
B
T
DIET
IL-10
D
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a
a
b
C
B
T
DIET
IL-6
E
1.4
1.2
1
0.8
0.6
0.4
0.2
0
C
B
T
DIET