Fish and Shellfish Immunology 87 (2019) 490–498

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Intestinal immunity of dogfish *Scyliorhinus canicula* spiral valve: A histochemical, immunohistochemical and confocal study

E.R. Lauriano[a], S. Pergolizzi[a], M. Aragona[b], G. Montalbano[b], M.C. Guerrera[b], R. Crupi[a], C. Faggio[a,*], G. Capillo[a]

[a] Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Viale F. Stagno d'Alcontres 31, 98166, Messina, Italy
[b] Department of Veterinary Sciences, University of Messina, Zebrafish Neuromorphology Lab, Italy Polo Universitario Annunziata, 98168, Messina, Italy

ARTICLE INFO

Keywords:
Scyliorhinus canicula
GALT
Immunity
Spiral valve

ABSTRACT

The present study describes histochemical and immunohistochemical characteristics of the spiral valve and its associated lymphoid tissue (GALT) in the dogfish *Scyliorhinus canicula*. The mucosal surface of the spiral valve represents the first line of defense against pathogens coming from the external environment through food. Epithelial, mucus and immune cells play a key role in controlling the inflammatory response. Valve intestine of *S. canicula* had many folds lined by simple columnar cells and goblet cells, which later reacted positive to PAS, AB and AB-PAS, histochemical stains differentiated the different types of mucins; lectin histochemistry (PNA and WGA), detected neutral and acid mucins secreted that plays an important role in protection against invading pathogens. Integrin α5β1 was expressed in enterocytes that line the valve's folds with greater marking in the apical part of the cells. Laminin was found on the apical side of the epithelium, in fibrillar and cellular elements of the lamina propria and in the muscularis mucosa. In the spiral valve gut-associated lymphoid tissue (GALT) has been studied. For the first time, massive leucocytes aggregates were identified by confocal immunofluorescence techniques, using the following antibodies: TLR2, S100, Langerin/CD207. Our results expand knowledge about Dogfish valve intestine giving important news in understanding comparative immunology.

1. Introduction

Anatomy and morphology of the fish gastrointestinal tract, as in other vertebrates reflect in phylogeny and ontogeny, diet and environment. Generally, carnivores have shorter intestines compared to herbivores. The gastrointestinal tract of the common dogfish, *Scyliorhinus canicula* (Linnaeus, 1758), order Carcharhiniformes, comprises a spiral valve intestine that represents a character of primitiveness in fishes phylogeny. Among Agnates only the lampreys have valvular intestine that consists of loose coils of epithelium [1]. Spiral valve intestine is a feature common to some species of Carcharhinidae [2], other cartilaginous fish as rays (sawfish) [3] and holocephalans, some primitive bony fishes as sturgeons [4] and paddlefish [5], sarcopterygian lungfishes *Neoceratodus forsteri* [6], and *Protopterus annectens* [7] and in the living fossil, coelacanths [8]. In most studied fish, the spiral valve, which is "screw-like and symmetrical shaped", is short, located in the post duodenal intestine and increases surface area to enhance digestion and absorption. The number of turns in the spiral valve reflects the diet of the species and may be as low as two or three (*Chimaera monstrosa*) or as many as fifty (*Cetorhinus maximus*) [9]. The spiral valve of *S. canicula* is located within the intestine end is composed of twenty turns, present decreasing diameter and increasing length, proceeding in cephalo-caudal direction [10]. The wall of the valve is formed by intestinal mucosa and submucosa infoldings. The mucosa of the intestine is lined by a simple columnar epithelium which possesses a brush-like border of microvilli (apical plate) typical of absorptive tissues end mucus-secreting goblet cells occur scattered among the epithelial cells. The mucosal immune system of vertebrates plays a significant role in fish immunology [11–14]. Recognition of pathogens occurs not only through the specialized antigen presenting cells (APCs), but also by epithelial cells that constitute the primary cellular barrier. There is a strong relationship between epithelial cells and T cells, which play an important role in the intestinal immunity [15]. Mucus is an essential component of mucosal innate immunity [16–18]. Mucins, serve to protect the epithelial lining of the digestive tract from mechanical and chemical damage, furthermore the mucin secretion plays an important role in protection against invading pathogens and facilitates transfer of nutrients and water through enterocytes [1,19–22].

* Corresponding author. Department of Chemical, Biological, Pharmaceutical and Environmental Sciences. University of Messina, Viale Ferdinando Stagno d'Alcontres 31, 98166, S.Agata, Messina, Italy.
E-mail address: cfaggio@unime.it (C. Faggio).

https://doi.org/10.1016/j.fsi.2019.01.049
Received 13 November 2018; Received in revised form 25 January 2019; Accepted 30 January 2019
Available online 31 January 2019
1050-4648/

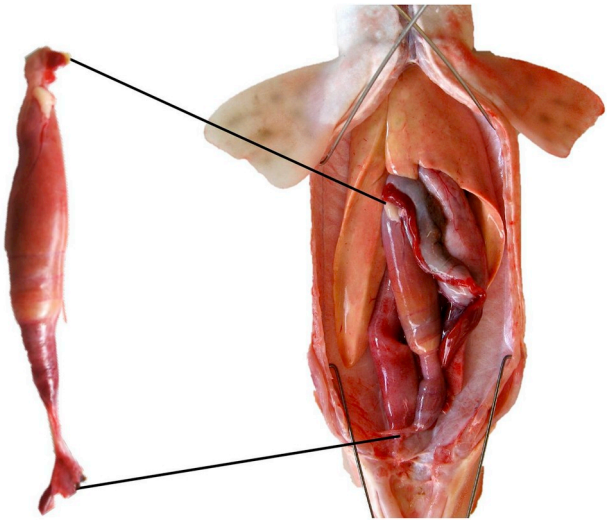

Figure 1. Dissection of the gastrointestinal tract of the dogfish *S. canicula*. On the left, spiral valve intestine.

Laminin and Integrin α5β1, are glycoproteins involved in Cell-cell and cell-matrix interactions, creating and maintaining the polarity of intestinal epithelial cells, contribute to the formation of an intrinsic barrier against microbial invaders [23,24] and tissue repair under inflammatory conditions [25]. Hart et al. [26], highlighted the existence of gut-associated lymphoid tissue (GALT) in spiral valve of *S. canicula,* describing morphology and ultrastructure. The presence of GALT implies the involvement of this region in the immune system with two main populations of immune cells: *lamina propria-submucosa* leukocytes (LPLs), which include a variety of effector cells, such as macrophages, granulocytes, dendritic cells, lymphocytes, and plasma cells; and the intraepithelial lymphocytes (IEL), composed mostly of T cells and a few B cells located among epithelial cells [27]. Sharks as also jawless fishes evolved adaptive immune responses in addition to the innate immune system. One of the highly-conserved components of the innate immune system is the toll-like receptor (TLR), which plays a critical role in early innate immunity detecting pathogens microorganisms' invasion. These evolutionary conserved receptors, homologues of the Drosophila Toll gene, recognize highly conserved structural motifs only expressed by microbial pathogens, called pathogen-associated microbial patterns (PAMPs) [28]. TLR2 is expressed in intestinal spiral valve of shark *Chiloscyllium* sp. [29]. Langerhans cells (LCs) are a distinct population of immature dendritic cells (DCs) that play a sentinel role through their specialized function in antigen uptake and capture, in the epidermis of vertebrates [30,31]. In mammals, mucosal DCs are able to directly sample antigens from the gut lumen and uptake both commensals and pathogens [16]. Langerhans like cells were identified by using Langerin/CD207 and S100 in spleen, kidney and gut in several bony fish species [32–34]. However, there is no in literature, evidence of the existence of DCs in elasmobranchs. The aim of this study is to describe immunity features of Dogfish *S. canicula* spiral valve.

2. Materials and methods

2.1. Experimental protocol

Three adult specimens of *S. canicula*, were caught in the Strait of Messina, transported to the laboratory and transferred in large tanks equipped with running sea water for 1 week before the sacrifice. Specimens of *S. canicula* were anesthetized with MS-222 (tricaine methanesulfonate) to 0.01% and fragments from spiral valve were isolated (Fig. 1). Protocols of fish and experimentation were reviewed and approved in accordance with the standards recommended by the Guide for the Care and Use of Laboratory Animals and Directive 86/609 CEE.

2.2. Histology and histochemistry

Tissues were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) 0.1 mol/l (pH 7.4) for 2 and 4 h, dehydrated in graded ethanol, cleared in xylene, embedded in Paraplast® (McCormick Scientific, St. Louis, MO, USA) and cut into 5 μm sections. The sections were stained with hematoxylin and eosin (H&E) [35], Galgano's trichrome, Toluidine blue and AB pH 2.5 staining followed by PAS (PAS/AB) was performed to distinguish between neutral glycoconjugates (magenta-stained by PAS) and acidic glycoconjugates (blue stained by AB). Sections were examined under a Zeiss Axioskop 2 plus microscope equipped with a Sony Digital Camera DSC-85.

2.3. Lectin histochemistry

The lectins used are reported in Table 1. The PNA, WGA, lectins were HRP-conjugated. They have been obtained from Sigma Chemicals Co (St. Louis, MO, USA). Deparaffinized and rehydrated tissue sections were immersed in 3% H_2O_2 for 10 min to suppress the endogenous peroxidase activity, rinsed in 0.05 mol/l Tris-HCl buffered saline (TBS) pH 7.4 and incubated in lectin solution at appropriate dilutions (Table 1) for 1 h at room temperature (RT). After rinsing 3 thrice in TBS, the peroxidase activity was visualized by incubation in a solution containing 0.05% 3,30-diaminobenzidine (DAB) and 0.003% H_2O_2 in 0.05 mol/l TBS (pH 7.6) for 10 min at RT before dehydration and mounting. Controls for lectin staining included: 1) substitution of the substrate medium with buffer without lectin; 2) incubation with each lectin in the presence of its hapten sugar (0.2–0.5 mol/l in Tris buffer).

2.4. Immunoperoxidase method

Serial sections were incubated overnight at 48C in a humid chamber with following antibodies: Human TLR2, Human Integrin α5β1 and Mouse Laminin (Table 2).

Then, the sections were washed in phosphate buffered saline (PBS) and incubated for 60 min with a goat anti-rabbit IgG-peroxidase conjugate (Table 3). Peroxidase activity was visualized by incubation of the sections for 1–5 min in at room temperature in a solution 0.02% diaminobenzidine (DAB) and 0.015% hydrogen peroxide, as reported by Montalbano et al. [36]. After rinsing in PBS, sections were dehydrated, mounted and examined under a Zeiss Axioskop.

Table 1
Lectins used, their sugar specificities and inhibitory sugars used in control experiments.

Lectin abbreviation	Source of lectin	Concentration (μg/ml)	Sugar specificity	Inhibitory Sugar
PNA	*Arachis hypogea*	20	Terminal Galβ1,3GalNAc	Galactose
WGA	*Triticum vulgaris*	15	Terminal and internal βGlcNAc_NeuNAc	GlcNAc

Table 2
Primary antibodies used in this study.

Primary antibody	Immunogen/host	Supplier	Antibody ID	Dilution
S100 antibody	rabbit	Sigma-Aldrich, St.Louis, MO, USA	AB_10013383	1:100
Langerin/CD207	rabbit	Santa Cruz Biotechnology, Inc	AB_2074213	1:100
Toll-like Receptor 2 Antibody	rabbit	Active Motif, La Hulpe, Belgium, Europe	AB_2750977	1:125
Anti-Integrin α5β1	mouse	Chemicon international	AB_11211991	1:50
Anti-Laminin antibody	rabbit	Sigma-Aldrich, St.Louis, MO, USA	AB_477163	1:25

Table 3
Secondary antibodies used in this study.

Secondary antibody	Supplier	Antibody ID	Dilution
Donkey anti-Rabbit IgG Secondary Antibody, Alexa Fluor 594	Thermo Fisher Scientific	AB_2556547	1:100
Goat anti-rabbit IgG-peroxidase conjugate	Sigma-Aldrich, St.Louis, MO, USA	AB_257896	1:100

2.5. Confocal immunofluorescence

Serial sections were deparaffinized and rehydrated, rinsed several times in PBS and blocked in 10% normal goat serum for 1 h. Following polyclonal antibodies: Human TLR2, Human Langerin/CD207 [37] and Bovine S100, were diluted in a permeabilizing solution (PBS, 0.2% Triton X-100, 0.1% sodium azide) according to the optimal dilutions (Table 2) and placed on the slides to incubate overnight at room temperature. Sections were then treated with fluorescent labeled secondary antibody diluted in PBS Alexa Fluor 594 donkey anti-rabbit IgG TRITC conjugated (Table 3) and left to incubate at room temperature for 2 h in the dark. Control experiments were performed excluding primary antibody.

The storage dilution for three different antibodies are reported in Table 2. After washing, the sections were mounted with Vectashield (Vector Labs, Burlingame, CA, USA) to prevent photobleaching, and cover-slipped.

2.6. Laser confocal immunofluorescence

Sections were analyzed and images acquired using a Zeiss LSMDUO confocal laser scanning microscope with META module (CarlZeiss Micro Imaging GmbH, Germany). Each image was rapidly acquired in order to minimize photodegradation. Digital images have been cropped and the figure montage prepared using Adobe Photoshop7.0 (Adobe Systems, SanJose, CA, USA).

2.7. Western blot analyses

Western blot analyses were performed on the spiral valve intestine; the samples were homogenized in lysis buffer. Briefly, spiral valve intestine from each specimen were suspended in extraction buffer A containing PMSF 0.2 mM, pepstatin A 0.15 mM, leupeptin 20 mM, sodium orthovanadate 1 mM, homogenized for 2 min, and centrifuged for 4 min at 4 °C at 12,000 rpm. The pellets, containing enriched nuclei, were resuspended in buffer B containing 1% Triton X-100, NaCl 150 mM, Tris–HCl pH 7.4 10 mM, EGTA 1 mM, EDTA 1 mM, PMSF 0.2 mM, leupeptin 20 mM, and sodium orthovanadate 0.2 mM. After centrifugation 10 min at 12,000 rpm at 4 °C, the supernatants containing the nuclear protein were stored at −80 °C for further analysis. Protein concentrations were calculated by the Bio-Rad protein assay using bovine serum albumin as standard.

Samples were heated at 100 °C for 5 min, and equal amounts of protein were separated on SDS-PAGE gel and transferred to PVDF membrane. The filters were blocked with 1x PBS and 5% (w/v) non-fat desiccated milk for 40 min at room temperature and successively probed with one of the following primary antibodies: rabbit polyclonal anti-Langerin (1:500; Santa Cruz Biotechnology, CA, USA), rabbit polyclonal Toll Like Receptor 2 (TLR2) (1:500, Active Motif) and rabbit polyclonal S100 (1:500, Sigma-Aldrich, St.Louis, MO, USA) at 4 °C overnight in 1 × PBS, 5% (w/v), nonfat dried milk, and 0.1% Tween-20. Membranes were incubated with peroxidase conjugated goat anti-rabbit IgG or peroxidase conjugated bovine anti-mouse IgG secondary antibody (1:2000, Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at room temperature. To assess that blots were loaded with equal volumes of protein lysates, they were probed with either a mouse monoclonal β-actin antibody (1:5000; Santa Cruz Biotechnology, CA, USA) for cytosolic proteins. Signals were detected with Super Signal West Pico Chemiluminescent Substrate according to the producer's instructions (Pierce Thermo Scientific, Rockford, IL, USA). The relative expression of protein bands was quantified by densitometric scanning of the X-ray films utilizing a GS-700 Imaging Densitometer (GS-700, Bio-Rad Laboratories, Milan, Italy) and a software (Image J), and standardized to β-actin.

3. Results

3.1. Histological and histochemical description

Results showed that intestinal valve wall in *S. Canicula,* consisted of: mucosa, submucosa, muscularis and serosa (Fig. 2A). The mucosal surface of valve intestine had numerous long folds, obliquely oriented, which in section showed a conformation similar to villi (Fig. 2B). The valve folds were lined by a columnar epithelium composed of enterocytes (Fig. 2C) presenting an apical brush-like border strongly PAS positive; basal cells were also present. Among epithelial cells there were numerous goblet cells, more abundant along the folds, showing a high secretory activity; abundant mucous secretion was visible in the gut lumen (Fig. 2D). Alcian Blue pH 2.5 PAS double staining highlighted the existence of two mucous cells populations. One of these showed cells positive to neutral mucins (magenta), while other mucous cells presented both mucin types, neutral and acidic mucins (purple) (Fig. 2E). Migratory lymphocytes, with a typical roundish shape nucleus containing condensed chromatin and basophilic cytoplasm positive to Toluidine blue stain, were observed (Fig. 2 F). Basal lamina was slightly PAS positive (Fig. 2D). The *Lamina propria* formed a continue layer below the adjacent folds consisting of a network of collagen fibers and smooth muscle; in the connective tissue were seen abundant blood vessels and scattered immune cells such as: granulocytes, lymphocytes, mast cells and eosinophils (Fig. 2G). Furthermore, massive aggregates of immune cells, above all lymphocytes constituting a lymphoid tissue associated with intestinal mucosa (GALT) in the *lamina propria* (Fig. 2H and I) were present. *Muscularis mucosae* presented two different layers of smooth muscles: the inner circular and the outer longitudinal. The two muscular layers were separated by connective tissue.

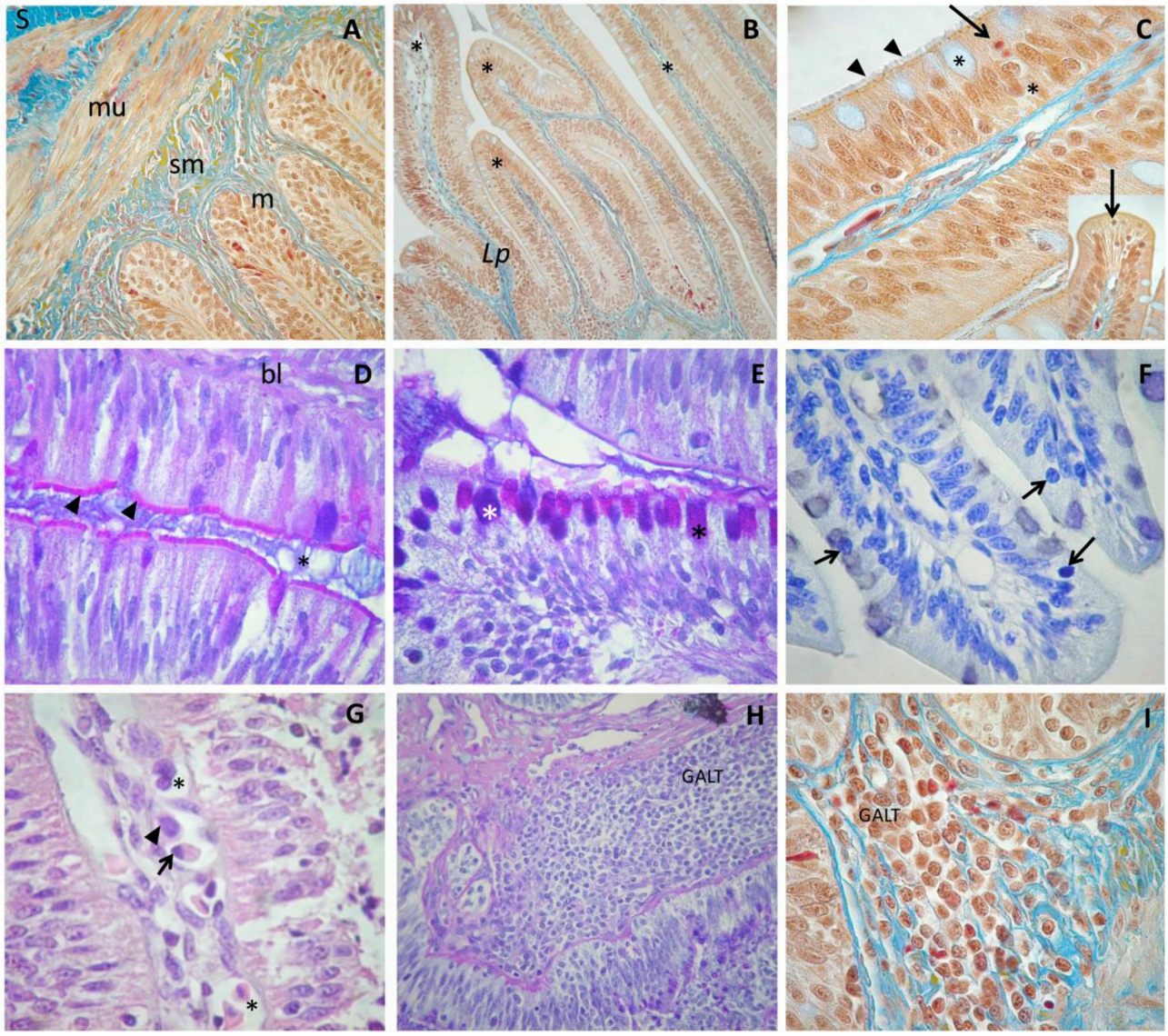

Fig. 2. Transverse section of *S. canicula* intestinal valve: (A-B and C, Galgano's trichrome) in A mucosa (m), submucosa (sm), muscolaris (mu) and serosa (s), (B) long folds (*) with central core formed by Lamina propria (Lp) (C), fold intestinal valve lined of enterocytes with brush-like border (arrow heads) and goblet cells (*), among enterocytes, migratory lymphocytes (black arrows, and insert); (D and E Alcian Blue pH 2.5 PAS) showing in (D) brush-like border PAS positive (arrow heads) and abundant mucous secretion in gut lumen (*), basal lamina (bl) slightly PAS positive, in (E) mucous cells mucins positive (magenta-black *), and acidic mucins (purple-white *); (F) Toluidine blue stain migratory lymphocytes (black arrows); (G and H hematoxylin and eosin), in G numerous blood vessels (*) and scattered immune cells: granulocyte (arrow), lymphocyte (arrow heads) are present, in (H), aggregates of immune cells form a lymphoid tissue associated with intestinal mucosa; (I) Galgano's trichrome also show GALT. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.2. Lectin histochemistry

The PNA stained intensely the brush-like band of enterocytes and some mucosal cells. In addition, lectin localized some cells, with granular content corresponding to blood elements in the *lamina propria* (Fig. 3A). The WGA weakly colored some large cells; there was no positivity around the vessels or to other intestinal structures (Fig. 3B).

3.3. Immunohistochemistry

Integrin α5β1 was expressed in the *muscularis mucosa* and in enterocytes that line the valve's folds with greater marking in the apical part of the cells (Fig. 4A and B). Laminin was found on the apical side of the epithelium, in fibrillar and cellular elements of the *lamina propria* and in the *muscularis mucosa* (Fig. 4C and D). TLR2 was expressed in the cytoplasm and in the apical membrane of the epithelial columnar cells that in particular lined the fold tip; goblet cells were negative. Abundant intraepithelial lymphocytes (like cells) TLR2 positive, with thin rim of cytoplasm were scattered among epithelial cells (Fig. 4 E), furthermore several lymphoid like cells, were strongly TLR2 positive mainly localized in GALT and in the *lamina propria* (Fig. 4 F).

Fig. 4 Showing Immunohistochemistry results, (A) Integrin is expressed in *muscularis mucosa* (mm) and around vessels (arrows); (B) enterocytes immunoreactive to Integrin with greater marking in the apical part of the cells (arrows); (C) Laminin marked *muscularis mucosa* (mm), vessels (*), fibers and cells (arrows) of *lamina propria* connective tissue and in (D) apical side (arrows) of the epithelium; TLR2 has marked the thin rim of lymphocytes –like cytoplasm present among

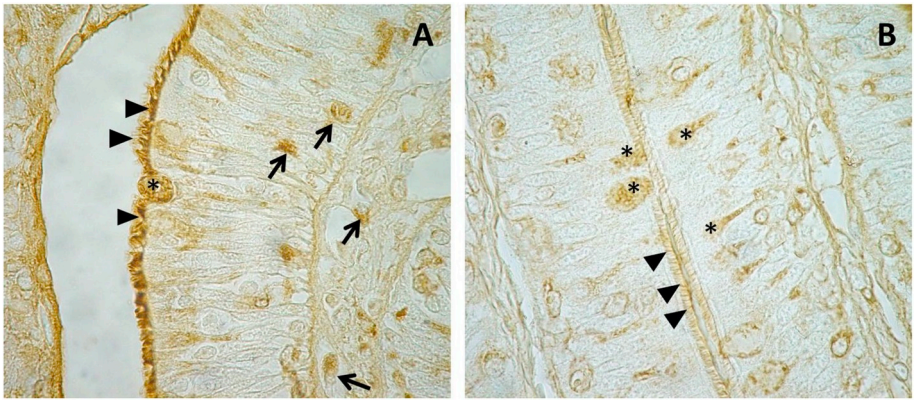

Fig. 3. Lectin histochemistry, in A brush-like border (arrow heads) is strongly stained with PNA lectin that also stains a mucous cell (*) and some blood cells (arrows) in the lamina propria and among enterocytes; in B WGA lectin weakly stain some mucous cells (*) and brush-like border (arrow heads). (100x).

epithelial cells (E) (arrows) and in GALT (F), furthermore the apical membrane of the epithelial columnar cells, in particular in the fold tip was strongly TLR2 positive (E) (arrow heads).

3.4. Confocal scanning laser microscopy

Visualization of the immunohistochemical staining by confocal microscopy revealed that lymphoid tissue associated with spiral valve mucosa (GALT) was strongly positive with Langerin, S100 and TLR2 antibodies. Langerin and S100 reactive cells (dendritic-like cells), were found in the basal epithelium, *lamina propria* and GALT; cells showed a round to ovoid irregular morphology and no cytoplasmic processes could be observed (Fig. 5A and B). This finding was in agreement with published works [32] which also showed that Langerhans-like cells (dendritic cells) did not possess dendritic processes [34]. TLR2 confocal results replied those obtained with immunohistochemical peroxidase investigations (Fig. 5C). Immunofluorescence with TLR2 confirmed the presence of intraepithelial lymphocytes (like cells) scattered in the gut epithelium among enterocytes (Fig. 5D).

Expression of antibodies used in immunofluorescence has been confirmed by western-blot analyses (Fig. 6A and B-C), see densitometric analyses (Fig. 6 D).

Fig. 5 (A) Abundant Langerin/CD 207 positive dendritic-like cells, were present in the basal epithelium (e), *lamina propria* (*lm*) and GALT (insert); (B) S100 positive Langerhans –like cells in *lamina propria* showed few and short cytoplasmic processes (arrow); (C) TLR2 labeled lymphocytes in epithelium (e) and *lamina propria* (*lm*) confirming immunohistochemical peroxidase investigations results. (D) Intraepithelial lymphocytes (arrows), showing immunoreactivity to TLR2. Fig. 5A–C are provided also with bright fields.

4. Discussion

The shark's digestive tract can be divided in: proximal, spiral, and distal intestine. The spiral intestine plays a double role in increasing the intestine surface area without increasing intestinal length, and slowing down the food passage speed [38–40]. The spiral intestine of elasmobranchs has been studied for over 130 years [41,42], but the absorptive properties of the valve are still little explored [40]. Studies about *S. canicula* spiral valve intestine have been performed by Hart and coworkers [26], that described the histological and ultrastructural features of the gut associated lymphoid tissue. Our study confirmed the presence of GALT in spiral valve of *S. canicula* and also characterized immunohistochemically some lymphoid and macrophage like cells, using confocal immunofluorescence and immunohistochemical staining. Furthermore, the present finding described histochemical and immunohistochemical features of the *S. canicula* spiral valve. The histology of the spiral valve intestine in *S. canicula* is otherwise essentially similar to that of other fish studied: *Himantura signifier* [43], *Protopterus annectens* [7]; *Neoceratodus forsteri* [6]. The valve folds are lined by a columnar epithelium that presents enterocytes with an apical brush-like border strongly PAS positive, that implies nutrient absorption role of these cells. Abundant neutral and acid mucins produced by goblet cells scattered between the epithelial cells facilitates the absorption of digested substances and acts as lubricant for food and intestinal mucosa [7,43]. Furthermore, mucins are known to play an important role in protecting the mucosa against toxins and pathogens and physical/chemical damage [44]. Goblet cells and brush border were also positive to the PNA and WGA lectins; these molecules play an important role in the recognition and elimination of pathogens [45]. The main carbohydrate residues typically present in mammalian mucins, fucose, galactose, *N*-acetylglucosamine (GlcNAc), *N*-acetyl galactosamine (GalNAc) and neuraminic acid have been also observed in the digestive tract of several fish species [46–50]. The PNA identifies in particular the galactose residues that characterize serous cells; so, the presence of these residues in the mucous cells lead to the characterization of these as a type of glandular cells that has been defined serous mucus [51]. In spiral valve of *S. canicula*, we did not observed crypts of Lieberkuhn, the secretion of NaCl and fluid in the intestine, like in all Elasmobranches, is carried out by rectal gland, that we have studied in a previous work [52]. The present study represents the first report that concerned the localization of integrin and laminin in the *S. canicula* and in general in cartilaginous fishes spiral valve. Integrins are a large family of cell-surface receptors binding to basement membrane molecules such as the laminins and the type IV collagens. Integrin α5β1 is normally limited to the basolateral domains of nonspecialized intestinal epithelial cells and is involved in proliferation and differentiation of intestinal cells [53]. Despite this, laminins are not exclusively localized to basal lamina [54]. Reports of laminins in tissue locations different to basement membranes are increasingly frequent. In the brain, laminins have been observed not only within the basement membrane of capillaries, but also at other sites not conceptualized as basement [54,55]. Our results showed integrin α5β1and laminin around the vessels and muscle tissue of the spiral valve but unexpectedly these glycoproteins were strongly expressed also in the apical cell surface of intestinal epithelium. In literature, it has been reported that during intestinal inflammation, the loss of intestinal epithelial barrier and polarity functions induces the redistribution of basolateral membrane proteins such as integrin α5β1 to the apical cell surface [24]. Clusters of lymphocyte infiltrations have been reported in the spiral valve of sharks, rays and dogfish *S. canicula* [56]. In this study, we have characterized abundant intraepithelial lymphocytes TLR2 positive, that are involved in immunological

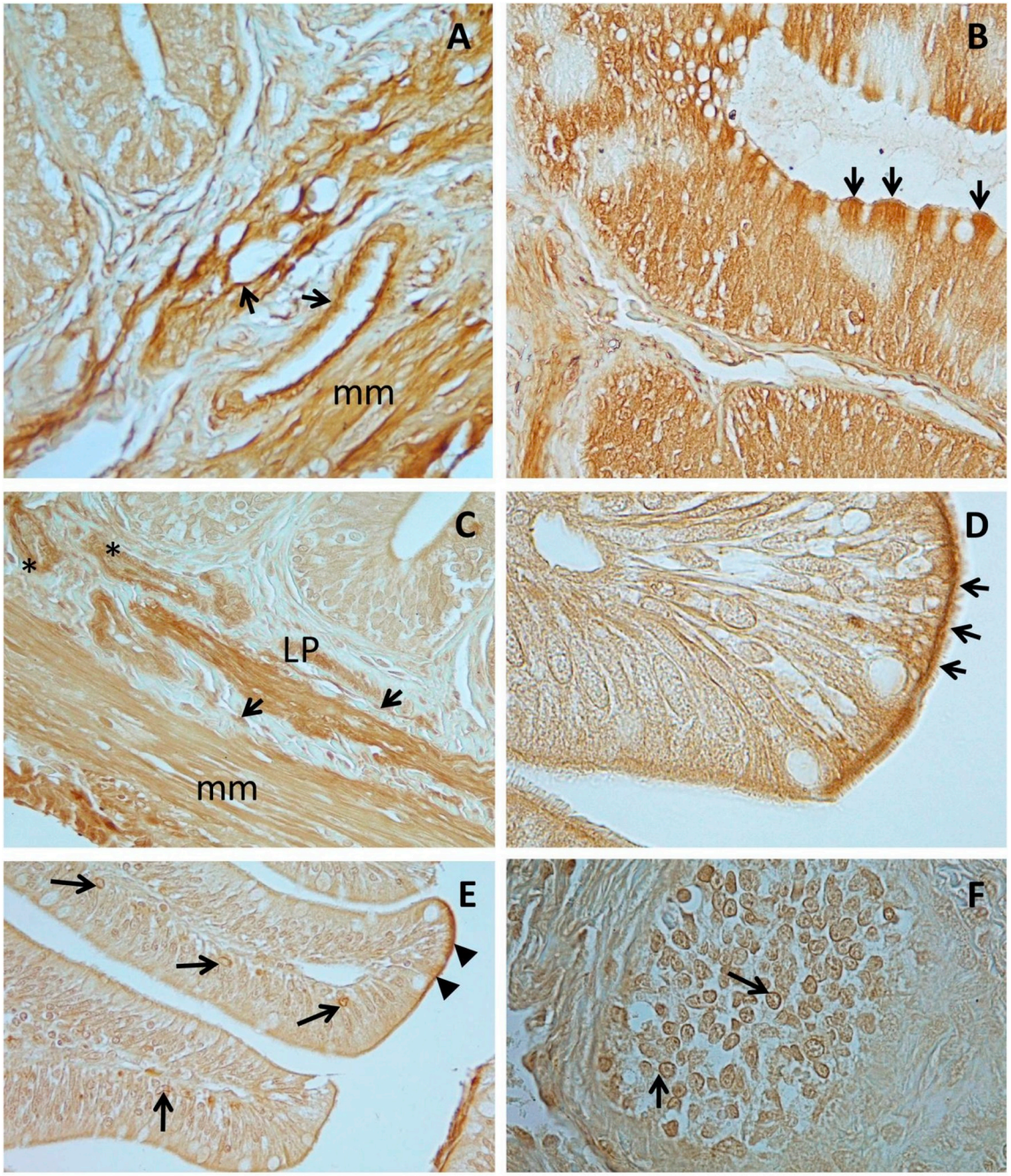

Fig. 4. Immunohistochemistry results: (A) Integrin is expressed in muscularis mucosa (mm) and around vessels (arrows); (B) enterocytes immunoreactive to Integrin with greater marking in the apical part of the cells (arrows); (C) Laminin marked muscularis mucosa (mm), vessels (*), fibers and cells (arrows) of lamina propria connective tissue and in (D) apical side (arrows) of the epithelium; TLR2 has marked the thin rim of lymphocytes–like cytoplasm present among epithelial cells (E) (arrows) and in GALT (F), furthermore the apical membrane of the epithelial columnar cells, in particular in the fold tip was strongly TLR2 positive (E) (arrow heads).

surveillance. Migratory lymphocytes represent a common feature in the intestine of many species of fish [57,58]. According to Anandhakumar et al. [29], we have found high level of TLR2 expressed by lymphoid like cells in gut-associated lymphoid tissue. TLR2 was also present in the cytoplasm of enterocytes, where can plays a role in maintaining epithelial barrier function in response to enteric pathogens and parasites. Toll-like receptors strongly expressed on the surface of epithelial cells that lined the fold tip act as sensors for invading pathogens. In some studies, it has been shown that certain fish GALT epithelial cells display morphological similarities with mammalian M cells [59,60]. We could hypothesize that the epithelial cells lining the extremity of the fold are more specialized in recognizing the antigens and acting M-like cells. Toll like receptors are involved in recognition of pathogens through the specialized antigen presenting cells (APCs), but also by epithelial cells, which constitute the primary cellular barrier [16,61]. For the first time in this study we have immunohistochemical characterized by confocal microscopy staining, dendritic-like cells in *S. canicula* GALT and in *lamina propria* with Langerin/CD207 and S100 antibodies. The mucosal immune system of vertebrates comprises innate and adaptive immune cells and molecules that act in concert to protect

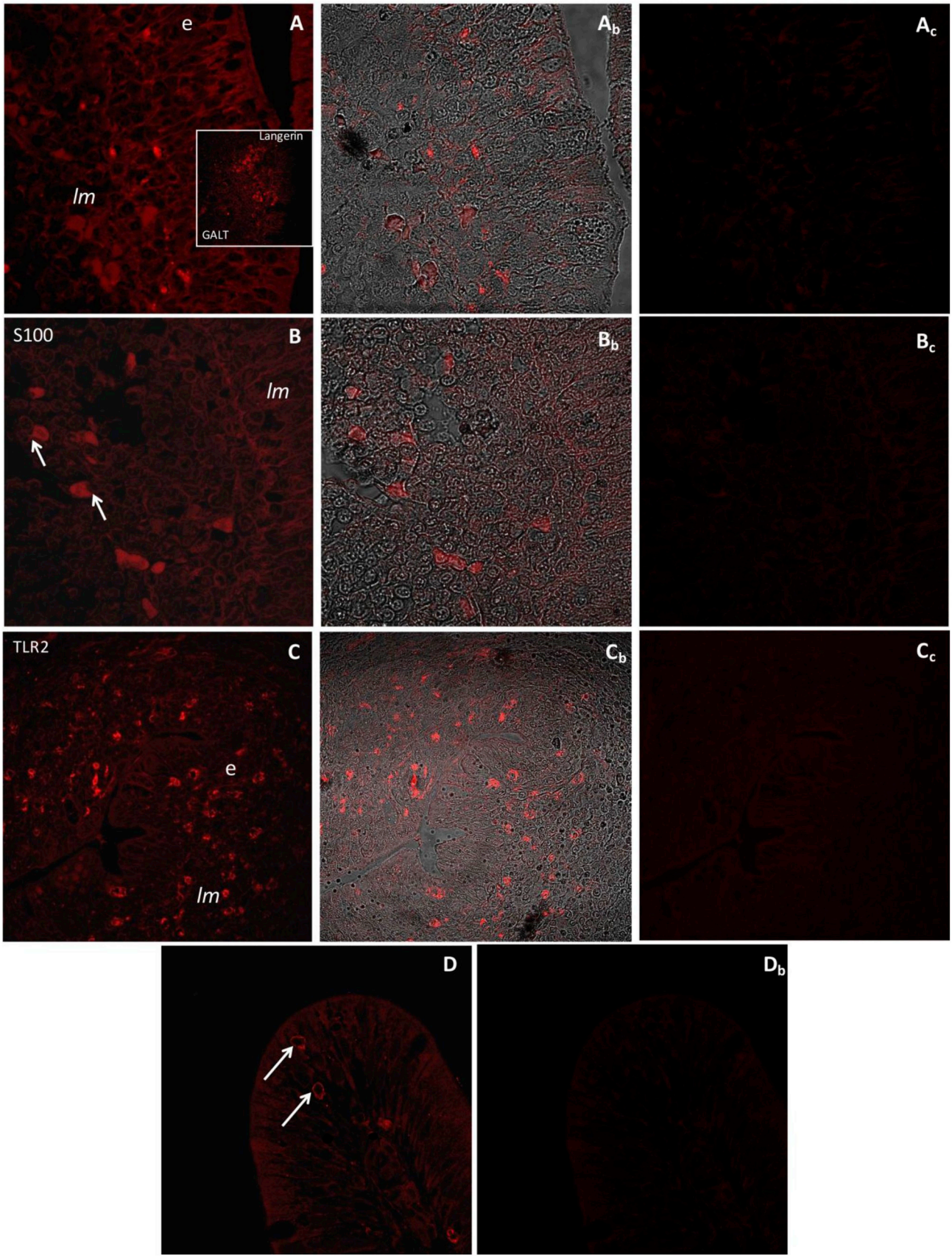

Fig. 5. (A) Abundant Langerin/CD 207 positive dendritic-like cells, were present in the basal epithelium (e), lamina propria (lm) and GALT (insert); (B) S100 positive Langerhans –like cells in lamina propria showed few and short cytoplasmic processes (arrow); (C) TLR2 labeled lymphocytes in epithelium (e) and lamina propria (lm) confirming immunohistochemical peroxidase investigations results. (D) Intraepithelial lymphocytes (arrows), showing immunoreactivity to TLR2. Fig. 5A–C are provided also with bright fields (A_b, B_b, C_b). No-primary antibody controls are provided in $5A_c$-B_c-C_c-D_b.

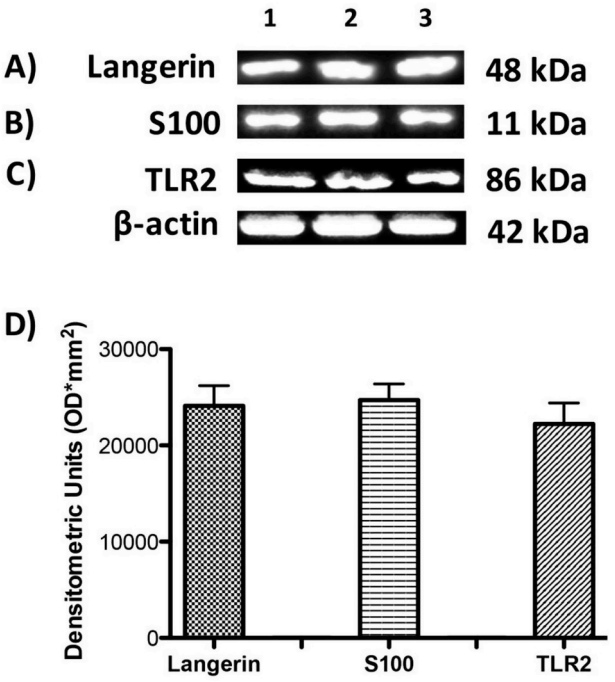

Fig. 6. Western Blot analyses of the expression of (A) Langerin, (B) S100 and (C) TLR2. A representative blot of homogenates of spiral valve intestine lysates obtained is shown and densitometry analysis is reported (D).

the host against pathogens. The gut mucosal epithelia play a significant role in fish immunology [62]. Epithelial surveillance via these innate immune pattern recognition receptors, can then be transmitted to the underlying cell populations of the *lamina propria* and GALT composed of macrophages, lymphocytes and plasma cells [16]. Future studies could be oriented to characterize the different molecules and cells of the intestinal mucosa of the elasmobranches to deepen the phylogeny of the immune system in these interesting and primitive vertebrate.

References

[1] N.A. Theodosiou, D.A. Hall, A.L. Jowdry, Comparison of acid mucin goblet cell distribution and Hox13 expression patterns in the developing vertebrate digestive tract, J. Exp. Zool. B Mol. Dev. Evol. 308 (4) (2007) 442–453.

[2] G.L. Crow, J.C. Howe, S. Uchida, S. Kamolnick, M.G. Wisner, J.N. Caira, Protrusion of the valvular intestine through the cloaca in sharks of the family Carcharhinidae, Copeia 1990 (1) (1990) 226–229.

[3] A.D. Henningsen, B.R. Whitaker, I.D. Walker, Protrusion of the valvular intestine in captive smalltooth sawfish and comments on pristid gastrointestinal anatomy and intestinal valve types, J. Aquat. Anim. Health 17 (3) (2005) 289–295.

[4] E. Cataldi, C. Albano, C. Boglione, L. Dini, G. Monaco, P. Bronzi, S. Cataudella, Acipenser naccarii: fine structure of the alimentary canal with references to its ontogenesis, J. Appl. Ichthyol. 18 (4-6) (2002) 329–337.

[5] A. Peterman, L. Petrie-Hanson, Ontogeny of American paddlefish lymphoid tissues, J. Fish. Biol. 69 (2006) 72–88.

[6] M. Hassanpour, J. Joss, Anatomy and histology of the spiral valve intestine in juvenile Australian lungfish, *Neoceratodus forsteri*, Open Zool. J. 2 (2009) 62–85.

[7] J.M. Icardo, W.P. Wong, E. Colvee, F. Garofalo, A.M. Loong, Y.K. Ip, The gut of the juvenile African lungfish *Protopterus annectens:* a light and scanning electron microscope study, J. Morphol. 272 (7) (2011) 769–779.

[8] Y. Suyehiro, Some views on the dissected specimens of coelacanth, Bienn Rep Keikyu Aburat Mar Park Aquar 12 (1983) 12.

[9] D.G. Ragnar Fänge, 4 - digestion, in: D.J.R.W.S. Hoar, J.R. Brett (Eds.), Fish Physiology, 1979, pp. 161–260.

[10] D.R. Khanna, Biology of Fishes, Discovery Publishing House, 2004.

[11] H.W. Dickerson, The biology of teleost mucosal immunity, Fish Def. 2 (2010) 11–52 CRC Press.

[12] H. Van Doan, S.H. Hoseinifar, C. Faggio, C. Chitmanat, N.T. Mai, S. Jaturasitha, E. Ringø, Effects of corncob derived xylooligosaccharide on innate immune response, disease resistance, and growth performance in Nile tilapia (*Oreochromis niloticus*) fingerlings, Aquaculture 495 (2018) 786–793.

[13] A. Iswarya, B. Vaseeharan, M. Anjugam, N. Gobi, M. Divya, C. Faggio, β-1, 3 glucan binding protein based selenium nanowire enhances the immune status of *Cyprinus carpio* and protection against Aeromonas hydrophila infection, Fish Shellfish Immunol. 83 (2018) 61–75.

[14] V. Aliko, M. Qirjo, E. Sula, V. Morina, C. Faggio, Antioxidant defense system, immune response and erythron profile modulation in gold fish, *Carassius auratus*, after acute manganese treatment, Fish Shellfish Immunol. 76 (2018) 101–109.

[15] E.R. Lauriano, S. Pergolizzi, G. Capillo, M. Kuciel, A. Alesci, C. Faggio, Immunohistochemical characterization of Toll-like receptor 2 in gut epithelial cells and macrophages of goldfish *Carassius auratus* fed with a high-cholesterol diet, Fish Shellfish Immunol. 59 (2016) 250–255.

[16] D. Gomez, J.O. Sunyer, I. Salinas, The mucosal immune system of fish: the evolution of tolerating commensals while fighting pathogens, Fish Shellfish Immunol. 35 (6) (2013) 1729–1739.

[17] G. Bosi, B.S. Dezfuli, Responses of Squalius cephalus intestinal mucous cells to *Pomphorhynchus laevis*, Parasitol. Int. 64 (2) (2015) 167–172.

[18] S.H. Hoseinifar, S. Yousefi, G. Capillo, H. Paknejad, M. Khalili, A. Tabarraei, H. Van Doan, N. Spanò, C. Faggio, Mucosal immune parameters, immune and antioxidant defence related genes expression and growth performance of zebrafish (*Danio rerio*) fed on *Gracilaria gracilis* powder, Fish Shellfish Immunol. 83 (2018) 232–237.

[19] A. Lugea, A. Salas, J. Casalot, F. Guarner, J. Malagelada, Surface hydrophobicity of the rat colonic mucosa is a defensive barrier against macromolecules and toxins, Gut 46 (4) (2000) 515–521.

[20] W. Khan, Physiological changes in the gastrointestinal tract and host protective immunity: learning from the mouse-Trichinella spiralis model, Parasitology 135 (6) (2008) 671–682.

[21] J. Kim, W. Khan, Goblet cells and mucins: role in innate defense in enteric infections, Pathogens 2 (1) (2013) 55–70.

[22] G. Zaccone, D.S. Fudge, T.M. Winegard, G. Capillo, M. Kuciel, K. Funakoshi, E.R. Lauriano, Confocal imaging and phylogenetic considerations of the subcutaneous neurons in the Atlantic hagfish *Myxine glutinosa*, Acta Zool. 96 (2) (2015) 209–217.

[23] M. Lepage, A. Seltana, M.-P. Thibault, É. Tremblay, J.-F. Beaulieu, Knockdown of laminin α5 stimulates intestinal cell differentiation, Biochem. Biophys. Res. Commun. 495 (1) (2018) 1510–1515.

[24] M.A. Charania, H. Laroui, H. Liu, E. Viennois, S. Ayyadurai, B. Xiao, S.A. Ingersoll, D. Kalman, D. Merlin, Intestinal epithelial CD98 directly modulates the innate host response to enteric bacterial pathogens, Infect. Immun. 81 (3) (2013) 923–934 IAI. 01388-12.

[25] C. Spenlé, O. Lefebvre, J. Lacroute, A. Méchine-Neuville, F. Barreau, H.M. Blottière, B. Duclos, C. Arnold, T. Hussenet, J. Hemmerlé, The laminin response in inflammatory bowel disease: protection or malignancy? PLoS One 9 (10) (2014) e111336.

[26] S. Hart, A. Wrathmell, J. Harris, Ontogeny of gut-associated lymphoid tissue (GALT) in the dogfish *Scyliorhinus canicula* L, Vet. Immunol. Immunopathol. 12 (1–4) (1986) 107–116.

[27] I. Estensoro, I. Mulero, M.J. Redondo, P. Alvarez-Pellitero, V. Mulero, A. Sitjà-Bobadilla, Modulation of leukocytic populations of gilthead sea bream (*Sparus aurata*) by the intestinal parasite *Enteromyxum leei* (Myxozoa: Myxosporea), Parasitology 141 (3) (2014) 425–440.

[28] M. Flajnik, L. Rumfelt, The Immune System of Cartilaginous Fish, Origin and Evolution of the Vertebrate Immune System, Springer, 2000, pp. 249–270.

[29] C. Anandhakumar, V. Lavanya, G. Pradheepa, K. Tirumurugaan, G.D. Raj, A. Raja, N. Pazhanivel, C. Balachandran, Expression profile of toll-like receptor 2 mRNA in selected tissues of shark (*Chiloscyllium sp.*), Fish Shellfish Immunol. 33 (5) (2012) 1174–1182.

[30] E. Lauriano, G. Silvestri, M. Kuciel, K. Żuwała, D. Zaccone, D. Palombieri, A. Alesci, S. Pergolizzi, Immunohistochemical localization of Toll-like receptor 2 in skin Langerhans' cells of striped dolphin (*Stenella coeruleoalba*), Tissue Cell 46 (2) (2014) 113–121.

[31] E. Lauriano, C. Faggio, G. Capillo, N. Spanò, M. Kuciel, M. Aragona, S. Pergolizzi, Immunohistochemical characterization of epidermal dendritic-like cells in giant mudskipper, *Periophthalmodon schlosseri*, Fish Shellfish Immunol. 74 (2018) 380–385.

[32] J. Lovy, G.M. Wright, D.J. Speare, Comparative cellular morphology suggesting the existence of resident dendritic cells within immune organs of salmonids, Anat. Rec. 291 (4) (2008) 456–462.

[33] A.O. Kordon, M.A. Scott, I. Ibrahim, H. Abdelhamed, H. Ahmed, W. Baumgartner, A. Karsi, L.M. Pinchuk, Identification of Langerhans-like cells in the immunocompetent tissues of channel catfish, *Ictalurus punctatus*, Fish Shellfish Immunol. 58 (2016) 253–258.

[34] D.M. Zaghloul, A.E. Derbalah, C.S. Rutland, Unique characterization of Langerhans cells in the spleen of the African catfish (*Clarias gariepinus*), Matters Select 3 (7) (2017) e201703000005.

[35] P. Licata, R. Tardugno, S. Pergolizzi, G. Capillo, M. Aragona, A. Colombo, T. Gervasi, V. Pellizzeri, N. Cicero, M. Calò, In vivo effects of PCB-126 and genistein on vitellogenin expression in zebrafish, Nat. Prod. Res. (2018) 1–8.

[36] G. Montalbano, G. Capillo, R. Laurà, F. Abbate, M. Levanti, M.C. Guerrera, E. Ciriaco, A. Germanà, Neuromast hair cells retain the capacity of regeneration during heavy metal exposure, Ann. Anat. Anat. Anzeiger 218 (2018) 183–189.

[37] S. Pergolizzi, V. D'Angelo, M. Aragona, P. Dugo, F. Cacciola, G. Capillo, G. Dugo, E.R. Lauriano, Evaluation of antioxidant and anti-inflammatory activity of green coffee beans methanolic extract in rat skin, Nat. Prod. Res. 3 (2018) 1–7.

[38] P. Jhaveri, Y.P. Papastamatiou, D.P. German, Digestive enzyme activities in the guts of bonnethead sharks (*Sphyrna tiburo*) provide insight into their digestive strategy and evidence for microbial digestion in their hindguts, Comp. Biochem. Physiol. Mol. Integr. Physiol. 189 (2015) 76–83.

[39] C. Bucking, Feeding and digestion in elasmobranchs: tying diet and physiology together, Fish Physiol. (2015) 347–394 Elsevier.

[40] S.C. Leigh, Y. Papastamatiou, D.P. German, The nutritional physiology of sharks, Rev. Fish Biol. Fish. 27 (3) (2017) 561–585.
[41] P. TJ, On the intestinal spiral valve in the genus Raja, Zool. Soc. Lond. Trans. 11 (1885) 49–61.
[42] J. Wilson, L. Castro, Morphological diversity of the gastrointestinal tract in fishes, Fish Physiol. (2010) 1–55 Elsevier.
[43] K. Chatchavalvanich, R. Marcos, J. Poonpirom, A. Thongpan, E. Rocha, Histology of the digestive tract of the freshwater stingray *Himantura signifer* Compagno and Roberts, 1982 (Elasmobranchii, Dasyatidae), Anat. Embryol. 211 (5) (2006) 507–518.
[44] N. Salamat, R. Darbeh, N.E. Majd, A.R. Safahieh, Histological aspects of gut associated lymphoid tissue in *Acanthopagrus latus*, Glob. Vet. 7 (4) (2011) 381–385.
[45] M.J. Redondo, P. Alvarez-Pellitero, Carbohydrate patterns in the digestive tract of *Sparus aurata* L. and *Psetta maxima* (L.)(Teleostei) parasitized by *Enteromyxum leei* and *E. scophthalmi* (Myxozoa), Parasitol. Int. 59 (3) (2010) 445–453.
[46] C. Domeneghini, R.P. Straini, A. Veggetti, Gut glycoconjugates in *Sparus aurata* L. (Pisces, Teleostei). A comparative histochemical study in larval and adult ages, Histol. Histopathol. 13 (2) (1998) 359–372.
[47] C. Sarasquete, E. Gisbert, L. Ribeiro, L. Vieira, M. Dinis, Glyconjugates in epidermal, branchial and digestive mucous cells and gastric glands of gilthead sea bream, *Sparus aurata, Senegal sole, Solea senegalensis* and *Siberian sturgeon, Acipenser baeri* development, Eur. J. Histochem. 45 (3) (2009) 267–278.
[48] A. Fiertak, W. Kilarski, Glycoconjugates of the intestinal goblet cells of four cyprinids, Cell Mol. Life Sci. CMLS 59 (10) (2002) 1724–1733.
[49] V. Pedini, P. Scocco, A. Gargiulo, P. Ceccarelli, S. Lorvik, Glycoconjugate characterization in the intestine of *Umbrina cirrosa* by means of lectin histochemistry, J. Fish. Biol. 61 (6) (2002) 1363–1372.
[50] V. Pedini, C. Dall'Aglio, F. Parillo, P. Scocco, Glycoconjugate distribution in gastric fundic mucosa of *Umbrina cirrosa* L. revealed by lectin histochemistry, J. Fish. Biol. 66 (1) (2005) 222–229.
[51] M. Rojo, M. Blanquez, M. Gonzalez, A histochemical study of the distribution of lectin binding sites in the developing branchial area of the trout *Salmo trutta*, J. Anat. 189 (Pt 3) (1996) 609.
[52] E. Lauriano, S. Pergolizzi, J. Gangemi, M. Kuciel, G. Capillo, M. Aragona, C. Faggio, Immunohistochemical colocalization of G protein alpha subunits and 5-HT in the rectal gland of the cartilaginous fish *Scyliorhinus canicula*, Microsc. Res. Tech. 80 (9) (2017) 1018–1027.
[53] J.-F. Beaulieu, Integrins and human intestinal cell functions, Front. Biosci. 4 (4) (1999) 310–320.
[54] A. Abd-Elmaksoud, Comparative expression of laminin and smooth muscle actin in the testis and epididymis of poultry and rabbit, J. Mol. Histol. 40 (5–6) (2009) 407–416.
[55] M. Koch, P.F. Olson, A. Albus, W. Jin, D.D. Hunter, W.J. Brunken, R.E. Burgeson, M.-F. Champliaud, Characterization and expression of the laminin γ3 chain: a novel, non-basement membrane–associated, laminin chain, J. Cell Biol. 145 (3) (1999) 605–618.
[56] A. Zapata, M. Torroba, R. Sacedon, A. Varas, A. Vicente, Structure of the lymphoid organs of elasmobranchs, J. Exp. Zool. Part A: Ecological Genetics and Physiology 275 (2-3) (1996) 125–143.
[57] S.B. Khojasteh, F. Sheikhzadeh, D. Mohammadnejad, A. Azami, Histological, histochemical and ultrastructural study of the intestine of rainbow trout (*Oncorhynchus mykiss*), World Appl. Sci. J. 6 (11) (2009) 1525–1531.
[58] M. Carrassón, A. Grau, L. Dopazo, S. Crespo, A histological, histochemical and ultrastructural study of the digestive tract of *Dentex dentex* (Pisces, Sparidae), Histol. Histopathol. 21 (4/6) (2006) 579.
[59] B. Fuglem, E. Jirillo, I. Bjerkås, H. Kiyono, T. Nochi, Y. Yuki, M. Raida, U. Fischer, E.O. Koppang, Antigen-sampling cells in the salmonid intestinal epithelium, Dev. Comp. Immunol. 34 (7) (2010) 768–774.
[60] I. Salinas, Y.-A. Zhang, J.O. Sunyer, Mucosal immunoglobulins and B cells of teleost fish, Dev. Comp. Immunol. 35 (12) (2011) 1346–1365.
[61] K.M. Nutsch, C.-S. Hsieh, T cell tolerance and immunity to commensal bacteria, Curr. Opin. Immunol. 24 (4) (2012) 385–391.
[62] W. Meyer, U. Seegers, A. Schnapper, H. Neuhaus, W. Himstedt, E. Toepfer-Petersen, Possible antimicrobial defense by free sugars on the epidermal surface of aquatic vertebrates, Aquat. Biol. 1 (2) (2007) 167–175.