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Granulomatous enteritis in rainbow trout (*Oncorhynchus mykiss*) associated with soya bean meal regardless of water dissolved oxygen level

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Abstract

This study investigated morphological changes associated with soya bean meal-induced enteritis (SBMIE) in distal intestine (DI) of rainbow trout (*Oncorhynchus mykiss*) fed a soya bean meal (SBM)-based diet and exposed to normoxia or hypoxia created by optimal and low water flow rates, respectively. A 28-day adaption period was followed by a 42-day challenge period where 600 fish were subjected to dietary challenge and/or hypoxia. Twelve tanks each containing 50 juvenile trout were assigned randomly in triplicate to each treatment. Histopathological and immunohistochemical evaluation revealed pathological features that have not previously been described in association with SBMIE. Vacuolar degeneration of epithelial cells mainly at the base of mucosal folds, epithelial cysts, epithelial dysplasia, necrosis, shedding of necrotic cells, and granulomatous inflammation including infiltration of enlarged, sometimes finely vacuolated or "foamy" macrophages, multinucleated giant cells and increased proliferation of fibroblasts were observed. Acid-fast bacteria were not detected in enlarged macrophages; however, these cells contained AB-PAS- and sometimes cytokeratin-positive material, which was interpreted to be of epithelial/goblet cell origin. Hypoxia did not affect the morphological changes in DI. These results suggest that SBM was associated with a granulomatous form of enteritis in DI of rainbow trout regardless of water oxygen level.

KEYWORDS
foamy macrophages, granulomatous enteritis, hypoxia, rainbow trout, soya bean meal

1 | INTRODUCTION

The inclusion of soya bean meal (SBM) in salmonid feeds is known to affect intestinal homeostasis adversely through the development of a chronic inflammation referred to as soybean meal-induced enteritis (SBMIE; Baeverfjord & Krogdahl, 1996). The development of SBMIE is attributed to the presence of antinutritional factors (ANFs). Soya saponins have been shown to increase gut permeability and to interact with other alcohol-soluble feed components to induce an enteritis in Atlantic salmon; however, the exact aetiology of the disease is not yet fully understood (Knudsen, Urán, Arnous, Koppe, & Frøkiær, 2007; Knudsen et al., 2006, 2008). SBMIE is a condition characterized by increased leucocyte accumulation in subepithelial tissues, atrophy of intestinal folds, increased number of goblet cells and changes in the morphology of epithelial cells including reduced supranuclear vacuolization (SNV), reduced cell height and increased cytoplasmic basophilia (Baeverfjord & Krogdahl, 1996; Bakke-Mckellep, Frøystad, et al., 2007; van den Ingh, Krogdahl, Olli, Hendriks, & Koninkx, 1991; Urán et al., 2009). SBMIE shares some morphological and immunological features with human intestinal inflammation as seen in inflammatory bowel disease (IBD) and coeliac disease (Dickson, Streutker, & Chetty, 2006; Geboes, Joossens, Prantera, & Rutgeerts, 2003;

Hisamatsu et al., 2013). These forms of intestinal inflammation are typified by leucocyte infiltration and proliferation in the lamina propria resulting in a thickening of mucosa, villus atrophy and loss of crypts with changes in morphology of epithelial cells. The composition of cells infiltrating the lamina propria differs to some extent among various types of enteritis. In addition to T cells that have been studied in SBMIE in Atlantic salmon (Bakke-Mckellep, Frøystad, et al., 2007; Lilleeng et al., 2009), population of macrophages are also involved in the mucosal immune response. Macrophages are one of the main agents of the innate immune system and their function is crucial to maintain tissue homeostasis. As in mammals, activated macrophages in fish perform phagocytic activity and produce pro-inflammatory cytokines, reactive oxygen species and nitric oxide (Forlenza, Fink, Raes, & Wiegertjes, 2011).

Inclusion of 200 g/kg SBM and higher in diets for Atlantic salmon (*Salmo salar* L.) has been shown to induce morphological changes in the distal intestine (DI) within the first week of consumption (Urán et al., 2009). Rainbow trout (*Oncorhynchus mykiss*) have been suggested to be more resistant to pathological effects of SBM than Atlantic salmon (Refstie et al., 2000); however, these effects are also evident for this species at high SBM inclusion levels (Mosberian-Tanha et al., 2016; Romarheim et al., 2008).

Adverse environmental conditions such as hypoxia may affect fish health and welfare, as reviewed elsewhere (Wu, 2002). Pathological changes may occur in intestinal tissue in response to environmental factors. For example, impaired intestinal barrier function along with morphological changes in proximal and DI (Sundh et al., 2010) and elevated mucosal neutrophil infiltration (Niklasson, Sundh, Fridell, Taranger, & Sundell, 2011) have been previously reported to occur in response to chronic hypoxia in Atlantic salmon. It has also been shown that the degree of diet-induced intestinal morphological changes was aggravated in Nile tilapia (*Oreochromis niloticus*) kept under hypoxic conditions (Tran-Ngoc et al., 2016).

It is not known whether adverse environmental conditions such as hypoxia may aggravate the effect of a dietary challenge such as SBM-based diet on DI morphological changes in salmonids. Thus, the purpose of this study was to investigate morphological changes associated with SBMIE in DI of rainbow trout under hypoxic conditions. Following histopathological investigations, variant features of SBMIE including granulomatous response were observed in addition to the commonly reported tissue response to SBM-based diet (Mosberian-Tanha et al., 2016; Romarheim et al., 2008). These changes were observed in a large fraction of fish with SBMIE. The findings necessitated a further detailed investigation of the intestinal pathology and the possible role of hypoxia.

2 | MATERIALS AND METHODS

2.1 | Fish rearing and experimental procedure

The experiment was performed in accordance with the Dutch law on the use of experimental animals and approved by the ethical committee of Wageningen University (DEC: 2014006.a).

At the start of the experiment, 600 juvenile rainbow trout with mean initial body weight (±*SE*) of 74.1 ± 0.3 g were randomly allocated among 12 tanks (50 fish per tank). Two isoenergetic and isonitrogenous diets were formulated (Table 1): one fishmeal-based control (FM) and one experimental diet containing 400 g/kg soya bean meal (SBM). Each of the four diet–environment combinations (Table 2) was assigned randomly to three tanks (200 L capacity), and the assigned diet was fed restrictively to the fish manually twice daily throughout the experiment at 9:00 and 16:00 hours for a maximum of 1 hr. Restrictive feeding was used to ensure that the fish in all treatment groups consumed the same amounts of feed and thus the same amount of SBM-based diet as a dietary challenge. The purpose was to eliminate the effect of feeding level on the degree of morphological changes.

The experiment was split into two periods: in period 1, the fish were adapted to FM or SBM for 28 days and were kept at normoxia by setting the water flow rate at 7.5 L/min resulting in a mean dissolved oxygen (DO) level of above 8 mg/L in the outlet (>78% saturation). Period 2 was a challenge period of 42 days where the fish were subjected to either a dietary challenge and/or exposed to hypoxia by reducing the water flow rate from 7.5 to 2.25 L/min resulting in a mean DO level of below 6 mg/L in the outlet (<55% saturation). The normoxic tanks, however, remained at the same water flow rate as used in period 1. If necessary, pure oxygen was injected into the inlet to maintain the intended DO level. The

TABLE 1 Diet formulation and chemical composition of experimental diets fed to rainbow trout (*Oncorhynchus mykiss*)

	FM	SBM
Ingredients (g/kg)		
Fish meal[a]	540.0	250.0
Soya bean meal[b]	–	400.0
Wheat flour[c]	170.0	140.0
Rapeseed oil	100.0	120.9
Fish oil[d]	40.0	40.0
Cellulose	143.4	30.0
Monocalcium phosphate[e]	–	10.0
DL-methionine[f]	–	2.5
Yttrium oxide	0.1	0.1
Vitamin/mineral premix	6.5	6.5
Proximate analysis		
Crude protein (g/kg)	430.0	427.0
Crude fat (g/kg)	206.0	220.0
Ash (g/kg)	79.0	76.0
Gross energy (MJ/kg)	23.0	23.2

FM, fishmeal; SBM, soya bean meal.
[a]TripleNine Fish Protein, Esbjerg, Denmark.
[b]Cargill, Amsterdam, The Netherlands.
[c]Meneba, Weert, The Netherlands.
[d]Coppens International, Helmond, The Netherlands.
[e]Tessenderlo Chemie, Rotterdam, The Netherlands.
[f]Evonik Industries AG, Hanau, Germany.

TABLE 2 Experimental design to evaluate morphological changes in the distal intestine of rainbow trout (*Oncorhynchus mykiss*) fed soya bean meal and exposed to hypoxic conditions

Treatment	Period 1		Period 2	Abbreviation
1	FM at Normoxia	→	FM at Hypoxia	FMNO → FMHY
2	FM at Normoxia	→	SBM at Hypoxia	FMNO → SBMHY
3	FM at Normoxia	→	SBM at Normoxia	FMNO → SBMNO
4	SBM at Normoxia	→	SBM at Hypoxia	SBMNO → SBMHY

FM, fishmeal; SBM, soya bean meal; NO, normoxia; HY, hypoxia. In period 2, fish were subjected to change in diet (FM to SBM) and/or oxygen level (NO to HY). Treatments 1 and 4 were only subjected to a change in the environment (NO to HY) and fed the same diets as used in period 1 (steady-state dietary condition).

minimum DO level in the outlet, however, was maintained above 3.8 mg/L in hypoxia tanks to avoid extreme reduction in feed intake and increased mortality. At the start of period 2, the feeding level was reduced from 1.5% to 1.25% of mean biomass of 12 tanks. Water DO level is the key limiting factor when the water flow rate is reduced; however, this treatment also leads to accumulation of metabolites or fish excretions such as ammonia. To simplify nomenclature, low water flow rate is termed hypoxia (HY) and optimal water flow rate is termed normoxia (NO). Throughout the experiment, the fish was reared at photoperiod of 12 L:12 D, water temperature of 14.0 ± 0.5°C, pH between 7.0 and 8.0, nitrate of <250 mg N/L and nitrite of <0.15 mg N/L. Total ammonium nitrogen (TAN) during week five of period 2 and daily water oxygen level were measured as reported previously (Saravanan et al., 2012). The average TAN level was 0.14 mg N/L under hypoxic conditions and 0.06 mg N/L at normoxia. The mean of DO level (mean ± *SD*) in the inlet water was 10.3 ± 0.3 mg/L.

The four treatments tested in this experiment are shown in Table 2. Treatment 1 was designed to evaluate whether exposure to hypoxia alone would induce morphological changes in DI. Treatments 2 and 3 were designed to evaluate whether a change from FM to SBM-based diet is more harmful to DI health at hypoxia than at normoxia, leading to an increase in the degree of morphological changes associated with SBMIE. Treatment 4 was designed to evaluate whether under steady-state dietary challenge (continuous exposure to SBM) any change in the environment from normoxia to hypoxia will aggravate SBM-induced morphological changes in DI.

2.2 | Sampling

During the experiment, DI was sampled at days 0, 7, 14, 21 and 42 of period 2. At each time point, three fish per tank were randomly selected, individually weighed and anaesthetized by 2-phenoxy ethanol (0.25 ml/L). Thus, for each of the four diet–environment combinations at each time point, nine fish were sampled. In total, for the experiment, 180 fish were sampled. The anaesthetized fish were killed by a blow to the head before DI tissue was collected for morphological evaluation and immunohistochemistry. Tissue samples were taken from the middle portion of DI, which is distinguished from the mid-intestinal region by the increased diameter, darker colouration and transverse folds of the mucosal surface. The DI tissue samples were dissected and cut lengthways prior to fixation in neutral buffered formalin (4% formaldehyde) for 48 hours. DI tissue samples were dehydrated in 70% ethanol and embedded in paraffin before tissue sections were stained by haematoxylin and eosin (H&E), Alcian blue/periodic acid-Schiff (AB-PAS) and Ziehl–Neelsen (ZN) staining following standard routines. One stained tissue section (H&E, AB-PAS) from the DI of each fish was examined. AB-PAS staining was performed to detect acidic (blue) and neutral (red) mucins of goblet cells. ZN staining was performed to detect acid-fast bacteria, and fish were selected for examination based on the presence of morphological changes determined from the examination of H&E sections.

2.3 | Histological evaluation

Blinded evaluation and scoring of the following morphological parameters were carried out on each DI tissue sample:

- **A** Subepithelial infiltration of leucocytes: increased accumulation of leucocytes in the subepithelial tissues down to stratum compactum.
- **B** Supranuclear vacuolization (SNV) of epithelial cells: reduced vacuolization of the epithelial cells.
- **C** Atrophy (shortening) of intestinal folds.
- **D** Vacuolar degeneration (VD) of the epithelial cells: increased VD at the base of the intestinal folds.
- **E** The presence of granulomatous response and the degree of such response: increased proliferation of fibroblasts and aggregation of enlarged macrophages and multinucleated giant cells (MGCs) along with lymphocytes in the subepithelial tissues.

A score was given to each parameter that ranged from 0 (no morphological change) to 3 (severe changes) with increments of 1. Score of 1 was given to slight changes that are still assessed as normal morphology while score 2 was given to moderate changes. For evaluation of granulomatous response, score of 1 was given to the tissue containing only a few enlarged macrophages and/or slight increase in the number of fibroblasts. Score 2 was given to tissue containing increased numbers of fibroblasts, enlarged macrophages and a few MGCs. Score 3 was given to tissue containing large numbers of foamy macrophages and increased numbers of MGCs. According to this protocol, a score of at least 2 should be given to parameters A, B and C to confirm the presence of a classic SBMIE.

2.4 | Immunohistochemistry

Paraffin sections were placed on glass slides and air-dried for 30 min at 58°C. The sections were then deparaffinized with xylene and rehydration. The sections were autoclaved in citrate buffer (pH 6.0) for 15 min at 121°C. Endogenous peroxidase was inhibited by incubation of the tissue sections for 10 min in 3% H_2O_2 (hydrogen peroxide) diluted in methanol. To prevent non-specific binding of antibodies, the sections were treated with goat serum containing 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS) for 20 min at room temperature. The sections were then subjected to primary antibodies and incubated for 1 hr at room temperature. For PCNA detection, mouse monoclonal IgG2α-κ antibody (diluted 1:25,000 in 1% BSA/TBS, M0879; Dako, Norge, Oslo, Norway) and for cytokeratin detection mouse monoclonal IgG1-κ antibody (pan, clone AE1/AE3, diluted 1:50 in 1% BSA/TBS, Zymed Laboratories; Dale, Tørud, Kvellestad, Koppang, & Koppang, 2009) were used. Sections without primary antibody incubation served as negative controls. The incubation for the peroxidase-labelled secondary antibody was performed with Labelled Polymer-HRP anti-mouse (Dako, Norge, Oslo, Norway) for 30 min. All incubations were performed in a humid chamber at room temperature. The peroxidase activity was developed with a 3-amino-9-ethyl carbazole kit (Dako, Norway) for 15 min. The sections were then counterstained with Mayer's haematoxylin for 20 seconds and mounted in Aquatex mounting medium (VWR International). The sections were washed three times, except for the treatment with goat serum, in PBS for 5 min between each step.

2.5 | Calculations and statistics

Quantification of PCNA reactivity of each DI tissue sample was measured as described elsewhere (Romarheim, Øverland, Mydland, Skrede, & Landsverk, 2011). Statistical analyses were performed using SAS 9.4 (SAS Institute 2012). All data were tested for normality and homogeneity by Kolmogorov–Smirnov and Bartlett tests. Data from morphological parameters violated the normal distribution assumption after log10-transformation; and thus, these data were subjected to nonparametric Kruskal–Wallis test followed by multiple pairwise comparisons (Dwass–Steel–Critchlow–Fligner) if the test was significant. PCNA reactivity score in period 1 was subjected to one-way analysis of variance (ANOVA) in GLM procedure to test the effect of diet. The effect of treatment and sampling time on PCNA reactivity score in period 2 was analysed using a two-way ANOVA in GLM procedure. Least square means comparison was used to determine which groups differed significantly in PCNA reactivity from each other. Differences were declared statistically significant if $p < .05$.

3 | RESULTS

3.1 | Histopathological evaluation of the distal intestine

Histopathological examination of the tissue sections revealed the presence of SBMIE in fish fed SBM-based diet (Figure 1). Under SBMIE conditions, the intestinal folds showed various degrees of atrophy. Epithelial changes were often pronounced with reduced SNV of epithelial cells. In most individuals, epithelial change also included VD often progressing to evident necrosis, which was recognized by shrinkage and condensation of chromatin and fragmentation of the nucleus. Acid and neutral mucins in goblet cells were readily identified with AB-PAS staining allowing distinction between goblet cells and cells with VD (Figure 2). The necrotic epithelial cells were extruded to the intestinal lumen resulting in denudation of the lamina propria. Apparent fusion of adjacent intestinal folds in some cases resulted in the formation of cysts filled with epithelial debris

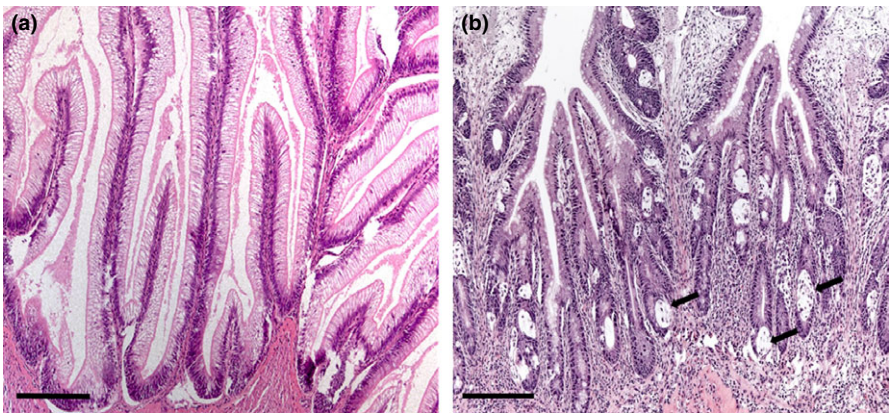

FIGURE 1 Morphology of the distal intestine in rainbow trout (*Oncorhynchus mykiss*) stained with haematoxylin and eosin (H&E) (bars = 200 μm). (a) Normal distal intestine (DI) of rainbow trout fed fish meal. Epithelial cells are regular, with a high columnar shape, and contain a finely vacuolated supranuclear cytoplasm. (b) DI with soya bean meal-induced enteritis after 42 days of feeding a diet containing 400 g/kg soya bean meal. DI shows atrophy of mucosal folds and heavy infiltration of leucocytes into the subepithelial mucosa. Epithelial cells have a darker supranuclear cytoplasm with reduced degree of supranuclear vacuolization. The height of the epithelial cells is also reduced. Cyst-like structures at the base of the mucosal folds are formed, in part outlined by epithelial cells, containing cellular debris (arrows) (bar = 200 μm)

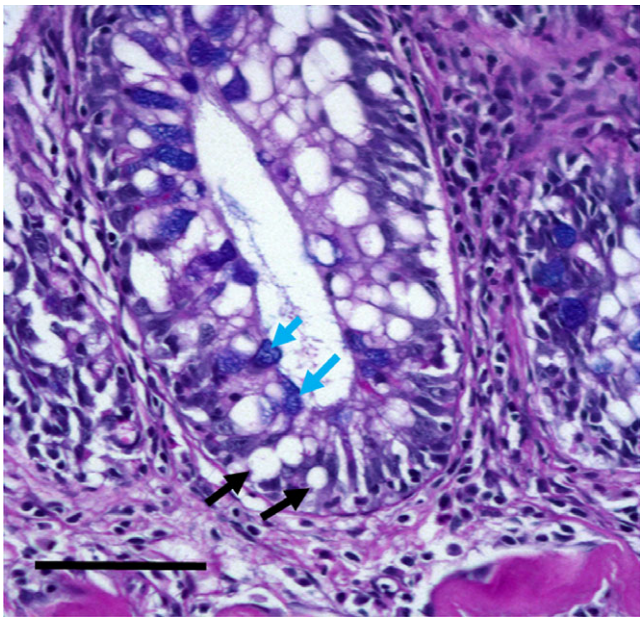

FIGURE 2 Distal intestine of rainbow trout with soya bean meal-induced enteritis stained with Alcian blue/periodic acid-Schiff (AB-PAS). Vacuolar degeneration of epithelial cells (black arrows) is identified, and goblet cells containing acidic (blue) mucins are readily distinguished (blue arrows) (bar = 50 μm)

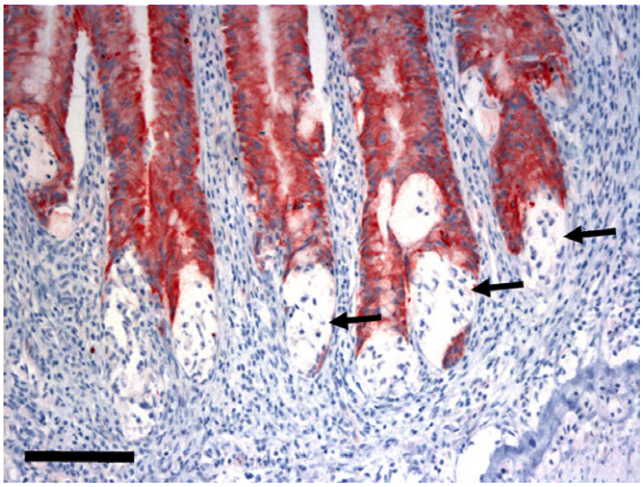

FIGURE 3 Immunohistochemistry using anticytokeratin antibody (AE1/AE3). Distal intestine of rainbow trout with soya bean meal-induced enteritis. Epithelial cells, easily distinguished by their red labelling, have largely disappeared from the base of the mucosal folds and replaced by cysts (arrows) containing necrotic debris (bar = 100 μm)

(Figure 3). The cysts were either in part outlined by epithelial cells or lacking an epithelial lining, which was a feature that could have been caused by rupture of the cysts. These changes were predominantly found at the base of the folds. Flattened epithelial cells covering or partly covering the lamina propria and regenerative reaction in the remaining epithelial cells were interpreted as signs of epithelial restitution (Figure 4). Irregular shape of the epithelial cells and their nuclei in the vicinity of these areas and the site of fusion of the

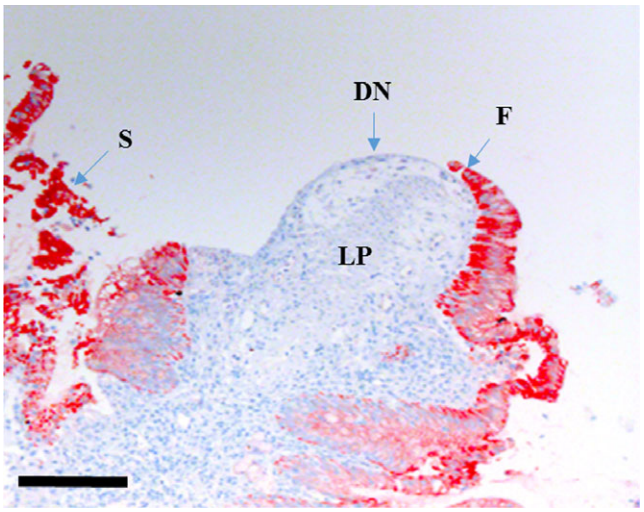

FIGURE 4 Lamina propria is denuded due to loss of epithelial cells. Distal intestine of rainbow trout with soya bean meal-induced enteritis. The section is immunolabelled with anticytokeratin antibody (AE1/AE3). In proximity to the denuded area (DN), the epithelial cells are flattening out (F), probably in the process of restitution to cover the denuded area. LP, lamina propria; S, shed necrotic cells (bar = 100 μm)

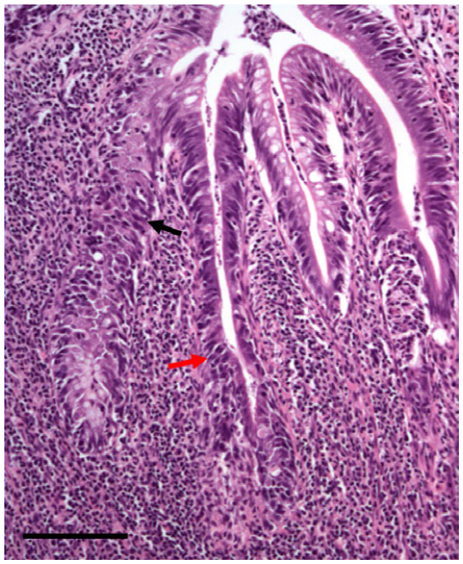

FIGURE 5 Changes in the epithelium of rainbow trout with soya bean meal-induced enteritis (SBMIE). We have chosen to use the term "dysplasia" for a particular epithelial change that was observed in 10% of individuals with SBMIE (and sampled during the last three weeks of period 2). This change is characterized by the irregularity of the shape of the epithelial cells, their organization and chromatin density within nuclei of the epithelial cells. In this micrograph, these changes are seen at a site where intestinal folds could be fusing (arrow) and in an adjacent site the epithelium shows similar irregularities (red arrow). Tissue stained with haematoxylin and eosin (H&E) (bar = 100 μm)

intestinal folds sometimes justified the use of the term "dysplastic" changes (Figure 5). Based on our evaluation protocol, 113 fish in total (fed SBM regardless of their environment) were diagnosed with

SBMIE during the entire experimental period. Of these, 12 fish (≈10%), during the last 3 weeks of period 2, showed dysplastic changes in the epithelium.

Granulomatous response to different degrees was evident in the lamina propria (Figure 6). The granulomatous response included prominent macrophage aggregates. Macrophages were often enlarged and sometimes finely vacuolated allowing the use of the term "foamy macrophages" (Figure 6). The foamy macrophages were positive for acid (blue) mucin and fewer were positive for neutral (red) mucin (Figure 7). In addition to macrophages, infiltration of lymphocytes, eosinophilic granular cells, neutrophils and proliferation of fibroblasts were evident in the lamina propria. In individuals with marked granulomatous response, a prominent presence of MGCs was evident, and sometimes, these cells were detected within the cysts. Ziehl–Neelsen stains were negative for acid-fast bacilli within the macrophages and MGCs of selected sections with granulomatous response (Figure 8). A search for fungi, such as mycelia and spores, in AB-PAS-stained sections was also negative. Thus, the structural properties of the AB-PAS-positive material within the macrophages and MGCs were consistent with that of mucin.

The mean scores of morphological changes are shown in Figure 9. These changes were characterized by reduced apical SNV, reduced height of simple and complex intestinal folds (partial atrophy), and increased numbers of leucocytes (e.g., lymphocytes, granulocytes and eosinophilic granular cells) in the lamina propria, and an increase in the degree of VD at the base of the folds and the degree of granulomatous response. Atrophy, SNV of epithelial cells and mucosal leucocyte infiltration have been characterized in many studies as morphological parameters associated with SBMIE (Baeverfjord & Krogdahl, 1996; Mosberian-Tanha et al., 2016; Romarheim, Hetland et al., 2013). Thus, these parameters are referred to as classic parameters/features of SBMIE in this paper. There was no adverse effect of hypoxia on morphological parameters in fish fed FM throughout the experiment and exposed to hypoxia during period 2 (FMNO → FMHY). Fish fed the SBM diet throughout the experiment (SBMNO → SBMHY), developed SBMIE in the DI during period 1 and scored highest on all morphological parameters compared with fish fed the FM diet ($p < 0.05$). During period 2, this group was exposed to hypoxia and no significant change in the degree of morphological changes was observed overtime, although the granulomatous response tended to reduce at day 14 ($p = 0.08$). The pattern of change in all morphological parameters was similar in the fish subjected to dietary change from FM to SBM under normoxia and hypoxia (FMNO→SBMHY and FMNO→SBMNO) during period 2. All SBM-fed fish, regardless of the environment (i.e., hypoxia or normoxia), reached the same degree of change in three of the classic parameters (A, B and C) by day 7 and in VD and granulomatous response by days 14 and 21, respectively (Figure 9).

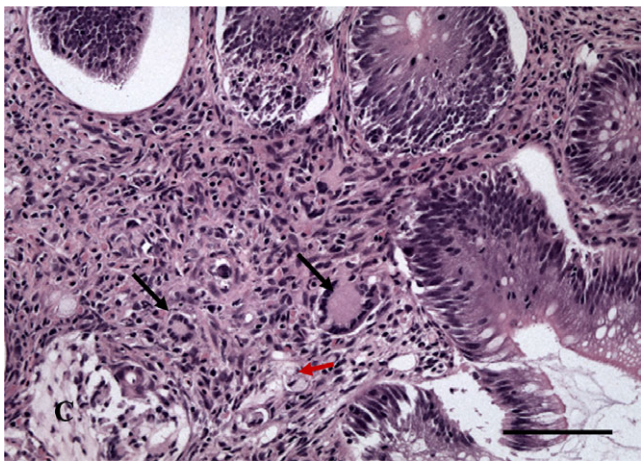

FIGURE 6 Granulomatous enteritis in rainbow trout with distal intestinal inflammation induced by soya bean meal. The change was characterized by the presence of multinucleated giant cells (black arrows), foamy macrophages (red arrow) and increased proliferation of fibroblasts in the lamina propria. Cyst-like structures (C) containing cell debris were also observed with or without outlining epithelial cells. Haematoxylin and eosin (H&E) (bar=50 μm)

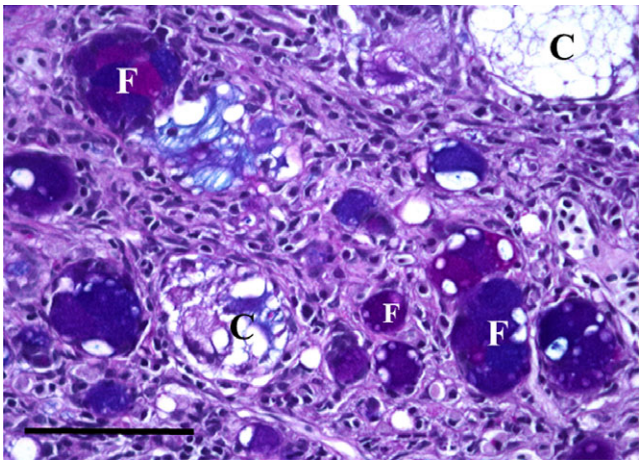

FIGURE 7 Foamy macrophages (F) in rainbow trout with granulomatous enteritis were mostly positive for acidic (blue) mucins; however, a few were also positive for neutral (red) mucins. In this micrograph, cyst-like structures (C) are also observed. AB-PAS (bar = 50 μm)

3.2 | Immunohistochemistry

Fish on a steady-state SBM-based diet (SBMNO→SBMHY) showed a higher degree of PCNA reactivity during period 1 than the groups fed FM-based diet ($P < 0.0001$) and the degree of PCNA reactivity in this group remained unchanged throughout period 2. In period 2, fish fed FM-based diet but exposed to hypoxia (FMNO→FMHY) generally showed the lowest degree of PCNA reactivity in the epithelium compared with other treatments (Figure 10). The fish subjected to change from FM- to SBM-based diets under normoxia (FMNO→SBMNO) and hypoxia (FMNO→SBMHY) in period 2 showed their highest degree of PCNA reactivity at days 7 and 21, respectively, reaching the same degree as achieved in the fish fed SBM throughout the experiment and exposed to hypoxia in period 2 (SBMNO-SBMHY). The degree of PCNA reactivity from day 21

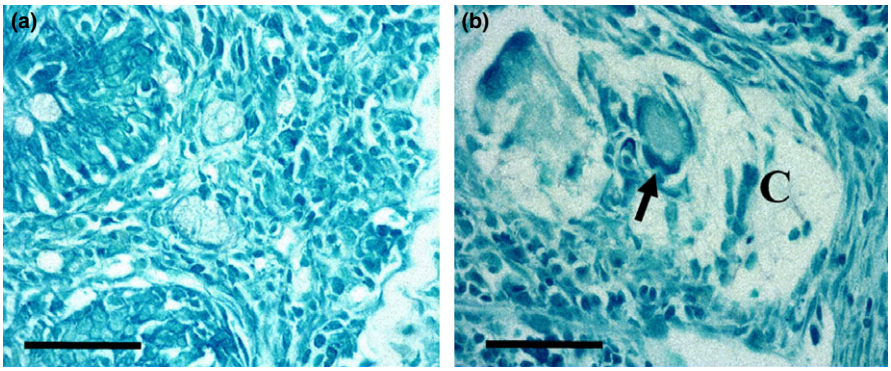

FIGURE 8 Distal intestinal tissue in rainbow trout with granulomatous enteritis was found to be negative for Ziehl–Neelsen staining. (a) Foamy macrophages and (b) multinucleated giant cells (black arrow) did not stain for acid-fast organisms. Positive controls were also included for evaluation (not shown) C: cyst-like structure. Note that there is no epithelial outlining of the cyst (bar = 50 μm)

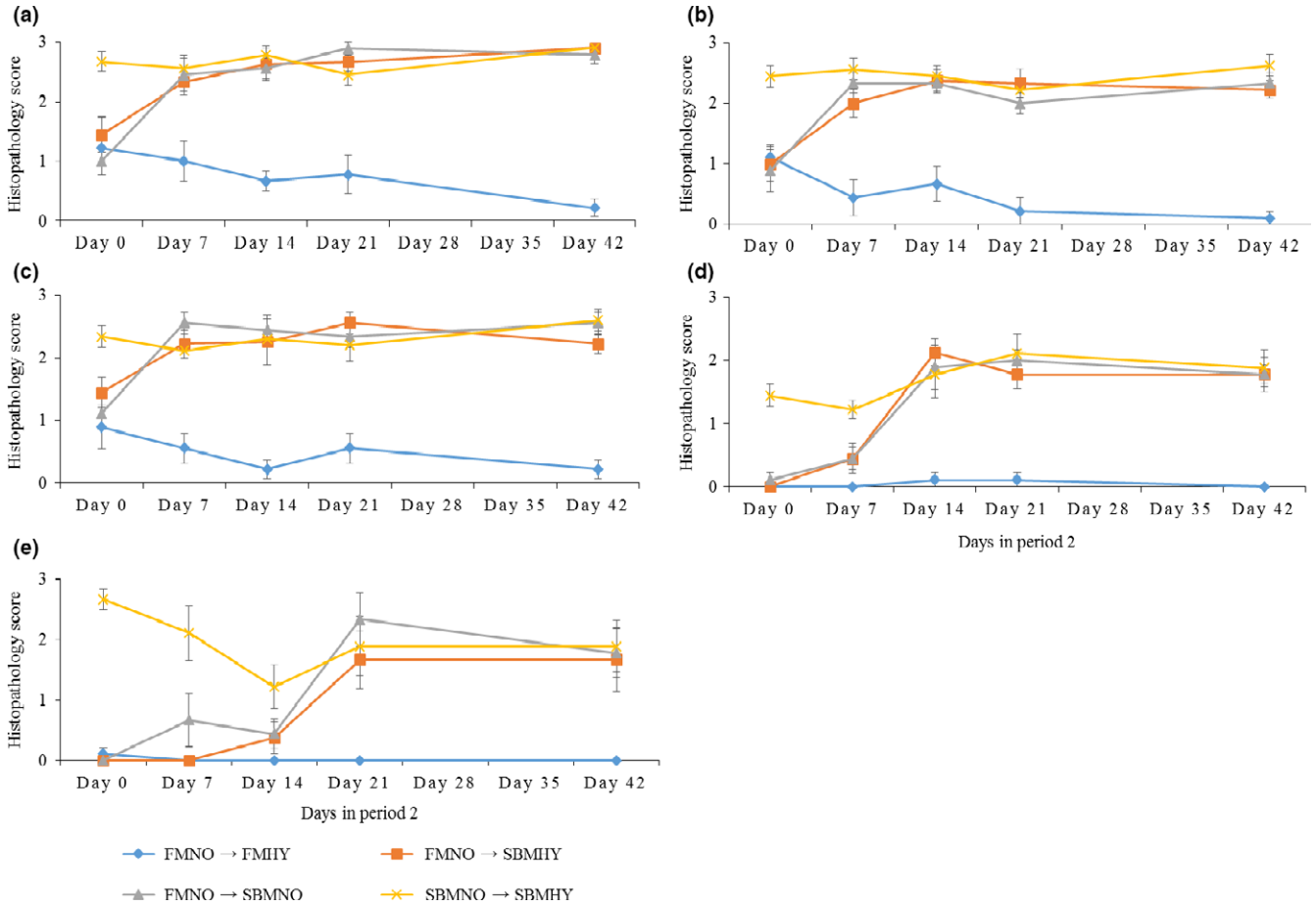

FIGURE 9 Morphological evaluation of distal intestine of rainbow trout fed fish meal or soya bean meal and exposed to hypoxia or normoxia for 42 days. The changes in subepithelial infiltration of leucocytes (a), supranuclear vacuolization of the epithelial cells (b), atrophy of intestinal folds (c), vacuolar degeneration of the epithelial cells at the base of the intestinal folds (d) and the subepithelial presence and degree of granulomatous response (e) are shown. Values are means (n = 9) ± standard errors represented by vertical bars. Fish were challenged with soya bean meal and/or hypoxia during period 2. FM, fish meal; SBM, soya bean meal; NO, normoxia; HY, hypoxia. Histopathological score of classic morphological parameters (a, b and c) in response to a soya bean meal (SBM)-based diet was significantly increased at the end of period 1 (Day 0). The degree of morphological parameters remained unchanged throughout period 2 for the fish under steady-state SBM feeding. In rainbow trout subjected to change from fish meal (FM) to SBM-based diet, regardless of water oxygen level, the scores of the classic morphological parameters were significantly increased after 7 days of SBM feeding in period 2. After day 7, there was no further change in the degree of these morphological parameters. The significant change in vacuolar degeneration of epithelial cells and granulomatous response was observed after 14 and 21 days, respectively, in period 2 in fish subjected to change from FM to SBM. After day 21, there was no further change in the degree of classic and variant morphological features among SBM-fed groups regardless of their environment

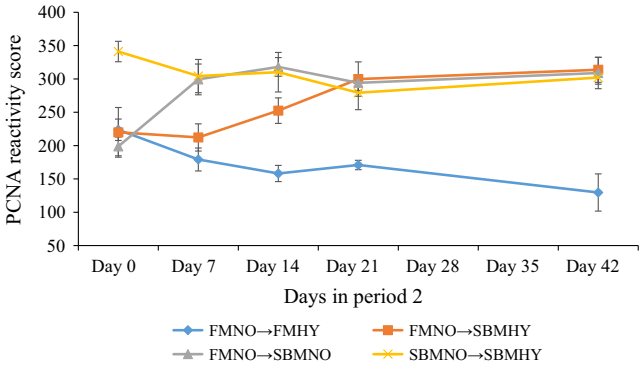

FIGURE 10 Proliferating cell nuclear antigen (PCNA) reactivity score of epithelium in the distal intestine of rainbow trout fed fish meal or soya bean meal-based diets and kept at normoxia or hypoxia for 42 days. FM, fish meal; SBM, soya bean meal; NO, normoxia; HY, hypoxia. Values are means (n = 9) ± standard errors represented by vertical bars. PCNA reactivity score was significantly increased in response to SBM-based diet by the end of period 1 (Day 0). The score remained unchanged during period 2 in the treatment group exposed to steady-state dietary challenge (i.e., SBM). In the group subjected to FM throughout the experiment, the PCNA reactivity score was significantly reduced at day 42. Change from FM to SBM without change in water oxygen level increased the score significantly after 7 days of SBM feeding and remained as high as the score observed in the group under steady-state SBM challenge. Change from FM- to SBM-based diets and simultaneously a change from normoxia to hypoxia resulted in significant increase in PCNA reactivity score after 21 days in period 2. After day 21, there was no significant difference in PCNA reactivity score among all groups fed SBM-based diet, regardless of water oxygen levels

onwards was not significantly different among the treatment groups fed SBM during period 2, regardless of their environment.

Following cytokeratin immunostaining, the epithelial cells were localized and the epithelial origin of the extruded cells into the lumen was confirmed (Figure 11). Cytokeratin reactivity in the cells that sometimes surrounded the cysts suggested the formation of cysts by fusion of layers of epithelial cells (Figure 12). The lack of cytokeratin-positive cells outlining many cysts could be due to either loss of the epithelial lining or rupture of the cysts. Macrophages in the lamina propria sometimes contained cytokeratin-positive material (Figure 12), which could result from the phagocytosis of epithelial cell debris released to the lamina propria following cyst rupture.

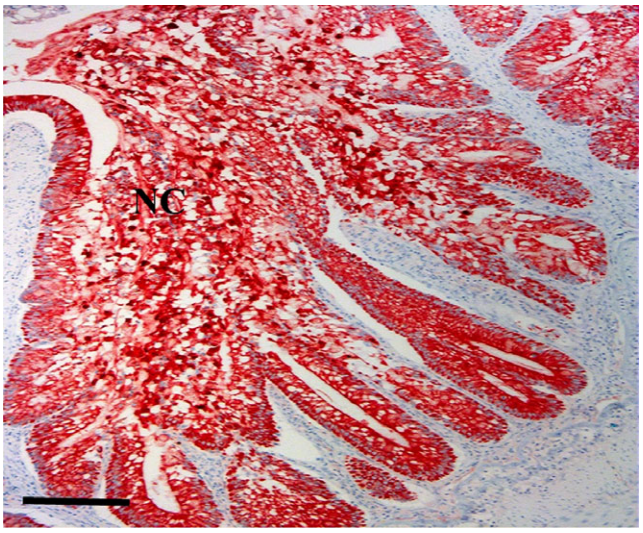

FIGURE 11 Extrusion of epithelial cells into the lumen of distal intestine of rainbow trout with soya bean meal-induced enteritis. The epithelial origin of extruded material was confirmed by immunohistochemistry using antibody (AE1/AE3) directed against cytokeratins. NC, necrotic cells (bar = 200 μm)

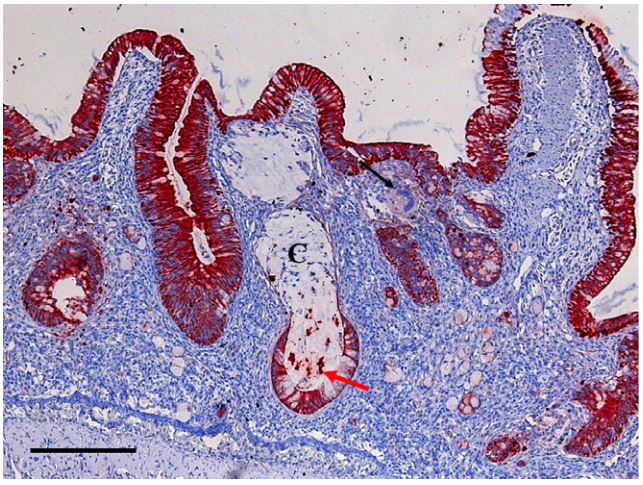

FIGURE 12 Cytokeratin reactivity was occasionally found within cyst-like structures (red arrow) in distal intestine of rainbow trout with soya bean meal-induced enteritis. The presence of this immunolabelling suggests accumulation of material of epithelial origin within these structures. Black arrow shows a multinucleated giant cell (bar = 200 μm)

4 | DISCUSSION

The objective of the present study was to investigate whether exposure to the environmental challenge of hypoxic conditions aggravated the effect of SBM on morphological changes associated with SBMIE in rainbow trout over time. The observed increase in morphological changes within the first week after SBM feeding was in agreement with the previous study in Atlantic salmon (Urán et al., 2009). However, in contrary to a previous study in rainbow trout (Romarheim et al., 2008), the current study found that most of the fish fed SBM, regardless of their environment, showed a significant increase in all classic parameters of SBMIE within seven days of dietary challenge. Hypoxia did not significantly affect the degree of morphological changes in DI of SBM-fed fish. It is possible that the severe changes induced by the SBM-based diet in this study concealed any additional effect of hypoxia. However, there was no adverse effect of hypoxia on morphology of DI in the fish fed FM-based diet. This finding may imply that hypoxia alone could not induce morphological changes in rainbow trout and is in contrast to previous studies in Atlantic salmon exposed to hypoxia (50% DO

saturation). These investigations reported atrophy of DI mucosal folds (Sundh et al., 2010) and infiltration of neutrophils into the mucosa of proximal intestine (Niklasson et al., 2011). The possible explanation is that rainbow trout may be more resistant to environmental challenges such as hypoxia than Atlantic salmon (Boutilier, Dobson, Hoeger, & Randall, 1988; Holeton & Randall, 1967). The lack of adverse effect from hypoxia was observed for all parameters including granulomatous response and VD of epithelial cells.

This study presents a variant feature of a diet-induced enteritis and the lack of hypoxia effect on development of this form of enteritis led to the hypothesis that the pathological condition was associated with SBM diet. Although there is a clear association with the diet in the present case, the possible contribution from microbial factors cannot be excluded. T-cell reactivity has been shown to be important in the pathogenesis of SBMIE in Atlantic salmon (Bakke-Mckellep, Frøystad, et al., 2007; Lilleeng et al., 2009); however, in the current study, the pronounced presence of macrophages forming MGCs was also evident in many individuals with SBMIE. As a result, the pathological feature was characterized as granulomatous enteritis.

Regarding pathogenesis, it is likely that initial epithelial changes caused by SBM resulted in an interruption of the intestinal mucosal barrier allowing the luminal contents including various types of microorganisms to interact directly with immune cells present in the subepithelial tissues. Increased intestinal mucosal barrier permeability and a greater incidence of bacterial translocation have been reported previously in rainbow trout with SBMIE (Mosberian-Tanha et al., 2016). The demonstrated absence of acid-fast organisms in tissue macrophages may indicate that mycobacteria were not the causative agent. However, it cannot be ruled out that ZN staining used in this study may not have detected certain species of mycobacteria possibly present in the environment. In an experimental infection trial, real-time PCR and immunohistochemical-based detection of various types of mycobacteria were found to be more sensitive than ZN (Zerihun, Hjortaas, Falk, & Colquhoun, 2011). It is also possible that other environmental factors, which could not be addressed directly in this experiment, may have contributed to the manifestation. One such factor is water temperature. In a previous experiment with rainbow trout kept at average temperature of 9°C (Mosberian-Tanha et al., 2016), inclusion of 375 g/kg of SBM resulted in only classic morphological changes commonly reported for SBMIE. However, in the current experiment, apart from hypoxia, water temperature was the other major environmental difference. Rainbow trout in this study were kept at relatively high temperature of 14°C. The higher temperature used in this experiment may have been a contributing factor in the manifestation of the variant features. Sealey, Barrows, Smith, Overturf, and Lapatra (2009) reported epithelial "cystic absorptive vacuoles," fusion of intestinal folds, and change in morphology of epithelial cell nuclei in rainbow trout fed 430 g/kg SBM at 14.8°C temperature. Burrells, Williams, Southgate, and Crampton (1999) observed increased vacuolization of epithelial cells and extrusion of mucosal material into the intestinal lumen in rainbow trout (of 5 grams body weight) fed 800–890 g/kg SBM at 14°C compared with the fish fed diets with lower levels of SBM inclusion. Increased immune cell infiltration, however, was the only subepithelial observation made and reported by the authors. Moreover, the degree of SBMIE was found to be more severe at 12°C than that at 8°C in Atlantic salmon fed 200 g/kg SBM, however, without a change in the form of inflammatory and tissue response (Uran et al., 2008). None of the above studies reported or observed a granulomatous form of enteritis. We hypothesize that the pathological effect of SBM on DI may be different at higher temperatures (at least in rainbow trout) and may explain the occurrence of the variant pathological manifestation, the granulomatous response. Temperature may also affect the function of immune system, which has a key role in the process of inflammation (Finn & Nielsen, 1971). In sockeye salmon (*Oncorhynchus nerka*), change in immune response pattern and a higher dependency on specific immune function have been shown to occur at higher temperatures (Alcorn, Murray, & Pascho, 2002).

Granulomatous enteritis involves a type 4 hypersensitivity reaction that may occur in association with chronic inflammation and is characterized by the occurrence of enlarged, activated macrophages (epithelioid cells), which sometimes are transformed into MGCs (Snyder, 2016). Granulomatous inflammation typically occurs following infection with mycobacteria but may also occur in association with neoplastic diseases and intoxications (Williams & Williams, 1983). Granulomatous inflammation has been reported also in fish at vaccination sites or in fish infected with mycobacteria (Koppang, Haugarvoll, Hordvik, Aune, & Poppe, 2005; Novotny et al., 2010). To our knowledge, there are no reports on the development of granulomatous enteritis in fish on a SBM diet. In human, a granulomatous regional enteritis of obscure aetiology is associated with Crohn's disease (Lee, Maguire, Obeidat, & Russell, 1997). Among mammalian species, the form of granulomatous enteritis that is best defined with respect to aetiology is paratuberculosis, which is commonly seen in ruminants (Arsenault et al., 2014). The aetiology of the disease is Mycobacterium avium spp. paratuberculosis and the bacteria can be detected within granulomas after ZN staining as previously shown (Abendaño et al., 2014).

The enlarged macrophages containing small vacuoles, or foamy macrophages seen in the present material, likely represent activated macrophages. Such macrophages may contain various types of materials (Sagaert, Tousseyn, De Hertogh, & Geboes, 2012). In this study, the presence, in foamy macrophages, of acidic and neutral mucins may imply that foamy macrophages engulfed mucins. Foamy macrophages were mostly positive for acidic (blue) mucin, which has been suggested to be an indication of mucin phagocytosis and presence of "muciphages," which are reported to occur in response to tissue injury (Sagaert et al., 2012). Positivity for neutral (red) mucin is an indication of a much broader array of disorders including phagocytosis of microorganisms and mucins (Sagaert et al., 2012). No indication was found in the present study that the contents of the macrophages contained material of microbial nature, including PAS-positive fungi. We are therefore suggesting that the AB-PAS-positive material was of endogenous nature, and most likely of goblet cell origin. Thus, necrotic goblet cells and their contents may have

become more easily available to the macrophages in the lamina propria by rupture of the cysts or loss of their outlining epithelial cells. A granulomatous reaction in response to release of mucins into the lamina propria has been reported in a colitis model of inflammation (Surawicz, Haggitt, Husseman, & Mcfarland, 1994).

Under SBMIE conditions, increased numbers of goblet cells have been reported (Urán et al., 2009), which may be confused with vacuoles of epithelial cells. In this study, however, AB-PAS staining allowed more accurate evaluation of this pathological feature in tissue samples. VD is a feature of reversible and non-lethal cell injury that occurs as a result of fluid accumulation in the cell or swelling of the endoplasmic reticulum. If cell injury is progressive, the cell will eventually become necrotic (Kumar, Abbas, Fausto, & Aster, 2010). Epithelial cells with VD were mainly observed at the mucosal fold bases where cysts were also formed. Fusion of intestinal folds containing cells with VD may have formed epithelial cysts, similar to the observations made previously in rainbow trout (Sealey et al., 2009). A lesser degree of maturation at base of the folds where proliferation occurs may in turn result in increased susceptibility of epithelial cells to various harmful agents. Cytokeratin reactivity within the cellular debris of the cysts further suggests that the debris was of epithelial origin.

Histopathology scores of VD and granulomatous response were significantly increased after 14 and 21 days, respectively, in period 2, which implies that these features required more time to develop than other morphological parameters (i.e., A, B and C). This delayed appearance may also imply that the classic SBMIE and the resulting disruption of epithelial integrity may have contributed to the development of these pathological features.

Increased epithelial cell proliferation as a compensatory response to cell loss under SBMIE condition has been shown previously (Bakke-Mckellep, Penn et al., 2007; Romarheim, Landsverk, Mydland, Skrede, & Øverland, 2013) and indicates an attempt to restore tissue homeostasis. Increased proliferation as indicated in this experiment by measurement of stretches of PCNA reactive showed a similar pattern as seen with the classic morphological changes (parameters A, B and C) and highlights the cellular proliferation in response to inflammation. Immunohistochemical detection of cytokeratin revealed the epithelial origin of extruded epithelial cells and contributed to explaining the cause of lamina propria denudation observed mainly at the tip of the intestinal folds. These observations indicate loss of epithelial cells and barrier damage, which in turn can cause increased cell proliferation (as indicated by increased PCNA reactivity) and exposure of the subepithelial tissues to luminal contents. At the edge of denuded areas, flattened epithelial cells were observed, which may indicate a rapid compensatory response referred to as restitution. Restitution has been reported to occur following severe epithelial damage in small intestine of rats (Matovelo, Sund, & Landsverk, 1989) and aims to cover the denuded areas and provide protection. Severe extrusion of epithelial cells has been suggested as a disturbing factor to the epithelial barrier integrity leading to inflammation (Gudipaty & Rosenblatt, 2016). Hypoxia did not increase PCNA reactivity, which implies that the tissue maintained homeostasis under this condition. The delayed increase in PCNA reactivity score in the fish challenged simultaneously to hypoxia and SBM-based diet may be an indication of a short-term effect of hypoxia on cell proliferation. Reduced SNV in the apical part of the intestinal folds under SBMIE could, at least partly, be a result of reduced maturity of the epithelium due to expanded proliferation zone.

Dysplastic changes (dysplasia) in areas expressing epithelial restitution and fusion of intestinal folds indicate abnormal cell proliferation and tissue growth under SBMIE conditions. The balance of cell death and proliferation is important to maintain tissue homeostasis. When the rate of cell proliferation exceeds that of cell death, the tissue may undergo abnormal growth with increased risk of tumorigenesis. Dysplasia is known to be associated with increased numbers of immature epithelial cells with changed morphology such as increased size and altered shape of the nuclei along with change in the orientation of these cells (Miller & Zachary, 2016). Increased risk of neoplasm due to abnormal growth of intestinal tissue has been reported in human with inflammatory bowel disease (Triantafillidis, Nasioulas, & Kosmidis, 2009). In salmonid fish, long-term exposure to a commercial diet has been shown to provoke an inflammation–dysplasia–carcinoma sequence that was considered similar to human colorectal cancer associated with inflammatory bowel disease (Dale et al., 2009). While carcinomatous changes were not evident in the rainbow trout after short-term dietary challenge, the presence of dysplasia is consistent with a sequence of development of dietary-induced cancer in salmonids. Further investigation is needed to determine the longer term outcome of granulomatous enteritis in rainbow trout.

In conclusion, hypoxic conditions neither induced inflammation nor aggravated the degree of SBMIE in rainbow trout. Simultaneous exposure to a SBM-based diet and hypoxia induced a delayed increase in PCNA reactivity score. Further to the commonly reported pathological features of SBMIE, the additional changes in a granulomatous response and vacuolar degeneration of epithelial cells were observed. These changes were associated with a more pronounced macrophage reaction. There was no indication that hypoxia affected the development of these changes. The variant pathological features reported in this study could potentially reveal new aspects of the pathogenesis of SBMIE.

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