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Mucosal barrier status in Atlantic salmon fed rapeseed oil and *Schizochytrium* oil partly or fully replacing fish oil through winter depression

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ABSTRACT

The study was designed to investigate the effects of replacing fish oil by algal oil and rapeseed oil on histomorphology indices of the intestine, skin and gill, mucosal barrier status and immune-related genes of mucin and antimicrobial peptide (AMP) genes in Atlantic salmon (*Salmo salar*). For these purposes, Atlantic salmon smolts were fed three different diets. The first was a control diet containing fish oil but no *Schizochytrium* oil. In the second diet, almost 50 % of the fish oil was replaced with algal oil, and in the third diet, fish oil was replaced entirely with algal oil. The algal oil contained mostly docosahexaenoic acid (DHA) and some eicosapentaenoic acid (EPA). The study lasted for 49 days in freshwater (FW), after which some fish from each diet group were transferred to seawater (SW) for a 48-h challenge test at 33 ppt to test their ability to tolerate high salinity. Samples of skin, gills, and mid intestine [both distal (DI) and anterior (AI) portions of the mid intestine] were collected after the feeding trial in FW and after the SW-challenge test to assess the effects of the diets on the structure and immune functions of the mucosal surfaces. The results showed that the 50 % VMO (Veramaris® algal oil) dietary group had improved intestinal, skin, and gill structures. Principal component analysis (PCA) of the histomorphological parameters demonstrated a significant effect of the algal oil on the intestine, skin, and gills. In particular, the mucosal barrier function of the intestine, skin, and gills was enhanced in the VMO 50 % dietary group after the SW challenge, as evidenced by increased mucous cell density. Immunolabelling of heat shock protein 70 (HSP70) in the intestine (both DI and AI) revealed downregulation of the protein expression in the 50 % VMO group and a corresponding upregulation in the 100 % VMO group compared to 0 % VMO. The reactivity of HSP70 in the epithelial cells was higher after the SW challenge compared to the FW phase. Immune-related genes related to mucosal defense, such as mucin genes [*muc2*, *muc5ac1* (DI), *muc5ac1* (AI), *muc5ac2*, *muc5b* (skin), and *muc5ac1* (gills)], and antimicrobial peptide genes [*def3* (DI), *def3* (AI), and *cath1* (skin)] were significantly upregulated in the 50 % VMO group. PCA of gene expression demonstrated the positive influences on gene regulation in the 50 % VMO dietary group. In conclusion, this study demonstrated the positive effect of substituting 50 % of fish oil with algal oil in the diets of Atlantic salmon. The findings of histomorphometry, mucosal mapping, immunohistochemistry, and immune-related genes connected to mucosal responses all support this conclusion.

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1. Introduction

Fish oil has been the primary lipid source in commercial aquafeeds for a long time [1]. Traditionally, high-quality marine fish oil is considered the primary source of energy and essential fatty acids [2]. However, due to the rise in demand for lipids and the high cost of fish oil, the aquaculture industry is now considering alternative feed ingredients [3]. For instance, plant meal and oils have emerged as substitute ingredients for aquafeeds. Moreover, microalgae have gained interest as a sustainable alternative to fish oil in aquafeeds. They can be used as the primary producers of EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid) in the marine environment, offering a low trophic solution to replace fish oil [4].

Numerous studies have documented the effects of substituting vegetable oil with algal oil in aquafeeds for many fish species, including salmonids [5]. Microalgae are rich in omega-3 PUFAs (polyunsaturated fatty acids), including DHA and EPA, and other essential micronutrients; therefore, they are considered good aquafeed ingredients [6].

Previous research suggests that the omega-3 found in aquafeeds can regulate the mucosal barrier response in Atlantic salmon [7]. Omega-3 fatty acids, including DHA, EPA, and ALA, play a significant role in activating the cellular components of both innate and adaptive immune systems. However, the mechanisms for such regulation are diverse [8]. For instance, omega-3 fatty acids promote macrophage activity, producing cytokines and chemokines [8]. Strengthening the different layers of skin and healing skin ulcers is also essential. These bioactive compounds are widely known for their anti-inflammatory properties [9]. Therefore, microbes that are rich sources of omega-3 PUFAs can enhance mucosal health and support the immune system. In addition, the presence of EPA and DHA in omega-3-containing diets influences the lipid metabolism during the smoltification of Atlantic salmon. Different fatty acids exert their influence by affecting the pro-inflammatory or anti-inflammatory pathways [10]. Fatty acid-containing diets are also essential for proper growth and metabolism in Atlantic salmon during smoltification [11]. Thus, it is crucial to investigate feed ingredients with specific fatty acids that play a pivotal role in critical life stages, such as the parr-smolt transition in salmon.

Schizochytrium spp. are microbes that stand out from others because of their high content of n-3 long-chain PUFAs (LC-PUFAs), various bioactive and antibacterial substances, and other nutrients [12]. Many studies have shown that *Schizochytrium*-derived oil, rich in omega-3 PUFAs, can be used alone or in combination with fish oil [4,13]. Hence, it can be considered a potential substitute for fish oil in salmonid feeds during smoltification. This alternative oil has the potential to impact mucosal health positively.

Mucosal epithelia are thin layers that cover the upper surface of fish skin, gills, and intestine [14]. They serve as defense barriers, protecting against external challenges like pathogens and contaminants. These barriers are coated with mucus produced by mucous cells. It contains lipids, globular proteins, DNA, salts, water, and mucins [15,16]. The mucus also contains important antimicrobial and anti-parasitic substances, including lysozymes, cytokines, and immunoglobulins that help protect against pathogens [17,18]. As mucosal surfaces play a vital role in protecting against unfavorable factors, assessing their immune components [19,20], which are closely linked to the immune system, is essential [21,22].

Atlantic salmon is an anadromous species that migrates from freshwater (FW) to seawater (SW) for feeding and growth before they return to FW for spawning and completing their life cycle [23,24]. The fish must adapt to the high concentration of ions in the SW by increasing their hypo-osmoregulatory ability, a process known as smoltification. This involves biochemical, physiological, morphological and behavioral changes. Manipulation of photoperiod is used in commercial farming to produce smolt all year. Short days (for example, light-dark cycle of 12:12 h for 8 weeks) simulate winter, followed by long days with 24 h of light triggering smoltification [25]. A winter signal (long night short day) is synchronizing the fish group so that smoltification can be completed within 350–400° days after the summer signal (24 h light) is given [26]. Recent research has shown great induvial variation in sea water readiness in light-manipulated fish groups [27]. There is a gap in knowledge if feed composition may have an impact during the winter signal. As part of a more extensive study, the present study examined the effects of replacing fish oil with Veramaris® algal oil (VMO), which is rich in EPA and DHA, on mucosal barriers of Atlantic salmon in fresh water under winter signal stimulation. However, the present study did not cover growth and smoltification indices, which will be presented in a separate publication.

2. Materials and methods

Ethical statement

The experiment and handling of animals were carried out according to the guidelines stated in national (LOV-2009-06-19-97) and European legislation (EU/2010/63) (approved application FOTS, ID-28278).

2.1. Experimental diets

All diets were formulated for the trial, and the experimental fish were fed 3 mm pellets (BioMar; Brande, Denmark). Three different diets were formulated: a control diet containing 5 % fish oil and 12 % plant oil (0 % VMO) and two additional diets in which either 50 % or 100 % of fish oil was replaced with Schizochytrium sp. derived oil (50 % VMO and 100 % VMO), respectively. The microbial oil was supplied by Veramaris® (Delft, Netherlands). The composition of fatty acid and algal oil product derived from *Schizochytrium* sp. microalgae are described in earlier literature by Santigosa et al. [4,28]. The ingredient and chemical composition of the experimental diets are given in Table 1.

2.2. Experimental design, fish and feeding management

The experiment used unvaccinated Atlantic salmon smolts obtained from Kvarøy AS in Mo i Rana, Norway. Before the experiment, the smolts were acclimated for 15 days in two freshwater tanks at Letsea's Land Research Facility in Dønna, Norway. The experiment was designed with three tanks per dietary group. Before the trial, 3000 smolts were weighed (46 ± 0.27 g), and their fork length was recorded. After acclimation, 1800 smolts were randomly distributed into nine 0.8 m3 tanks (3 groups × 3 replicates) in a recirculation aquaculture system (RAS). The dissolved oxygen, water temperature and salinity were maintained at ≈11.19 mg/L (105 % ± 12.2), 12.5 ± 1.5 °C, and 0 ppt, respectively. Water quality parameters such as ammonia, alkalinity, CO_2, pH, nitrates, and nitrites were assessed weekly and kept below threshold levels. During the experiment, the photoperiod regime was set for 12 h of light followed by 12 h of darkness (6000 lumens, LED module BH-MZ01, Philips 3030). Smolts were fed a commercial diet during the acclimatization period at LetSea following their well-established feeding protocol (BioMar, CPK 1.5 mm and CPK 2 mm). The experimental diets were nearly isoproteic, isolipidic, and isoenergetic based on the analysed crude lipid, protein, and energy values (Table 1). The feeding rate was maintained at 5–10 % overfeeding using mechanical band feeders (Mechanical belt feeders Pro-3kg/24t). The feeding trial was conducted for 49 days until the fish reached the size typically selected under farming conditions to initiate the transfer to seawater facilities.

2.3. Seawater challenge

To examine the seawater tolerance, the experimental fish were directly transferred without any stress from FW to SW, where they remained for 48 h before sampling. At the end of the feeding trial, six fish from each dietary treatment were transferred to land-based fiberglass tanks (0.8 m^3) with a continuous seawater supply (33 ppt).

Table 1
Ingredient composition, analysed chemical composition (%) and fatty acid composition (%) used in experimental diets.

Ingredients	0 % VMO	50 % VMO	100 % VMO
Marine origin	30	30	30
Soy protein concentrate	14.5	14.5	14.5
Wheat gluten	15	15	15
Wheat	12.5	12.5	12.5
Fish oil	**5.14**	**2.51**	–
Vegetable oil	**11.99**	**13.42**	**14.9**
Veramaris oil	–	**1.2**	**2.3**
Vitamin and mineral premix	0.43	0.43	0.43
Pigments	0.05	0.05	0.05
Other	10.4	10.4	10.4
Chemical composition			
Dry matter	93.79	93.80	92.62
% of dry matter			
Crude protein	42.99	43.11	43.07
Crude lipid	21.83	22.60	22.17
Ash	11.13	11.06	11.22
Energy (kJ/g)	21.68	21.42	21.47
Fatty acids (% of total fatty acids)			
Saturated fatty acids (SFAs)			
C14:0	2.1	1.5	0.8
C16:0	10.7	9.8	8.5
C18:0	2.6	2.4	2.1
∑SFAs	16.3	14.7	12.5
Monounsaturated fatty acids (MUFAs)			
C16:1	2.7	43.7	45.9
C18:1 n-9	41.7	9.83	9.98
C20:1 n-9	1.7	1.7	1.8
C22:1 n-11	1.8	1.8	1.8
∑MUFAs	48.2	49.3	50.5
Polyunsaturated fatty acids (PUFAs)			
C18:2 n-6 (LA)	16.9	18.1	19.6
C18:3 n-3 (ALA)	4.7	5.1	5.6
C20:5 n-3 (EPA)	5.1	4.2	3.2
C22:6 n-3 (DHA)	3.0	4.2	5.7
∑PUFA	31.6	33.4	35.9
∑n-3	14.3	14.7	15.6
∑n-6	17.3	18.7	20.3
∑n-3/∑n-6	0.83	0.79	0.77
EPA + DHA	8.1	8.4	8.9

Values are expressed as mean value of triplicate samples per diet. Σ SFA is the sum of saturated fatty acids includes C20:0, C22:0 and C24:00, Σ MUFA is the sum of monounsaturated fatty acids includes C24:1 n-9, Σ PUFA is the sum of polyunsaturated fatty acids, includes C18:4 n-3, C20:4 n-3, C20:4 n-6 and C22:5 n-3.

Throughout the 48 h seawater challenge, all fish were carefully monitored. At the end of the test, samples were collected and processed as described below.

2.4. Sample collection

At the end of the feeding trial, the fish were starved for 48 h before collecting samples. Both in the FW and SW phases, six fish from each dietary treatment were randomly collected and killed with an overdose of Finquel Vet (140 mg/L). Gills (second arch), dorsal skin (below dorsal fin), and mid intestine, anterior (AI) and distal (DI) segments (around 1.5 cm in length) were collected and used for gene expression and histomorphometric evaluation, as described in an earlier study [29]. For the histological assessment (histomorphometry/mucosal mapping), samples were cut, immediately transferred to 10 % neutral buffered formalin, and kept at room temperature for 24 h. For gene expression, skin, gill, and intestine were collected from each fish and immediately placed in RNA later®. Samples were then transferred to −80 °C until further analysis.

2.5. Histomorphometry and mucosal mapping

The histology samples were processed following standard histology procedures and embedded in paraffin, as described in a previous publication [30]. Briefly, after fixation, the tissue samples were dehydrated and embedded in paraffin. These samples were sectioned with a microtome (SHANDON, FINESSE ME; SI: FN1021M9812) and sections of approximately 5 μm were placed onto microscopic glass slides, preparing two slides per sample. One set of slides was stained with an automatic multi-stain machine (Leica ST5020) using PAS-Alcian blue (pH 2.5), mounted, and dried overnight. All images were captured using Leica Microsystems Framework Software connected to a digital microscopic camera and light digital microscope [31]. The captured images were then used for the histomorphometric analysis and mucosal mapping using ImageJ software (version 1.53).

For the quantitative assessment of the intestine, the following histomorphometric indices were measured: intestinal fold height, tunica muscularis width, lamina propria width, submucosa width and area of mucous cells were measured both in the anterior and distal part of the mid intestine. For each histological section, 15 intact intestinal folds, which were clearly visible in the whole length, were considered for the intestinal fold height. The intestinal fold height was considered from the bottom to the top of each intestinal fold. Lamina propria is the core connective tissue layer of each intestinal fold. Lamina propria was measured at 15 different points for each intestinal fold and the average value was finally registered. Submucosa is the loose connective tissue layer, containing a large number of blood vessels and lymph vessels. Clearly visible 15 different points were considered from each tissue section and the average value was finally registered. Tunica muscularis, is the muscular layer organized by an inner circular and an outer longitudinal layer of smooth muscle fibers. Randomly selected 15 different points were considered from each section to measure the thickness and the average value was registered. Mucous cells were quantified from 10 intestinal folds randomly selected using ImageJ software and the average value was finally registered. The indices for the skin histomorphology were: epidermis thickness (EP), scale thickness (SC), stratum compactum thickness (ST), number of epidermal mucous cells (MC), and relative area of epidermal mucous cells (AM). For the gills, indices were primary lamellae length (PL), secondary lamellae length (SL), secondary lamellae width (SW), basal epithelium thickness (BT) and number of mucous cells in lamellae (MC). The value of each index was measured randomly in different 15 regions, and the average value of each index was registered. The histomorphometric indices were measured and assessed as described in previous publications [31].

For the mucosal mapping of the intestine, skin and gill, the total number of mucous cells and the total area of mucous cells were measured according to the following equations [19]:

(a) Mean MC volumetric density (%) $= \frac{\text{Mucous area} \times \text{mucous number}}{\text{Epithelial area}} \times 100$

(b) Barrier status $= \frac{1}{\text{Mucous area} \times \text{Mucous cell density}} \times 1000$

2.6. Immunohistochemical assessment of mid intestine

The upregulation of HSP70 is a good indicator for this purpose [32]. Thus, in the current study, we used immunohistochemistry (IHC) to evaluate the increased localization of the protein in the epithelial area of the intestine. In brief, tissue sections (5 μm) from the intestine, prepared as described in the previous section, were placed on glass slides coated with Poly-L-lysine. The slides were dewaxed in xylene and dehydrated through an alcohol series of 100 %, 70 %, 30 %, and 30 %, respectively. All slides were then placed in phosphate-buffered saline (PBS). For antigen detection, the slides were first immersed in 3 % H_2O_2 in methanol for 30 min and then washed with running tap water for 5 min. The slides were placed in a citric buffer and heated in a microwave oven (1000 W) for 10 min. All slides were then placed carefully into the Sequenza™ cover-plate tray (model Ref: 72110017), a system for immunolabeling sections on glass slides. For serum blocking, 25 % normal goat serum (NGS) was prepared, and 100 μl was added to each chamber and

incubated at room temperature for 30 min. The slide reservoirs were filled with tris buffered saline (TBS) wash buffer and drained before adding 100 μl of primary antibody (mouse anti-HSP70, DAKO EnVision + system-Single Reagent, HRP. Rabbit; Life Diagnostic, Inc.) (diluted 1/500 in TBS) to each reservoir and incubating overnight at 4 °C. The next day, the slides were washed twice with TBS buffer before adding 100 μl of secondary antibody (DAKO EnVision + system-HRP labelled polymer anti-mouse, Product Ref: K4001) to each reservoir and incubating at room temperature for 30 min. Subsequently, all reservoirs were filled twice with TBS buffer, and the slides were washed many times before incubating with 100 μl of vector ImmPACT® DAB substrate (Product Ref: SK-4105) solution for 10 min, according to the manufacturer's instructions. The slides were washed multiple times with TBS before staining for 20 s with vector® hematoxylin (ref: H-3401). After the slides were air-dried, they were cover-slipped. Randomly, 120 microphotographs were taken from the tissues of each dietary group using a 5X macro-objective lens (500 magnification) connected with a digital light microscope and camera using Leica Microsystem Framework software [31]. All captured images were analysed using ImageJ software (Version 1.53). In brief, the whole area of intestinal mucosal folds was demarcated by the 'Freehand' selection tool, and then the area was measured using the 'Analyze' menu in ImageJ. Subsequently, the positively stained area for HSP70 was assessed using 'Colour Threshold' in the ImageJ, and then 'Brightness' was reduced gradually until the shaded areas were visible. Then the 'Thresholding method' was set to 'Default', and again 'Threshold colour' turned red and 'Colour space' to HSB (hue, saturation, and brightness). After that, tissue areas were selected, and the measurements of interest were taken. These values were used to calculate the ratio of the positively stained cells to the total area of mucosal folds. The calculated values were expressed as the reactivity of positive cells.

2.7. Gene expression analysis

2.7.1. RNA extraction

The expression of the immune-related genes mucin 2 (*muc2*), mucin 5ac1 (*muc5ac1*), mucin 5ac2 (*muc5ac2*), mucin 5b (*muc5b*) and the antimicrobial peptide (AMP) genes defensin1 (*def1*), defensin 2 (*def2*), defensin 3 (*def3*), cathelicidin 1 (*cath1*) were assessed in the skin, gills anterior and distal mid intestine. All primers were acquired from Eurofins Genomics (Luxembourg, Luxembourg), and detailed information about the sequences of all target and reference genes can be found in a previous publication [30]. However, a total of 60 μl RNA sample was extracted (DI, AI, skin, and gill) using the E-Z 96 Total RNA Kit (Omega Bio-Tek, USA). The whole procedure of RNA extraction was followed the manufacturer's protocol as briefly described in earlier literature [30].

2.7.2. cDNA synthesis and qPCR analysis

The extracted RNA was checked and purification confirmed using the Nanodrop test (NanoDrop One C, 58595, Thermo Fisher Scientific, USA). Then extracted RNA was quantified using the Qubit™ RNA broad-range kit (Life Technologies, Carlsbad, USA). Subsequently, cDNA was synthesized from 1000 ng of total RNA using the QuantiTect™ Reverse Transcription kit (Qiagen GmbH, Hilden, Germany), following the manufacturer's protocol. The resulting cDNA was diluted ten times with nuclease-free water and used as the qPCR template. Thermocycling was performed on a LightCycle® 96 instrument (Roche Life Science) under the following conditions: an initial enzyme activation and cDNA denaturation step at 95 °C for 1 min, followed by 45 cycles at 95 °C for 15 s, 58–61 °C for 15 s, and 72 °C for 15 s. A final standard dissociation protocol was employed to get the melting profiles. Finally, to measure the relative expression levels of target genes acquired, all data were calculated following the delta-CT method considering the *ribosomal protein S29* (*rps29*), *ribosomal protein L13 (rpl13),* and *ubiquitin (ubi)* as reference genes (Supplementary Table 1) .

2.8. Statistics and Principal Component Analysis (PCA)

R (version 4.2.2) and R Studio (2022.12.0 + 353) for Windows 10 (Version 22H2; OS Build 19045.3208) were used to perform statistical analyses of the data. All acquired data were checked for normality using the Shapiro-Wilk normality test. Kruskal Wallis test was employed for non-parametric data analysis. Dunn's multiple comparison analysis was performed to identify the significant variation among the dietary groups. Levene's test was performed to evaluate the homogeneity of variance. Nested ANOVA [$Y = \mu + diet_i + tank_{ij} + \varepsilon_{ijk}$] was employed for dietary treatment comparisons of each observed variable regarding histomorphometric indices (DI, AI, skin and gill) and their rearing tanks. Principal Component Analysis (PCA) of the histomorphometric indices revealed the histology changes-based differences in the dietary groups. The first principal component (PC1) values were extracted to correlate with the condition factor (CF), and nested ANOVA was used to identify the differences between the dietary treatments after checking the normality and homogeneity test, as suggested by a previous study [33]. Subsequently, a Pearson's correlation was applied to correlate between PC1 values and CF. Pearson's correlation analyses were carried out using the 'R' package "ggplot2". All variables in both PCAs were scaled and centered. PC1 gene values were retrieved and employed in a Pearson's correlation to correlate between gene expression and the mucosal barrier. Tukey's *post hoc* multiple comparison test assessed the significant variation among the experimental dietary groups. Statistically significant differences were expressed as: '*', $p < 0.05$; '**', $p < 0.01$; '***', $p < 0.001$; 'NS', Non-significant. A priori power analysis, using G* power 3.1.9.2 analysis [34] indicated that with the given sample size (6 fish per group), a good power (>0.85) would be obtained for effect size $f > 0.9$ [35].

3. Results

3.1. Growth parameters

Although a separate paper will present growth performance and smoltification-related parameters, some results are reported here. After the 49-day feeding trial, the final body weight, body length, and weight gain were numerically ($p > 0.05$) higher in the 50 % VMO dietary group (see Supplementary Table 2). The final weight, body length, and weight gain in the SW challenge were also higher in the 50 % VMO group ($p > 0.05$). Additionally, no differences in CF were observed among the feeding groups in both the FW and SW stages.

3.2. Histomorphometry

3.2.1. Histomorphometry of intestine

The examined histomorphological indices for distal mid intestine are presented in Table 2, Fig. 1A. The intestinal fold height (DV) appeared significantly higher ($p < 0.001$) in the 50 % VMO group compared to the other two diets. No significant differences were noted between the three groups for the width of tunica muscularis (DM), the lamina propria width (DL), and the submucosa width (DS). However, the area of goblet cells (DG) increased significantly ($p < 0.05$) in the 50 % VMO dietary group.

Following the SW challenge test, DVS and DLS appeared significantly higher in the 50 % VMO group, while DMS and DGS showed no significant differences between the other dietary groups (Table 2). The value of DSS decreased significantly in the 50 % VMO group compared to the other dietary groups.

The assessment of the different histomorphometric indices of the anterior mid intestine is presented in Table 3, Fig. 1B. The AV was significantly higher ($p < 0.01$) in the 50 % VMO dietary group compared to the other two groups. No statistical differences ($p > 0.05$) were observed between the three dietary groups for the AM, AL, or AS; however, the AG increased significantly ($p < 0.01$) in the 50 % VMO

Table 2
Mean ± SD values of distal mid intestine histomorphological indices measured in both freshwater and saltwater challenge phase. Different significance levels are indicated as: '*' $p < 0.05$; '***' $p < 0.001$; 'NS' Non Significant.

Parameters (μm)	Experimental diet				
	0 % VMO	50 % VMO	100 % VMO	p-value	Significance
Fresh water stage					
Intestinal fold height (DV)	600.44 ± 13.48[a]	720.88 ± 56.07[b]	553.11 ± 38.68[a]	0.0001	***
Tunica muscularis width (DM)	104.10 ± 4.66[a]	113.87 ± 22.49[a]	97.52 ± 11.27[a]	0.289	NS
Lamina propria width (DL)	11.03 ± 1.26[a]	12.32 ± 2.15[a]	12.23 ± 1.55[a]	0.471	NS
Submucosa width (DS)	41.33 ± 10.30[a]	42.87 ± 10.83[a]	39.33 ± 9.46[a]	0.876	NS
Area of goblet cells (DG)	9.23 ± 1.19[a]	11.65 ± 1.54[b]	9.53 ± 1.20[ab]	0.0364	*
Seawater challenge					
Intestinal fold height (DVS)	640.31 ± 27.74[a]	685.32 ± 22.23[b]	658.87 ± 27.98[ab]	0.0374	*
Tunica muscularis width (DMS)	107.85 ± 12.64[a]	124.76 ± 9.97[a]	123.10 ± 20.74[a]	0.164	NS
Lamina propria width (DLS)	11.84 ± 2.68[a]	17.00 ± 2.87[b]	15.39 ± 1.68[ab]	0.0107	*
Submucosa width (DSS)	42.67 ± 3.03[a]	38.85 ± 4.62[b]	46.03 ± 3.31[a]	0.0202	*
Area of goblet cells (DGS)	11.45 ± 1.83[a]	14.00 ± 2.65[a]	11.91 ± 2.40[a]	0.0364	NS

dietary group.

Following the SW challenge, there were no significant changes in AVS, AMS, ALS, ASS, or AGS values among the dietary groups (Table 3).

3.2.2. Histomorphometry of skin

The different histomorphometric indices for skin, such as epidermis layer thickness (EP), scale thickness (SC), stratum compactum layer thickness (ST), number of mucous cells (MC), and area of mucous cells (AM) are presented in Table 4, Fig. 1C. The value of EP and AM appeared significantly higher ($p < 0.01$) in the 50 % VMO group compared to the other two dietary groups. No significant differences were observed for SC between the dietary groups. ST and MC values increased significantly ($p < 0.001$) in the 50 % VMO group compared to the other two diets.

After the SW challenge test, EPS and AMS appeared significantly higher in the 50 % VMO group, whereas no significant difference was observed for SCS among the different dietary groups. The value of STS and MCS appeared significantly higher ($p < 0.001$) in the 50 % VMO dietary group.

3.2.3. Histomorphometry of gills

The histomorphometric indices of gills are presented in Table 5, Fig. 1D. No significant differences were observed for primary lamellae length (PL), secondary lamellae length (SL), secondary lamellae width (SW), and basal epithelium thickness (BT) between the different diets. However, the number of mucous cells in the lamellae (MC) appeared statistically different ($p < 0.05$) in the 50 % VMO dietary group compared to the other two dietary groups.

After the SW challenge test, no statistical differences were observed for PLS, SLS, and MCS among the different dietary groups. However, SWS and BTS appeared significantly higher ($p < 0.05$) in the 50 % VMO dietary group.

3.3. Mucosal mapping

3.3.1. Intestine

In the distal mid intestine, at the end of the feeding trial phase in the FW stage, the average mucous cell density varied between 5.9 and 10.8 %. The 50 % VMO dietary group exhibited the highest value compared to the other two dietary groups (Fig. 2A). However, there were no significant differences between the 0 % and 100 % VMO dietary groups. After the SW challenge, the mucous cell density range increased from 6.6 to 11.0 %, whereas the 50 % VMO group exhibited a statistically higher value ($p < 0.05$) compared to the 0 % VMO group (Fig. 2B).

The mucosal barrier status, based on the mucous cell density, ranged from 0.622 to 0.811 in the FW phase (Fig. 2C). The highest barrier status was obtained for the 50 % VMO group compared to 0 % VMO ($p < 0.01$). In comparison, no statistical difference ($p > 0.05$) was observed between the 0 % and 100 % VMO dietary groups. After the SW challenge, the barrier status values ranged from 0.625 to 1.14, while the status of the 50 % VMO group appeared statistically higher ($p < 0.01$) compared to the other two dietary groups (Fig. 2D). In contrast, no significant changes were detected between the 0 % and 100 % VMO groups.

For the anterior mid intestine, the average mucous cell density in the FW stage varied from 7.07 to 11.1 %, and the 50 % VMO dietary group exhibited the highest value (Fig. 3A). However, there was no significant difference ($p > 0.05$) between the 0 % and 100 % VMO dietary groups. After the SW challenge test, the mucous cell density ranged between 7.2 and 11.3 %, while the value of the 50 % VMO group appeared significantly higher ($p < 0.01$) compared to the other dietary groups (Fig. 3B).

The mucosal barrier status, based on the mucous cell densities, varied from around 0.690 to 0.819, while the highest barrier status ($p < 0.05$) was noticed in the 50 % VMO dietary group (Fig. 3C). After the SW challenge, the mucosal barrier status values varied from 0.655 to 0.894, with the barrier status appearing significantly higher ($p < 0.01$) in the 50 % VMO dietary group (Fig. 3D). No statistical difference was observed between the 0 % and 100 % VMO dietary groups.

3.3.2. Skin

For the skin, the average mucous cell density range in the FW stage was between 6.9 % and 9.50 %, with the 50 % VMO group exhibiting the highest value among the three dietary groups (Fig. 4A) and was significantly different ($p < 0.01$) compared to the control diet. After the SW challenge phase, the mucous cell densities ranged from between 8.7 % and 12.2, while the highest density of mucous cells was observed in the 50 % VMO group, which was significantly different ($p < 0.05$) compared to the 0 % VMO dietary group (Fig. 4B). No significant differences existed between the 0 % and 100 % VMO dietary groups.

Regarding the mucosal barrier in the FW phase, the values ranged from around 0.255 to 0.325. The 50 % VMO group appeared to have the highest mucosal barrier with a significant difference ($p < 0.05$) compared to the 0 % VMO group (Fig. 4C). After the SW challenge, the values of the mucosal barrier ranged from about 0.42 to 0.37. The value for 50 % VMO was significantly higher ($p < 0.05$) compared to the 0 % VMO dietary group (Fig. 4D).

3.3.3. Gill

In the gill, the average mucous cell density ranges varied from 2.3 % to 2.9 %, and the 50 % VMO group exhibited the highest value ($p > 0.05$) among the three dietary groups (Fig. 5A). After the SW challenge phase, the average mucous cell density values ranged from 2.6 % to 5.4 %. The highest value was observed in the 50 % VMO group, but the value didn't show a significant difference compared to the 0 % VMO group (Fig. 5B).

Regarding the mucosal barrier status in the FW phase, the values ranged from 0.388 to 0.427. The 50 % VMO group appeared to have the highest mucosal barrier value compared to the other dietary groups (Fig. 5C). After the SW challenge, the values of the mucosal barrier varied from 0.402 ± 0.068 to 0.467 ± 0.039. The 50 % VMO group exhibited the highest value among the three dietary groups. However, no statistical differences ($p > 0.05$) were observed among the dietary groups (Fig. 5D).

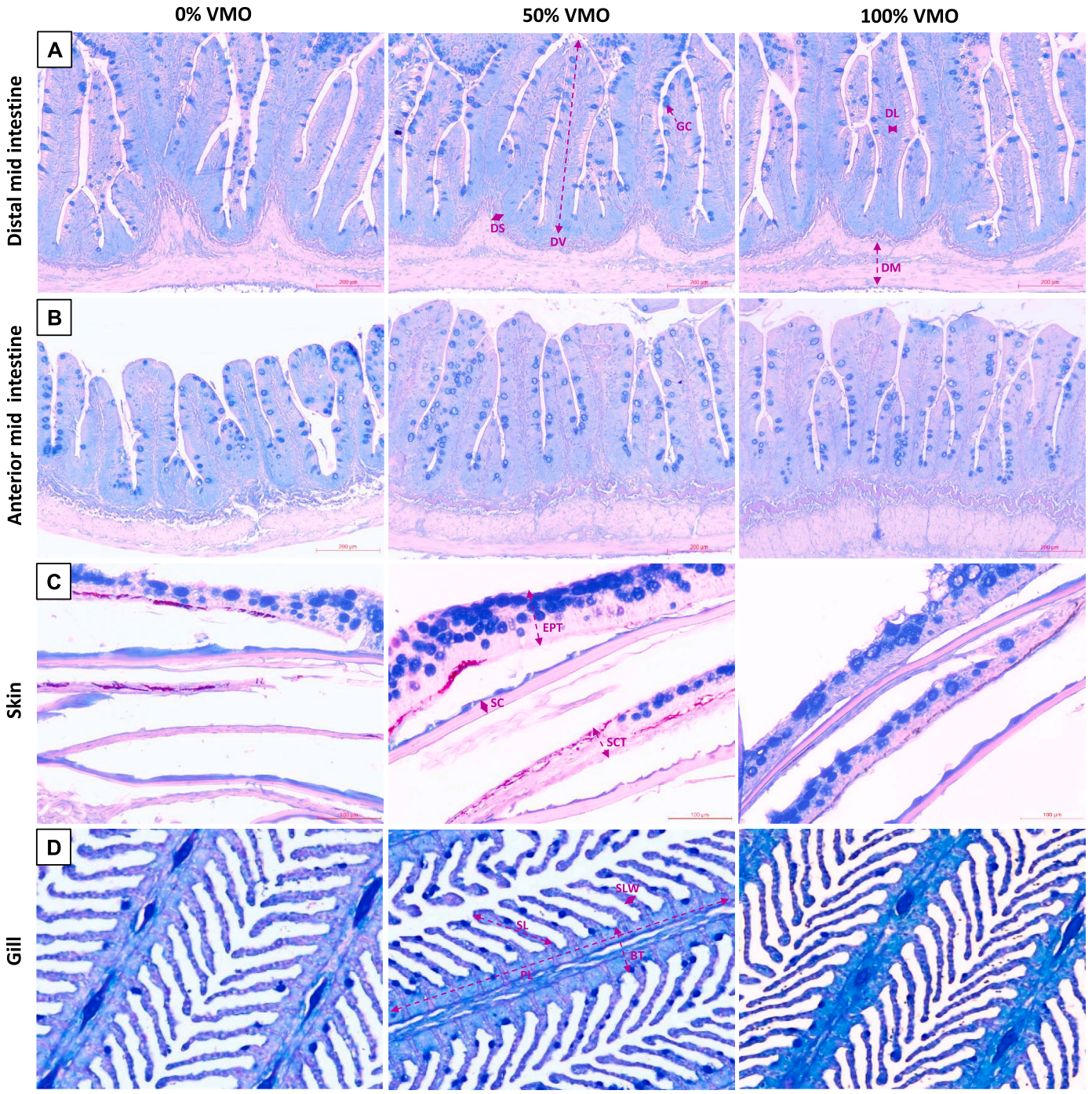

Fig. 1. Histomorphological assessment of different organs collected from Atlantic salmon fed algal oil as a replacement for fish oil in their diets, A. distal mid intestine; B. anterior mid intestine; C. skin; and D. gills. All images were captured with Leica camera fitted on a DM 3000 light microscope at 10X magnification for intestine and gill and 20X magnification for skin.

3.4. Immunohistochemistry of intestine

3.4.1. Distal mid intestine

The immunostaining of HSP70 positive cells in the distal mid intestine appeared predominantly in the cytoplasm of the epithelial area in the 50 % VMO group. Activated HSP70 positive cells were diffusely distributed in the intestinal villi (Fig. 6A). HSP70 positive cells decreased in the 50 % VMO group, though not significantly, compared to the 0 % VMO group (Fig. 6B). Following the SW challenge test, the HSP70 positive cells were distributed mainly in the epithelial area of the intestine. Activated HSP70 positive cells appeared to decrease in the 50 % VMO group, but there was no significant difference compared to the 0 % VMO group (Fig. 6C).

3.4.2. Anterior mid intestine

The number of HSP70 positive cells in the anterior mid intestine (Fig. 6D) decreased in the 50 % VMO group. Still, there was no significant difference compared to the 0 % VMO group (Fig. 6E). After the SW challenge test, the distribution of HSP70 positive cells decreased in the 50 % VMO group (Fig. 6F), with no significant difference compared to 0 % VMO group.

Table 3
Mean ± SD values of anterior mid intestine histomorphological indices measured in both FW and SW challenge phase. Different significance levels are indicated as: '**' $p < 0.01$; 'NS' Non Significant.

Parameters (μm)	Experimental diet				
	0 % VMO	50 % VMO	100 % VMO	p-value	Significance
Fresh water stage					
Intestinal fold height (AV)	457.64 ± 6.2[a]	569.04 ± 15.34[b]	538.91 ± 8.95[c]	0.001	**
Tunica muscularis width (AM)	138.06 ± 23.48[a]	142.06 ± 10.31[a]	121.02 ± 18.18[a]	0.191	NS
Lamina propria width (AL)	15.42 ± 2.63[a]	16.03 ± 2.52[a]	15.87 ± 0.95[a]	0.471	NS
Submucosa width (AS)	47.84 ± 3.56[a]	50.49 ± 4.65[a]	46.87 ± 5.80[a]	0.492	NS
Area of goblet cells (AG)	7.46 ± 0.24[a]	9.26 ± 1.22[b]	8.68 ± 0.51[b]	0.005	**
Seawater challenge					
Intestinal fold height (AVS)	517.94 ± 8.72[a]	548.77 ± 12.39[a]	525.69 ± 30.34[a]	0.088	NS
Tunica muscularis width (AMS)	137.85 ± 15.56[a]	144.00 ± 12.35[a]	126.43 ± 11.25[a]	0.174	NS
Lamina propria width (ALS)	14.01 ± 2.02[a]	15.12 ± 1.86[a]	15.35 ± 1.46[a]	0.513	NS
Submucosa (ASS)	42.82 ± 11.98[a]	44.58 ± 5.60[a]	40.48 ± 6.83[a]	0.779	NS
Area of goblet cells (AGS)	8.45 ± 1.26[a]	9.68 ± 0.95[a]	8.79 ± 1.06[a]	0.263	NS

Table 4
Mean ± SD values of skin histomorphological indices measured in both FW and SW challenge phase. Different significance levels are indicated as: '**' $p < 0.01$; '***' $p < 0.001$; 'NS' Non Significant.

Parameters (μm)	Experimental diet				
	0 % VMO	50 % VMO	100 % VMO	p-value	Significance
Fresh water stage					
Epidermis thickness (EP)	69.06 ± 4.23[a]	83.0455 ± 2.96[b]	77.46 ± 7.38[ab]	0.0017	**
Scale thickness (SC)	20.25 ± 2.62[a]	23.94 ± 3.20[a]	22.27 ± 2.50[a]	0.126	NS
Stratum compactum thickness (ST)	100.47 ± 5.40[a]	117.55 ± 5.27[b]	112.0 ± 4.50[ab]	0.0001	***
Number of mucous cell (MC)	55 ± 4.13[a]	84.83 ± 4.72[b]	65.67 ± 3.28[ab]	0.0001	***
Area of mucous cells (AM)	5.96 ± 0.14[a]	7.16 ± 1.02[b]	6.78 ± 0.21[b]	0.004	**
Seawater challenge					
Epidermis thickness (EPS)	74.448 ± 5.258[a]	86.07 ± 2.58[b]	81.40 ± 4.05[ab]	0.0011	**
Scale thickness (SCS)	25.51 ± 2.78[a]	25.60 ± 2.83[a]	21.85 ± 2.20[a]	0.0518	NS
Stratum compactum thickness (STS)	103.24 ± 3.97[a]	119.65 ± 5.31[b]	114.5 ± 3.66[ab]	0.0001	***
Number of mucous cell (MCS)	61.16 ± 3.63[a]	90 ± 4.08[b]	66.17 ± 4.44[ab]	0.0001	***
Area of mucous cells (AMS)	6.32 ± 0.22[a]	7.76 ± 1.45[b]	7.21 ± 0.13[b]	0.001	**

3.5. Gene expression

The results of mucins and AMP gene expression in both parts of the intestine (distal & anterior), skin, and the gill of Atlantic salmon are presented in Figs. 7 and 8.

Table 5
Mean ± SD values of gill histomorphological indices measured in both FW and SW challenge phase. Different significance levels are indicated as: '*' $p < 0.05$; 'NS' Non Significant.

Parameters (μm)	Experimental diet				
	0 % VMO	50 % VMO	100 % VMO	p-value	Significance
Fresh water stage					
Primary lamellae length (PL)	5024.29 ± 607.38	5376.53 ± 954.56	5142.98 ± 522.36	0.505	NS
Secondary lamellae length (SL)	149.55 ± 11.13	161.22 ± 7.61	151.82 ± 12.47	0.718	NS
Secondary lamellae width (SW)	10.29 ± 0.69	10.89 ± 0.67	10.14 ± 0.53	0.19	NS
Basal epithelium thickness (BT)	67.13 ± 9.06	70.30 ± 6.82	64.93 ± 6.15	0.12	NS
Number of mucous cells in lamellae (MC)	129.02 ± 13.91[a]	135.40 ± 18.38[a]	107.94 ± 3.51[b]	0.0108	*
Seawater challenge					
Primary lamellae length (PLS)	5178.51 ± 878.27	5858.67 ± 821.30	5364.13 ± 401.69	0.312	NS
Secondary lamellae length (SLS)	167.30 ± 11.07	175.15 ± 8.92	157.70 ± 16.87	0.11	NS
Secondary lamellae width (SWS)	11.65 ± 1.54[a]	12.64 ± 1.09[a]	10.58 ± 1.0[b]	0.0467	*
Basal epithelium thickness (BTS)	72.44 ± 8.17[a]	78.26 ± 7.37[a]	64.46 ± 5.90[b]	0.0212	*
Number of mucous cells in lamellae (MCS)	128.47 ± 9.59	142.47 ± 13.83	130.23 ± 4.65	0.0735	NS

3.5.1. Expression of mucin genes

3.5.1.1. Distal mid intestine. The diets affected the expression of *muc2* and *muc5ac1* genes in the distal mid intestine (Fig. 7A and B). The *muc2* and *muc5ac1* genes showed significantly higher relative expression in the 50 % VMO group, while the lowest expression level was observed in the 100 % VMO group. Other mucin genes, such as *muc5ac2* and *muc5b*, showed higher expression in the 50 % VMO group but were not significantly different (Supplementary Figs. 1A and B).

3.5.1.2. Anterior mid intestine. In the anterior mid intestine, the expression level of mucin genes was not significantly changed (Supplementary Figs. 2A, B, C), except for the *muc5ac1* gene (Fig. 7C), in the 50 % VMO group. The expression level of the *muc5ac1* gene in the 100 % VMO group was not significantly different from that of the 0 % VMO group.

3.5.1.3. Skin. In the skin, the two mucin genes, *muc5ac2* and *muc5B*, showed increased expression in the 50 % VMO group (Fig. 7D and E), whereas the mucin genes *muc5ac1* and *muc2* were not affected by the diets (Supplementary Figs. 3A and B). No statistical differences were observed between the 0 % VMO and the 100 % VMO diets.

3.5.1.4. Gill. In the gills, the *muc5ac1* showed significantly higher expression in the 50 % VMO group compared to the 0 % VM0 group. No

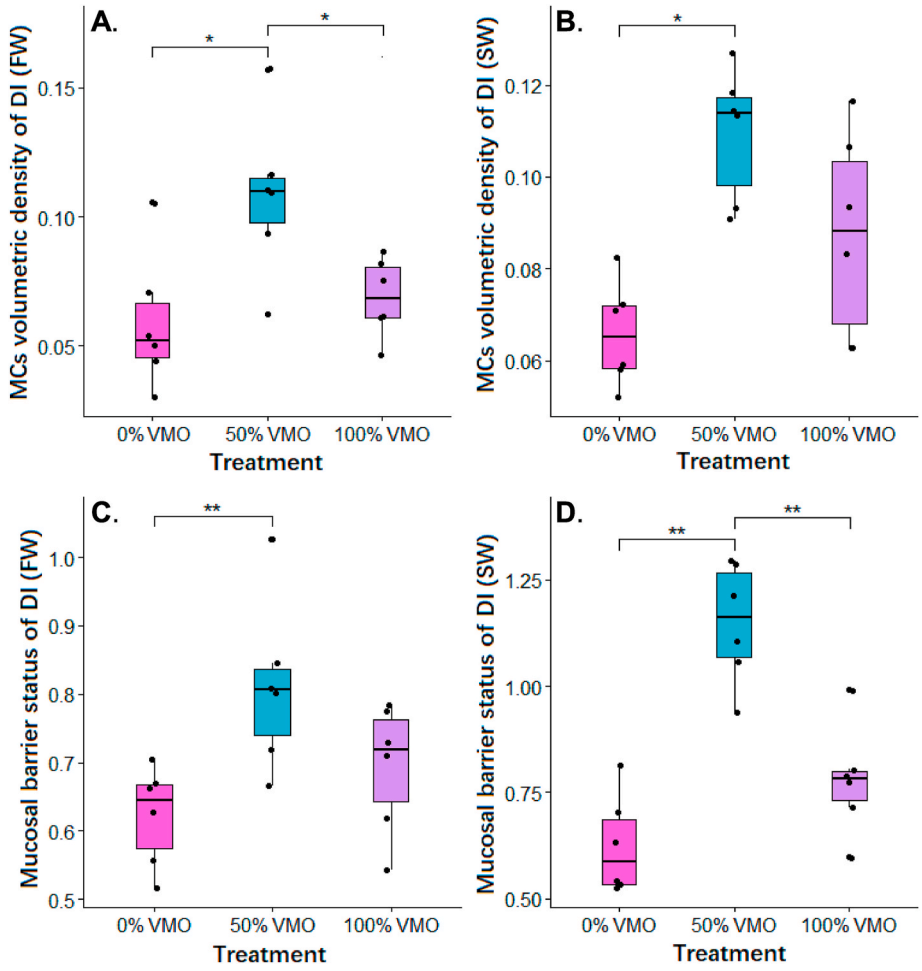

Fig. 2. Mucus cell-based analysis to assess the mucous cell density and mucosal barrier status in the distal mid intestine of different dietary groups of Atlantic salmon. A. DI: Mucous cells volumetric density in FW; B. DI: Mucous cells volumetric density in SW; C. DI: Mucosal barrier status in FW; D. DI: Mucosal barrier status in SW. Different significance levels are indicated as: '*' $p < 0.05$; '**' $p < 0.01$.

significant difference was observed compared to the 100 % VMO group (Fig. 7F). Other mucin genes *muc2*, *muc5ac2,* and *muc5b,* showed no significant differences between dietary groups (Supplementary Figs. 4A, B, C).

3.5.2. Expression of AMP genes

3.5.2.1. Distal mid intestine. In the distal mid intestine, the expression of the *def3* gene was significantly affected by the diet (Fig. 8A). The *def3* gene showed significantly higher expression in the 50 % VMO group. In contrast, for the 100 % VMO group, there was no statistical difference compared to the 0 % VMO group. Other AMP genes, such as *def1*, *def2,* and *cath1,* were expressed, but no significant differences were observed between dietary groups (Supplementary Figs. 1C, D, E).

3.5.2.2. Anterior mid intestine. Among the studied AMP genes, the expression of *def3* was up-regulated in the anterior mid intestine (Fig. 8B) in the 50 % VMO group, compared to the 0 % VMO group. Other AMP genes, such as *def1*, *def2,* and *cath1,* were expressed, though there were no statistical differences between dietary groups (Supplementary Figs. 2D, E, F).

3.5.2.3. Skin. AMP genes such as *def1*, *def2,* and *def3* were expressed, though there were no statistical differences between groups (Supplementary Figs. 3C, D, E), except for the *cath1* gene. Significantly higher expression of the *cath1* gene was observed in the 50 % VMO group (Fig. 8C) compared to the 0 % VMO group.

3.5.2.4. Gill. All AMP genes in gills, such as *def1*, *def2*, *def3,* and *cath1,* were expressed, but no significant differences were observed among the dietary groups (Supplementary Figs. 4D, E, F, G).

3.6. PCA analysis

3.6.1. Histomorphometric indices and correlation with condition factor

3.6.1.1. Intestine. The loadings of all the histomorphometric variables of the mid intestine, both distal and anterior, in the FW phase, are shown in Fig. 9A. The variance of the variables goblet cells (DG), intestinal fold height in distal (DV) and anterior (AV) segments were captured by PC1. loadings and clustered together. The variables DG and submucosa width (AS) loadings overlapped, but their variance was captured by the two Principal components, PC1 and PC2. (Supplementary Table 3). The other variables, including tunica muscularis width in distal (DM) and anterior (AM), submucosa width (DS), lamina propria width in distal (DL) and anterior (AL), and goblet cells in anterior (AG) captured by PC2 and account for only 16.4 % of the total variance of the data. The first principal component explained 29.1 % of the variance, and it was used to represent the overall health status of the intestine. The histological indices formed three overlapping clusters, indicating the variance is the same for all three diets. The least cluster was observed in the VMO 100 % dietary group, which is likely an oval shape; the cluster in the VMO 0 %

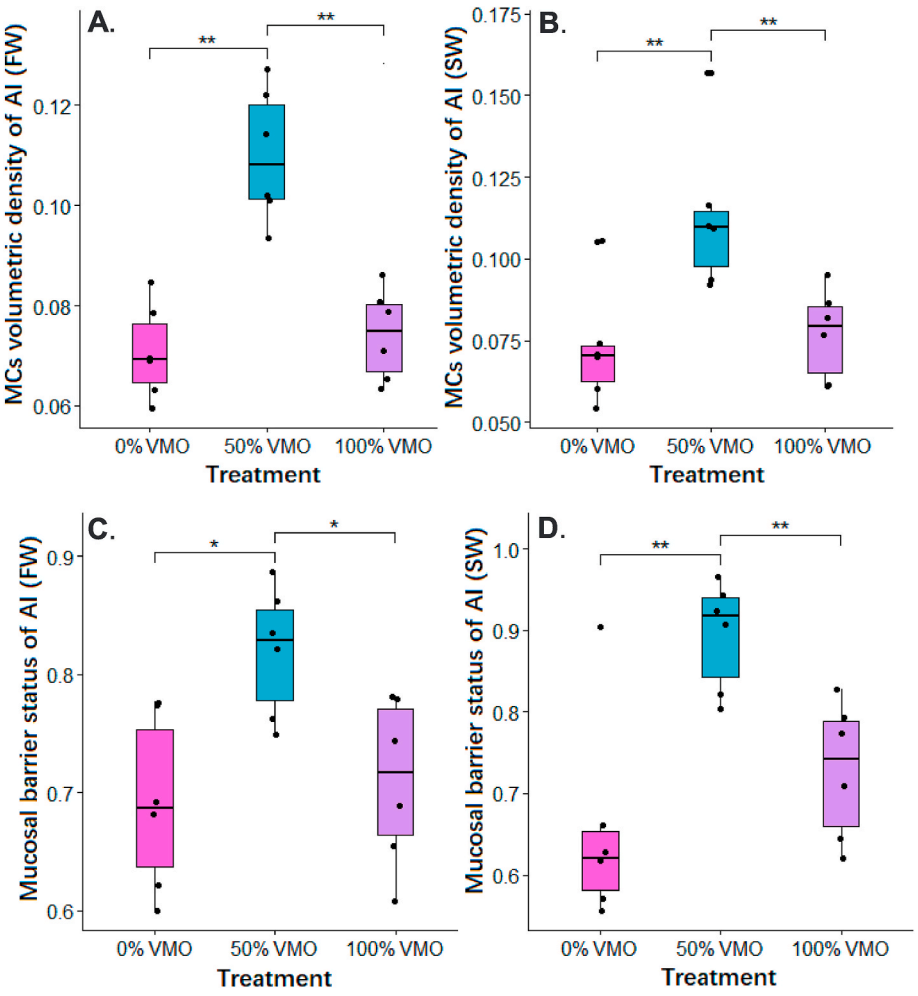

Fig. 3. Mucus cell-based analysis to assess the mucous cell density and mucosal barrier status in the anterior mid intestine of different dietary groups of Atlantic salmon. A. AI: Mucous cells volumetric density in FW; B. AI: Mucous cells volumetric density in SW; C. AI: Mucosal barrier status in FW; D. AI: Mucosal barrier status in SW. Different significance levels are indicated as: '*' $p < 0.05$; '**' $p < 0.01$.

dietary group showed comparatively bigger, and the largest cluster was observed in the VMO 50 % dietary group indicating a large variance in PC1 and PC2. Loadings of all variables were positively associated with each other in PC1. The nested ANOVA on 'PC1_intestinal health' demonstrated that diets in the treatment groups had no significant differences compared to the control group (Supplementary Fig. 5). Finally, 'PC1_intestinal health' was positively associated with CF, though not significantly (Fig. 9B).

3.6.1.2. Skin. Regarding the skin, all loadings of the histomorphometric variables are shown in Fig. 9C. PC1 and PC2 components explained 78.89 % of the variability of the data. Loadings of all variables were positively correlated on their PC1 axis, which explained 62.1 % of the variation in the data. Among all the loading variables, MC and ST explained most of the variance in PC1, and these variables showed a positive association with the other indices in PC1 (Supplementary Table 4). Other variables, including EP, AM, and SC, were explained by PC2, accounting for 16.8 % of the data variance. PCA biplot represented three different clusters of the dietary groups, while VMO 0 % and 50 % dietary groups showed more separation from each other. The skin histology indices of the VMO 50 % clustered together along the PC1 axis, while the VMO 100 % clustered together in the opposite direction along the PC1 axis. The remaining dietary group constituted an intermediate cluster in the PC1 axis. The nested ANOVA on 'PC1_skin health' demonstrated a positive association with CF, though not significantly (Fig. 9D).

3.6.1.3. Gill. All loadings of the histomorphometric variables for gill PCA are shown in Fig. 9E. The two principal components, PC1 and PC2, explained 62.6 % of the variance in the data. Loadings of all variables, such as PL, SL, SW, MC, and BT, showed positive associations along the PC1. SW appeared to be the main contributor to this loading pattern along the PC1 axis (Supplementary Table 5). PC1 axis explained 41.4 % of the variance and was used to represent the overall status of gills. The PCA biplot demonstrated three different clusters of the dietary groups VMO 50 % indices grouped in the positive zone of the PC1 axis and overlapped with the other two dietary clusters. The other two dietary clusters overlapped with each other. The nested ANOVA on 'PC1_gill health' showed no significant difference between the dietary groups. In addition, 'PC1_gill health' was not significantly positively associated with CF (Fig. 9F).

3.6.2. Mucin and AMP genes and correlation with condition factor

A PCA biplot representing all mucin and AMP genes and their loadings is shown in Fig. 10A. Two principal components, PC1 and PC2, explained more than 37.1 % of the variance in the data and provided information about the predominant association among the data. The 50 % VMO dietary group positively associated with the expressed genes. Mucin and AMP genes *muc5ac1* (DM1), *muc2* (DM2), *def1* (DD1), *def2* (DD2), *def3* (DD3) and *cath1* (DC1) in the distal intestine; *muc5ac1*

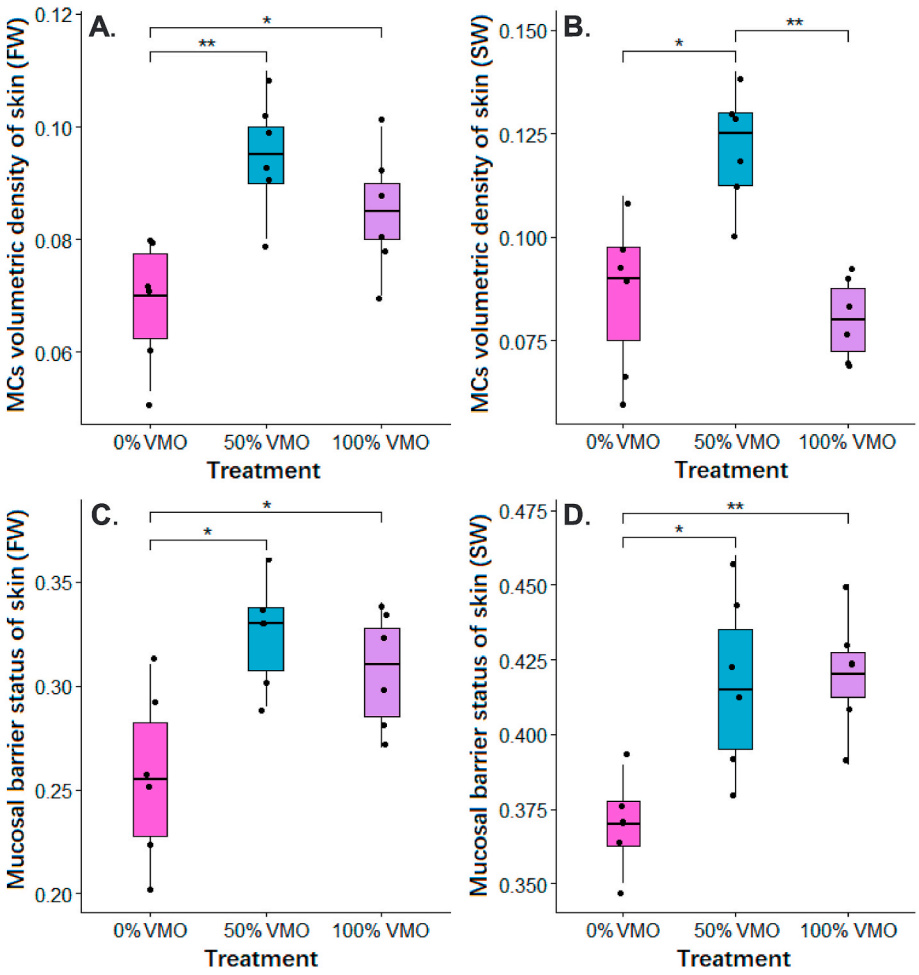

Fig. 4. Mucus cell-based analysis to assess the mucous cell density and mucosal barrier status in the skin of different dietary groups of Atlantic salmon. A. Mucous cells volumetric density in FW; B. Mucous cells volumetric density in SW; C. Mucosal barrier status in FW; D. Mucosal barrier status in SW. Different significance levels are indicated as: '*' $p < 0.05$; '**' $p < 0.01$.

(AM1), *muc2* (AM2), *def1* (AD1), *def2* (AD2), *def3* (AD3) and *cath1* (AC1) in anterior mid intestine; *muc2* (SM2), *muc5ac2* (SM3), *muc5b* (SM5), *def1* (SD1), *def2* (SD2), *def3* (SD3) and *cath1* (SC1) in skin; *muc5ac1* (GM1), *muc5ac2* (GM3), *muc5B* (GM5), *def3* (GD3) and *cath1* (GC1) genes in gill showed positively correlated expression compared to the other genes. Among all the loading genes, SM5 showed the highest loading on PC1. The arrangement of all the positive loading demonstrated 19.1 % variance in PC1, and the second principal component (PC2) explained 18.0 % of the variance in the data, which reflects an equal portion of the variance. Moreover, nested ANOVA on PC1 gene expression showed no significant difference among the dietary treatments (Supplementary Fig. 6). Finally, PC1 gene expression was positively associated with CF, though not significantly (Fig. 10B).

3.6.3. Correlation between the mucosal barrier and gene expression

3.6.3.1. Distal mid intestine. For the distal mid intestine, all loadings showed a positive correlation except DM3 (*muc5ac2*) on PC1 (Supplementary Fig. 7). PC1 explained 31.5 % of the observed variation among the expressed genes of the distal mid intestine. Loading of PC1 gene enteritis showed a significant positive association with the mucosal barrier (Fig. 11A).

3.6.3.2. Anterior mid intestine. Regarding the anterior mid intestine loading of each set variable on their PC1 (Supplementary Fig. 8) showed a positive correlation except AC1 (*cath1*). The orientation of the variables showed that the first principal component reflects a composite view of gene enteritis 32.89 % of the variation observed among the expressed genes in the anterior mid intestine. Finally, PC1 gene enteritis showed a significant positive relationship with the mucosal barrier (Fig. 11B).

3.6.3.3. Skin. For skin, loadings of all variables were positively correlated except SM1, SD1, and SD2 (*muc5ac1*, *def1*, *def2*) on their PC1 (Supplementary Fig. 9). The first principal component explained 30.5 % of the variance observed among all the expressed genes. PC1 gene enteritis showed a significant positive association with the mucosal barrier (Fig. 11C).

3.6.3.4. Gill. In gills, loadings of variables in the first principal component showed a positive correlation on their PC1 (Supplementary Fig. 10) except GD1 and GD2 (*def1*, *def2*). The first principal component explained 30.1 % of the variance observed among all the expressed genes. Finally, PC1 gene enteritis was significantly positively correlated with the mucosal barrier (Fig. 11D).

4. Discussion

In the current study, algal oil replaced 50 % and 100 % of the EPA and DHA from fish oil in the salmon diet. *Schizochytrium* oil is a good source of DHA and EPA [4,36], which can improve the overall health of fish [37]. Previous studies have shown that adding dietary omega-3 can

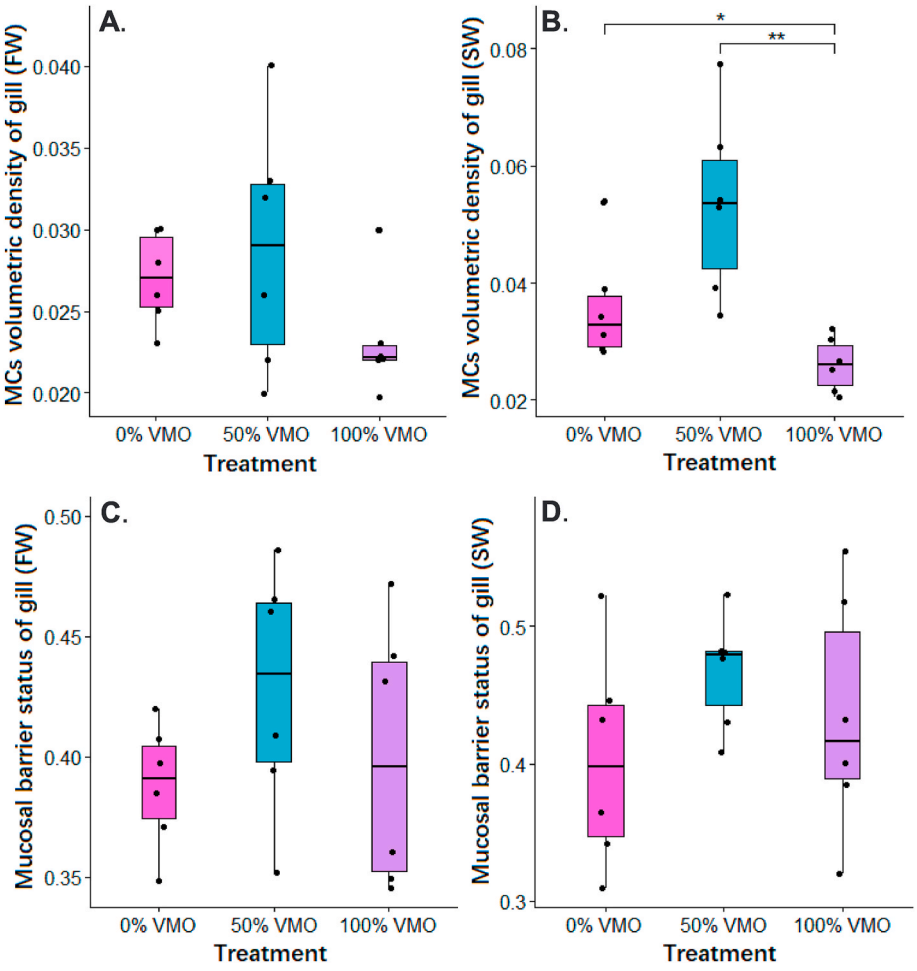

Fig. 5. Mucus cell-based analysis to assess the mucous cell density and mucosal barrier status in the gill of different dietary groups of Atlantic salmon. A. Mucous cells volumetric density in FW; B. Mucous cells volumetric density in SW; C. Mucosal barrier status in FW; D. Mucosal barrier status in SW. Different significance levels are indicated as: '*' $p < 0.05$; '**' $p < 0.01$.

improve the growth performance and internal organ health in different species, such as Atlantic salmon and Nile tilapia [7,12]. However, in this study, the condition factor of the fish fed omega-3 enriched diets was not different from that of the control diet fed to fish. It is also known that microbial oil contains various bioactive compounds, such as fatty acids, β-glucan, pigments, astaxanthin, polysaccharides, or β-carotene [6], that can modulate skin, gill and intestine structure and function. After the 49-days feeding trial, the study found that 50 % replacement of fish oil with microbial oil had significant positive effects on the mucosal barriers of the salmon. Interestingly, 100 % replacement did not have the same positive impact. Therefore, the current study investigated the effects of *Schizochytrium* oil on the microscopic structure of skin, gill and mid intestine and the expression of selected immune genes in salmon.

4.1. *Effects on intestinal mucosal barrier*

The mid intestine is responsible for nutrient absorption and acts as a protective barrier. In our study, after 49 days of *Schizochytrium* oil feeding (50 % VMO diet), the tunica muscularis, the lamina propria, and the submucosa widths increased slightly, while the intestinal fold height and the total epithelial area of goblet cells increased significantly in both intestinal parts examined. Similar changes were also observed in fish fed the 100 % VMO diet, though they were not that notable. Although little literature is available assessing the effects of algal oil on intestinal health, particularly the mucosal layer of fish, supplementation of DHA and EPA in a microalgal diet maintains the normal structure of the intestine [13]. In another study, Kousoulaki et al. observed similar findings with no adverse signs on the structure of the distal intestine [38]. Similarly, Atlantic salmon fed DHA and EPA-enriched microalgal biomass exhibited a normal structure of the mucosal fold and a number of goblet cells [39].

During the FW phase, it was observed that the distal mid intestine was more responsive to the experimental diet than the anterior mid intestine. At the end of the trial, the experimental diets enhanced the height and diameter of intestinal folds in both the distal and anterior parts of the mid intestine. Another study also reported a positive influence on intestinal fold height after administering a microalgal diet to European seabass [40]. Although the mechanism of dietary algal oil or microalgal administration on intestinal morphology has not been extensively elucidated, possible mechanisms could be the alteration of gut microbiota and their metabolites or an increase in the tight junction protein expression [41]. In the present study, supplementation of algal oil influenced the activity of HSP70 in epithelial cells in both the distal and anterior mid intestine. The activity of HSP70 was decreased in Atlantic salmon reared in freshwater and fed the 50 % VMO diet, while it was increased in the epithelial cells of fish fed the 100 % VMO diet. The activity of HSP70 increased in the epithelial cells of fish in the 100 % VMO dietary group and probably in a response to an oxidative stress in the intestine, to protect excessive amplification of the inflammatory activity [42]. Although, there is no previous knowledge of the regulation of HSP70 in any fish species after feeding *Schizochytrium* oil in diets, but microalgae-based compounds appeared to enhance HSP70 in shrimp

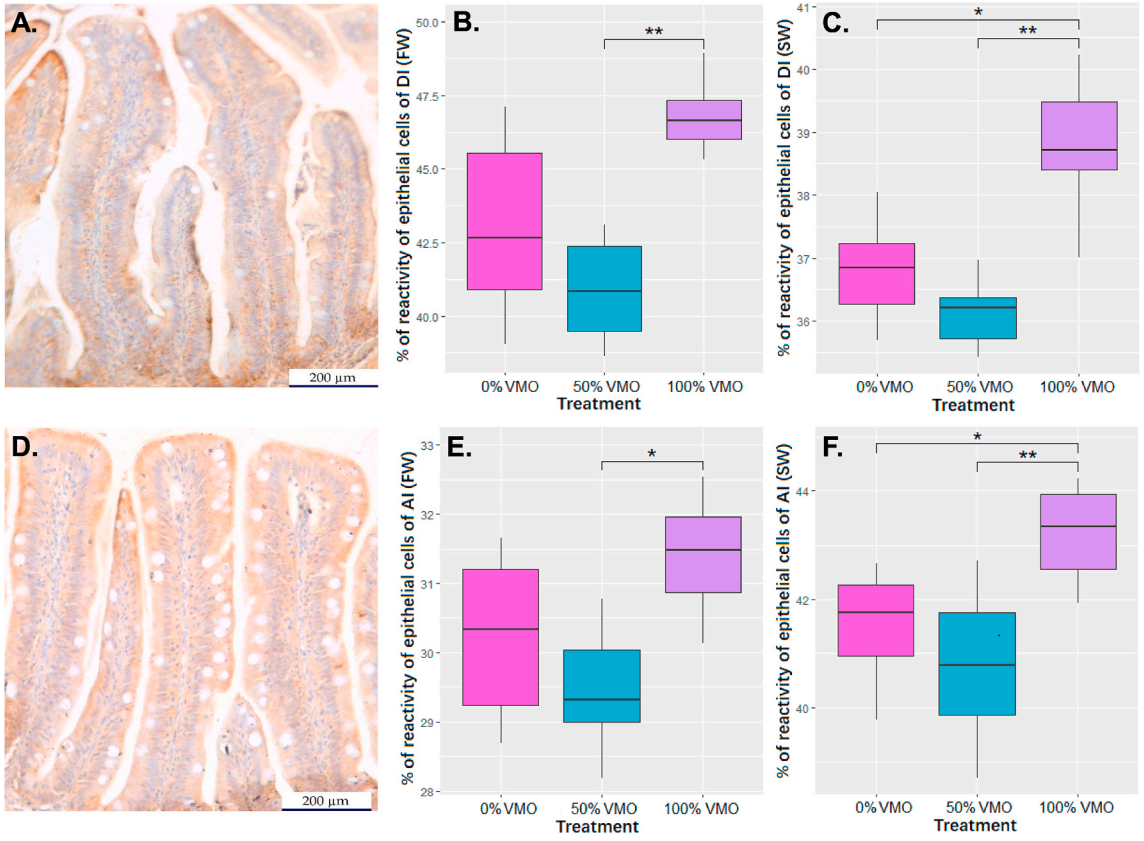

Fig. 6. Representative images illustrating the intestinal epithelial cells reactivity of heat shock protein (HSP70) (A & D) from the 50 % VMO dietary group. A. Distal mid intestine (FW); B. reactivity (%) of HSP70 in the distal mid intestinal epithelial cells (FW); C. reactivity (%) of HSP70 in the distal mid intestinal epithelial cells (SW); D. anterior mid intestine (FW); E. reactivity (%) of HSP70 in the anterior mid intestinal epithelial cells (FW); F. reactivity (%) of HSP70 in the anterior mid intestinal epithelial cells (SW). All values are expressed in % while n = 6 fish per dietary group. Different significance levels are indicated as: '*' $p < 0.05$; '**' $p < 0.01$.

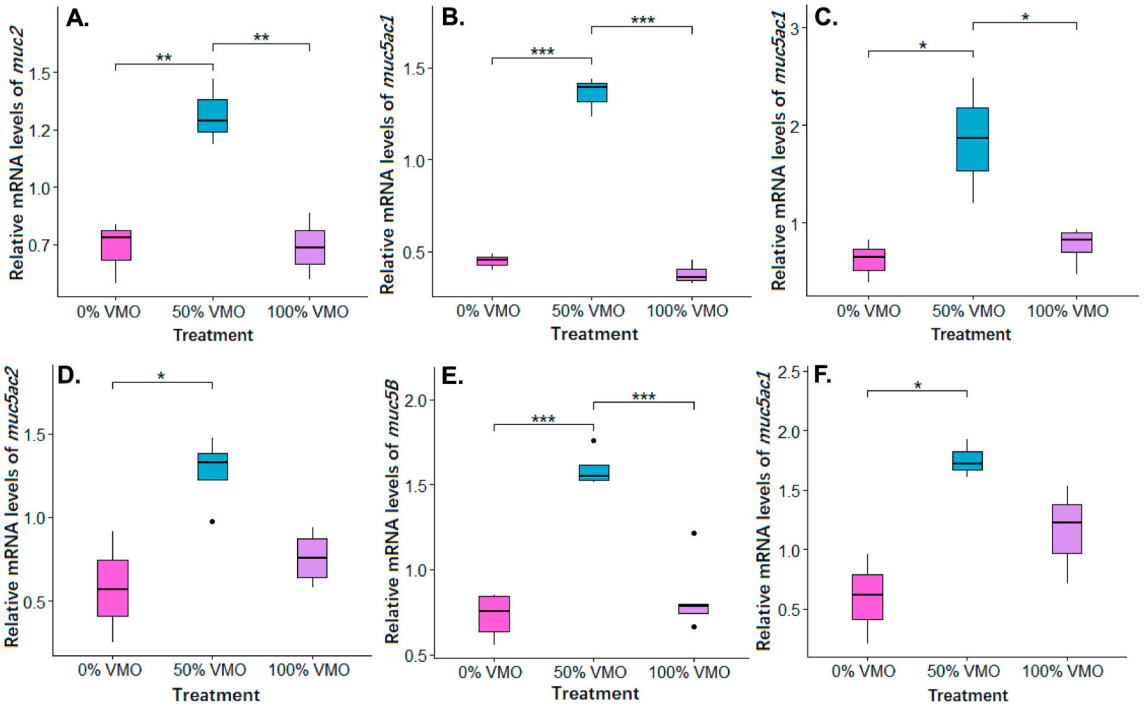

Fig. 7. Relative expression of immune-related mucin genes in the distal and anterior mid intestine, skin and gill of Atlantic salmon. A. Distal mid intestine, *muc2*; B. Distal mid intestine, *muc5ac1*; C. Anterior mid intestine, *muc5ac1*; D. Skin, *muc5ac2*; and E. *muc5b*; F. Gill, *muc5ac1*. Different significance levels are indicated as: '*' $p < 0.05$; '**' $p < 0.01$; '***' $p < 0.001$.

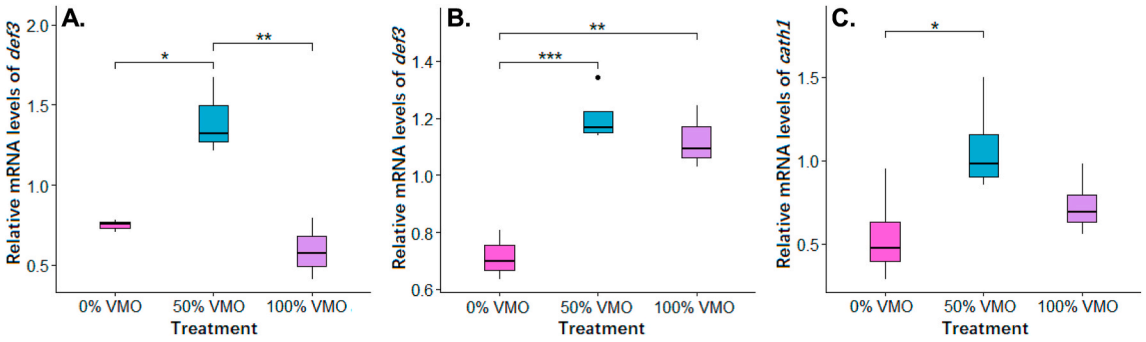

Fig. 8. Relative expression of immune-related antimicrobial peptide (AMP) genes in the distal and anterior mid intestine and in the skin of Atlantic salmon. A. Distal mid intestine, *def3*; B. Anterior mid intestine, *def3*; C. Skin, *cath1*. Different significance levels are indicated as: '*' $p < 0.05$; '**' $p < 0.01$; '***' $p < 0.001$.

[43]. After the SW challenge test, the expression of HSP70 in the epithelial cells was higher compared to the FW phase, both in the distal and anterior mid intestine, in all the dietary groups. This result indicated the activation of HSP70 in the intestinal epithelial cells after the stress response, and similar results were also observed in earlier studies on sea cucumber, *A. japonicus* [44].

The micromorphology of the mid intestine after the SW challenge test showed that the features in the two parts of the mid intestine differed. During smoltification, the anterior intestine responds better than the posterior intestine during SW adaptation. For example, the number of microvilli and absorptive perimeter increase, but the latter changes depend on the interaction between light and diet [45]. Our results align with those from other studies on Atlantic salmon, in which the anterior mid intestine responded more readily to dietary treatments. The findings also suggest that the adaption of the intestine to saltwater starts during the winter signal period. It should be noted that the intestinal folds of the anterior mid intestine are structurally developed for efficient absorption of nutrients from the digested food particles [46]. The increase in the fold height and, consequently, the absorptive surface is probably due to the increase in cell proliferation during smoltification and the SW adaptation, which happened along the entire mid intestine. These observations agree with an earlier study on wild Atlantic salmon, where increased fold height and absorptive surface were probably due to increased cell proliferation during the SW adaptation. Our results indicated that the 50 % VMO diet could alter the histomorphological indices along the entire mid intestine of salmon undergoing an SW challenge compared to the fish in FW. It has already been reported that morphological indices of the intestine (both anterior and posterior segments), including folds and wall thicknesses, increased during smoltification and adaptation in SW rather than in FW [45]. Other researchers have demonstrated substantial remodelling of the intestine during smoltification, and this change could assist salmonids during the development of salinity tolerance [47].

Mucous cells produce mucus, which helps to lubricate and protect the intestine from harmful external factors [48]. These cells also play a role in the immune system, as the mucus contains antimicrobial proteins, chemokines, cytokines, and immunoglobulins linked to adaptive immunity [18]. Our study found that the number and size of mucous cells in the mid intestine of fish increased significantly when fed the diet with the 50 % substitution by *Schizochytrium*. This suggests that the mucosal barrier was strengthened in the fish that consumed this diet. Other studies [38] have also shown that diets containing *Schizochytrium* oil can increase the number of mucous cells in the intestine of Atlantic salmon. Increased mucus production can indicate inflammation or a response against an immunostimulant [49]. In the second case, the intestinal mucosal barrier appears strengthened, providing greater protection against potential diseases [18,48].

Mucins are high-molecular-weight glycoproteins that comprise the mucus layer. Mucin genes, including *muc2*, *muc5ac1*, *muc5ac2*, and *muc5b*, are important in providing the viscoelastic nature of mucus. In our study, all mucin genes were expressed, but *muc2* and *muc5ac1* genes in the distal part of the mid intestine and *muc5ac1* gene in the anterior part of the mid intestine had significantly higher expression. Previous literature [50] suggests that *muc2* is the primary gel-forming mucin that contributes to developing the mucus barrier in the fish intestine. However, no other studies on algal oil have evaluated the mucin gene expression in the intestine of Atlantic salmon. Previous studies have shown that microalgae-based diets can affect the expression of the *muc2* gene in the distal intestine of Atlantic salmon [30]. Immunostimulants can also alter the expression of *muc2*, as observed in the intestine of carp and Atlantic salmon fed dietary glucan and probiotics, respectively [51].

The AMPs are essential defense molecules that play a significant role in disease resistance in fish. Increased AMPs production can stimulate the immune system and enhance disease resistance in fish [52]. Currently, there is no published information regarding the expression of AMP genes in fish fed microbial oil, although it has been demonstrated that AMP genes are affected by feed ingredients [30]. In our study, all AMP genes were expressed in both parts of the intestine, but the expression was not significantly affected, except for *def3*. Another study observed the upregulation of *def3* and *def4* genes in the intestine of Atlantic salmon following the administration of probiotics [53]. The upregulation of AMP genes, such as *def3*, *def4*, and *cathl*, suggests increased immune responses [54].

4.2. *Effects on skin mucosal barrier*

The skin is the largest fish organ, protecting the body from infections [55]. It is covered with mucus that contains various biologically active molecules. The Norwegian Fish Health Report has identified skin wounds as a major problem for on-growing salmonids [56]. These wounds can negatively affect the skin's functions and ultimately harm fish welfare. There is already ample evidence supporting the idea that different dietary ingredients can shape the architecture and function of fish skin [31]. For instance, omega-3 can strengthen different skin layers, potentially facilitating the healing of ulcers and preventing diseases linked to skin wounds [57]. In our trial, we found that the thickness of the epidermis and dermis, as well as the thickness of the stratum compactum, increased in fish fed with a diet containing *Schizochytrium* oil. Previous reports have already demonstrated that a diet rich in omega-3 (PUFAs) containing high DHA and EPA can affect the outermost layer, epidermis, and thickness of connective tissue of the skin in Atlantic salmon [58].

All skin histomorphological indices increased after the SW challenge test compared to the FW phase. Similar results have been found in earlier literature. In Atlantic salmon, the thickness of the epidermis and dermis increased approximately four months after transfer to SW, which coincided with growth [59]. However, further research is needed to clarify the mechanism that leads to this change in skin epidermis thickness in Atlantic salmon after SW transfer [59]. Researchers have observed that skin parameters, particularly the thickness of the skin, are increased

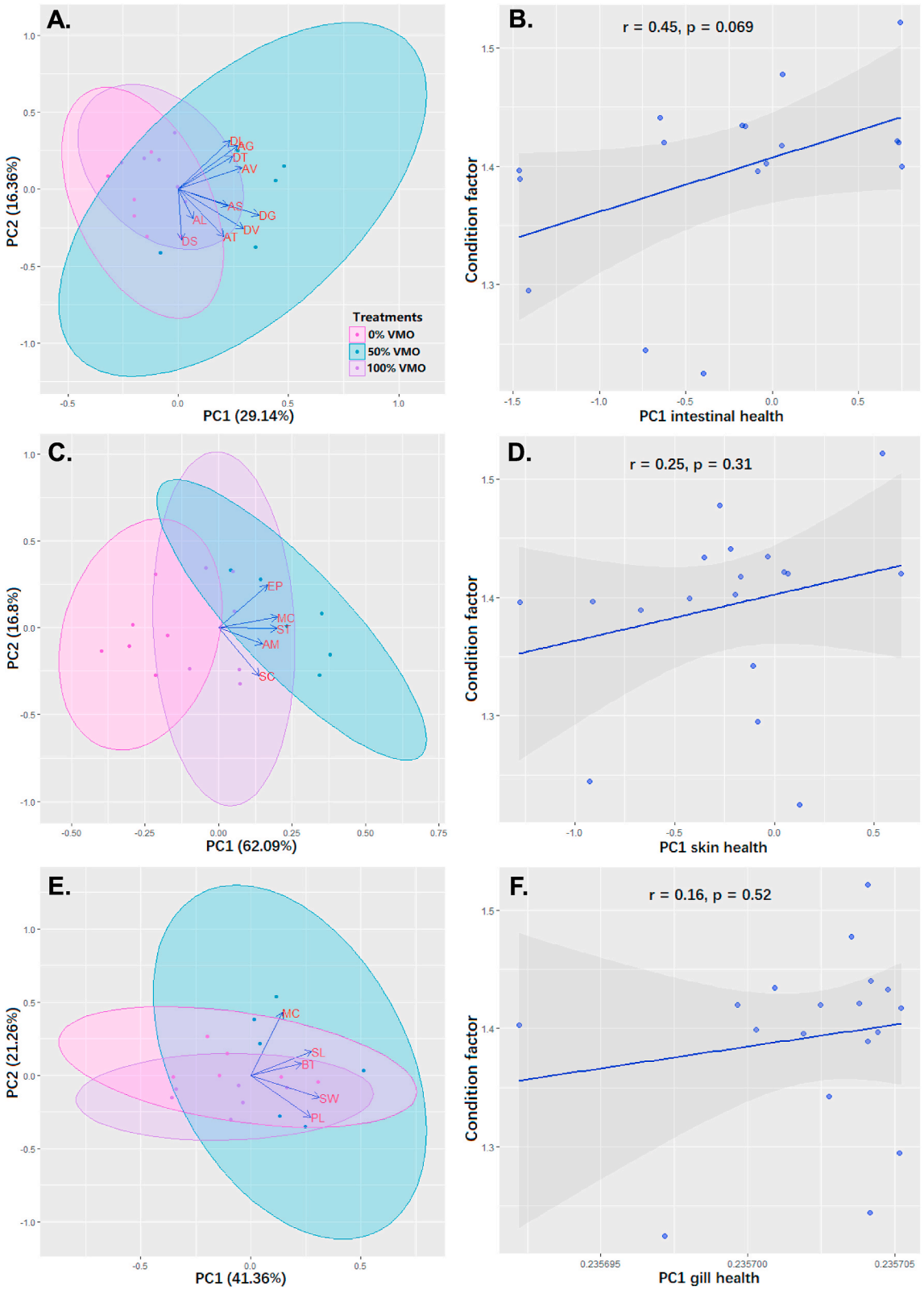

Fig. 9. PCA biplot presenting the histomorphometric data and Pearson's correlation coefficient of condition factor for the FW stage; A. Intestine (PC1_intestinal health); B. Pearson's correlation between 'PC1_intestinal health' and condition factor; C. Skin (PC1_skin health); D. Pearson's correlation between 'PC1-skin health' and condition factor; E. gill (PC1_gill health); F. Pearson's correlation between 'PC1_gill health' and condition factor.

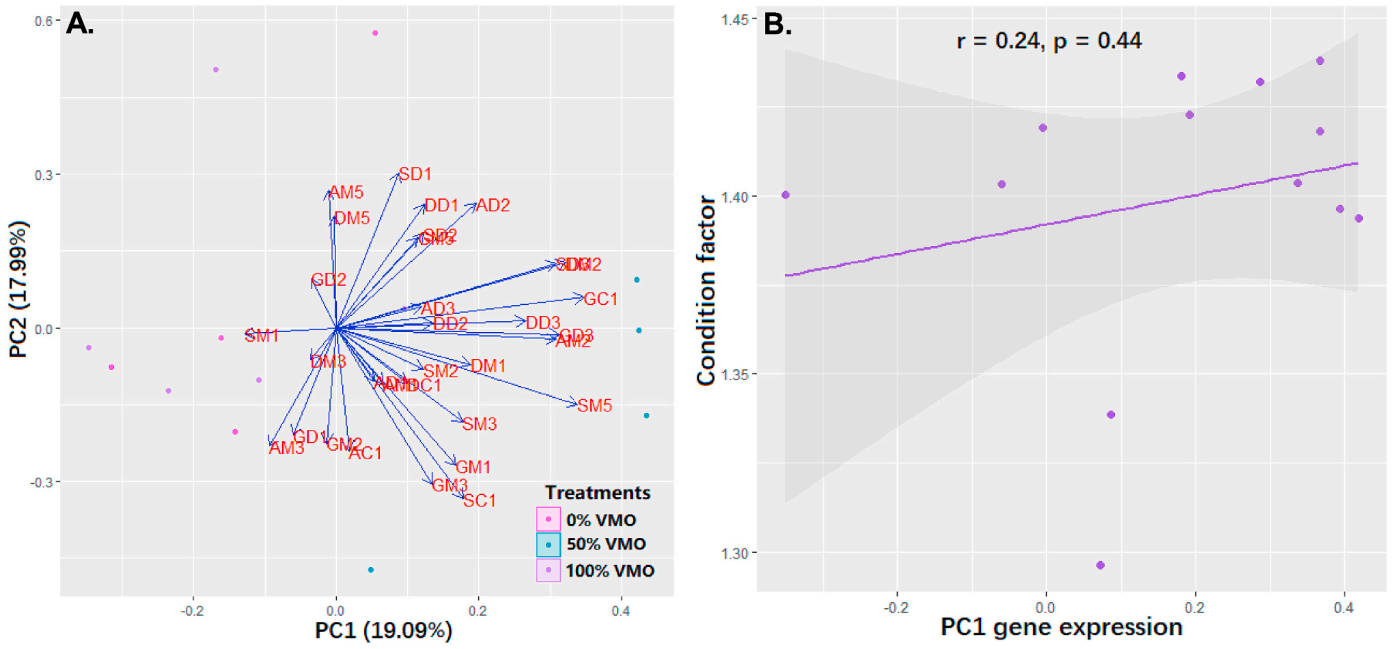

Fig. 10. A. PCA biplot presenting all the expressed mucin and AMP genes. The mucin genes are indicated as *muc2* (DM2), *muc5ac1* (DM1), *muc5ac2* (DM3), *muc5b* (DM5) in the distal mid intestine; *muc2* (AM2), *muc5ac1* (AM1), *muc5ac2* (AM3), *muc5b* (AM5) in the anterior mid intestine; *muc2* (SM2), *muc5ac1* (SM1), *muc5ac2* (SM3), *muc5b* (SM5) in the skin; *muc2* (GM2), *muc5ac1* (GM1), *muc5ac2* (GM3), *muc5b* (GM5) in the gill. All AMP genes are indicated as *def1* (DD1), *def2* (DD2), *def3* (DD3) and *cath1* (SC1) in the distal intestine; *def1* (AD1), *def2* (AD2), *def3* (AD3) and *cath1* (AC1) in the anterior mid intestine; *def1* (SD1), *def2* (SD2), *def3* (SD3) and *cath1* (SC1) in the skin and *def1* (GD1), *def2* (GD2), *def3* (GD3) and *cath1* (GC1) in the gill. B. Pearson's correlation between PC1 gene expression and condition factor.

after exposure to various acute stressors [60]. Transcription analysis indicated that gene expression changes are likely prioritized in salmon skin after SW transfer to enrich functional groups linked to skin development [59].

In the present study, skin mucous cell number and total mucous cell area increased in the *Schizochytrium* oil-fed fish. The presence of many skin mucous cells is a sign of stimulation of the skin's defence mechanisms against potential threats and during stressful situations [61]. The composition of the diet can influence the mucus composition. Marine-based diets offer an advantage due to the high levels of EPA and DHA, which can stimulate mucus production [38]. Although little information is available on the impact of diets on skin gene expression, some examples exist. For instance, the addition of β-glucans in the diet increased the expression of mucin and β-defensins in carp skin mucus [51]. The present study focused on the gel-forming mucin genes *muc2*, *muc5ac1*, *muc5ac2*, and *muc5b*, which are involved in mucosal barrier functions and innate immunity. Earlier studies have demonstrated that the expression of mucin genes can be used as markers for various diseases and disorders in humans and animals, including fish [62]. All the studied mucin genes were expressed in the skin of the fish in all groups, but only *muc5ac1* and *muc5b* genes showed significantly higher expression in the 1.2 % *Schizochytrium* oil-fed fish. An earlier study has reported higher expression of *muc5ac1* and *muc5b* in the skin of Atlantic salmon during stress conditions [63]. It is well known that diet can influence the expression of mucin genes in fish. Expression of several mucin genes, such as *muc5ac1*, *muc5ac2*, and *muc5b*, was affected, though not significantly, by dietary supplementation of probiotics (*L. fermentum* and *L. plantarum*) in the diet of Atlantic salmon [53]. Another study has also demonstrated the significant expression of the *muc5ac1* gene in Atlantic salmon following the incorporation of either marine or plant protein ingredients [30].

The AMPs are important for the immune response, and cathelicidins can directly attack bacteria [54]. In this study, four different AMP genes (*def1*, *def2*, *def3*, and *cath1*) were examined in the skin of Atlantic salmon. All these genes were expressed in the skin, but only *cath1* was significantly upregulated in the group that consumed a diet containing 50 % VMO. No information is available about dietary algal oil's effect on AMP genes. Nonetheless, a microalgae-based diet has significantly increased the expression of the immune-related gene β-defensin in seabream [64]. Previous research has also found significant expression of the *cath1* gene in Atlantic salmon [30]. Although the mechanisms by which algal oil affects AMP genes are still unknown, algae are directly associated with the innate immune system of fish and mammals [65].

4.3. *Effects on gill mucosal barrier*

A well-balanced diet strengthens the gill structure and enhances damage recovery [66]. Our research shows that algal oil can improve gill morphology and mucosal immunity. During the freshwater phase, the 50 % VMO dietary group slightly increased in different histomorphometric gill indices, such as primary and secondary lamellae length, secondary lamellae width, and basal epithelium thickness. Although there is limited information on the effects of diet on gill architecture, some studies have shown similar results. For example, Atlantic salmon showed no adverse effects on gill health after replacing FO with *Schizochytrium* sp. derived algal oil [67].

Fish are assumed to modify their gills in stressful conditions to adapt to the new physiological condition [68]. After the SW challenge test, all histomorphometric indices increased in the 50 % VMO group, while secondary lamellae width and basal epithelium thickness were significantly different compared to the control group. Interestingly, all values appeared to be higher after the SW challenge test compared to the FW phase. In agreement, morphological indices of gills increased rapidly in green sturgeon (*Acipenser medirostris*) in the SW phase compared to the FW phase. It has been demonstrated that chloride cell size, abundance, and content of Na+, K + -ATPase were increased in SW [69]. In addition, previous researchers have observed larger gill lamellae in rainbow trout after a transfer into SW [70].

As one of the mucosal barriers, fish gill is characterized by several humoral and cellular immune responses, and represents the first line defense to remove or inhibit the entrance of external threats, such as pathogens or toxins [71,72]. Following immunostimulation, increased

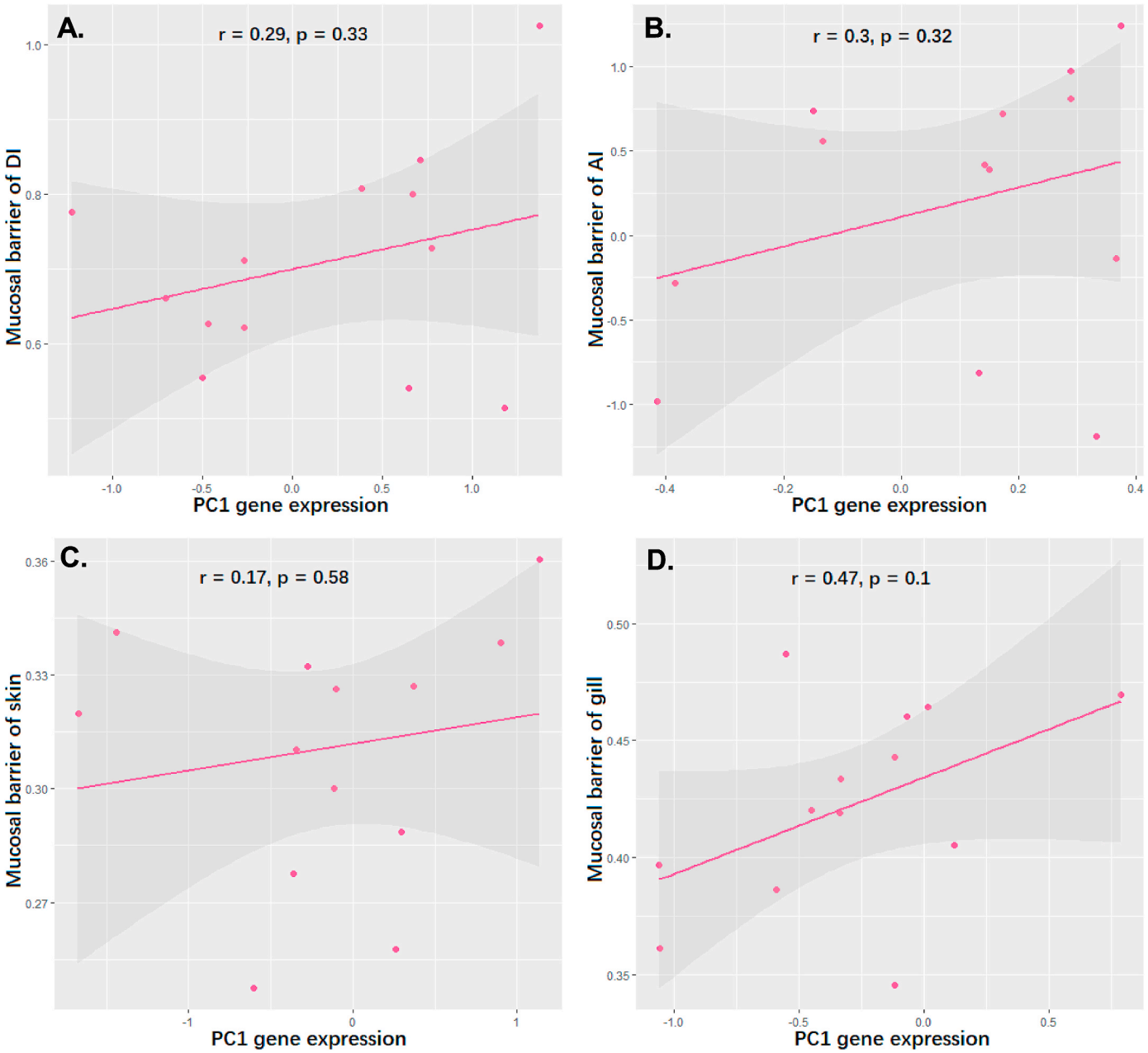

Fig. 11. Pearson's correlation between PC1 gene expression and mucosal barrier; A. distal mid intestine; B. anterior mid intestine; C. skin; D. gill.

mucus production can also occur, for example, after using probiotics [31]. This is considered a positive effect as the fish are better prepared to defend against possible threats. In our study, in the FW phase, the number of mucous cells in the gill lamellae increased significantly in the 50 % VMO group. Therefore, the mucosal barrier in the gills appeared activated, though not significantly. There is scarce information on the effect of diets on gills specifically in fish fed *Schizochytrium*-derived oil, so our results highlight the potential beneficial effect of this oil. All indices, particularly the number of mucous cells after the SW challenge test, were further increased in the 50 % VMO dietary group and the control group, but again, no statistical differences were observed between the groups. Similar results have been reported in previous studies [73], where mucous cells were increased in sockeye salmon (*Oncorhynchus nerka*) as a response to SW transfer. Interestingly, the author found an even higher number of mucous cells in the individuals who failed to adapt to SW, so the observation could also be related to the stress response. It is known that increased stress also leads to an increased number of mucous cells [74].

Several mucin and AMP genes, namely *muc2*, *muc5ac1*, *muc5ac2*, *muc5b*, *def1*, *def2*, *def3,* and *cath1,* were also used to evaluate the mucosal barrier function of the gills. All mucin and AMP genes were expressed, while only the *muc5ac2* gene appeared to be significantly affected in the 50 % VMO dietary group. Similarly, tissue-specific *expression* of mucin genes has previously been demonstrated in Atlantic salmon [30,53].

4.4. Algal oil (EPA + DHA) effects on the overall mucosal structure

The mucosal surfaces (intestine, skin and gill) of fish constitute a composite thin physical layer which provides a shelter against threats present in the intermediate environment [75,76]. These mucosal surfaces contain numerous immune cells and AMPs components responded by the various factors including diets [77,78]. For instance, microalgal oil is a prominent ingredient, rich in omega-3 PUFAs [4,28]. In the

present study, fish from the 50 % VMO dietary group appeared to have altered mucosal surfaces in the intestine, skin and gill, related to improved epithelial barrier functions. A previous study has demonstrated that algal oil significantly upregulated the concentration of tight junction protein and this protein are responsible to strengthen the intestinal barrier [79]. Additionally, EPA and DHA rich agal oil enhanced the abundance of beneficial gut microbiota connected to the production of short-chain fatty acids (SCFAs) [79,80] and these SCFAs contribute to the maintenance of intestinal mucosal barrier by promoting mucus production [81]. These SCFAs are also involved in regulating the intestinal mucin gene expression through interaction between myofibroblasts and intestinal epithelial cells [82].

Recent studies have demonstrated that fish skin mucus composition is also influenced by dietary ingredients [83,84]. Similar to intestine, SCFAs may affect skin through various pathways, as it has been shown in humans [85]. It is well known that fish skin and gut are both vascularized organs and highly innervated, and harbor numerous resident microorganisms showing similar functions such as immunity, inflammation or metabolism [86,87]. Additionally, various skin conditions are also inter-connected and altered by gut microbiome [88]. In the present study, skin mucus was significantly altered in the 50 % VMO dietary group. This skin mucus contains various amino acids (proline, histidine, lysine, threonine, glycine), metabolic components (nucleotides, nucleosides, analogues), immune cells and AMPs (defense peptides) molecules approximately 70 % AMPs are expressed [72]. The primary source of this class in skin mucus is epithelial cells either dead or dividing cells [89]. Although the actual mechanism for epithelial mucus changes due to specific diets is still unclear, various metabolic pathways were observed in other organs, such as in muscle and liver supplied nutritional components that influenced changes in skin mucus [90,91].

Similarly, gill mucosal surfaces are also influenced by diet, including the expression of the AMPs and mucins genes in Atlantic salmon [31]. In the present study, the number of mucous cells, the mucosal barrier and the mucin genes expression were affected by the 50 % VMO diet. Another study, also found an alteration of mucin composition in gilthead sea bream after feeding various dietary ingredients [92]. Although the actual mechanism is still unclear, dietary ingredients might have altered the intestinal metabolism and the metabolites including lipoprotein, amino acids, bile acids, and SCFAs might have indirectly or directly increased the mucus response, as mentioned earlier.

In the present study, partially or fully replaced fish oil by algal oil indicated the improvement of mucous cells and mucosal barrier in intestine, skin and gill; although 50 % VMO diet showed somewhat better response.

4.5. Interaction between condition factor and histomorphometry

The condition factor is a good indicator to understand the overall health and physiological condition of fish and is associated with the welfare of fish populations during different life stages [93]. In the present study, PCA was applied to assess the overall health condition of the intestine, skin, and gill, as well as mucous cell-based barrier status, in association with the condition factor. According to this, PC1 was correlated with CF to represent the overall effect of the treatments on each organ's structure. This approach has previously been used to assess the intestinal health of lumpfish [33], though not for gills and skin. The results of our study are quite interesting and showed that the health status of mucosal is positively associated with the condition factor, though the relation was not significant. Although further research is needed to examine the mechanisms and interactions between the organs, improving the structure and function of these organs contributes to improving the overall health status of fish.

5. Conclusion

Fish skin, gills, and intestine are important mucosal organs that protect the body from external threats and stressors. They form the physical barriers between the internal and external environments and act as the first line of defense against harmful agents. In our study, we used PCA analysis to observe the effects of dietary algal oil on the histological architecture of these mucosal organs. The results showed that when 50 % fish oil was replaced by algal oil, the histomorphology of the skin, gills, and intestine was improved without any observable adverse effects. Particularly in the 50 % VMO dietary group, there was an enhancement of the mucous cell production indicating better immune response and enhancement of the mucosal barriers of intestine, skin and gill. The activation of HSP70 in the intestine was also lower in the same dietary group, indicating less stress response in the intestinal epithelial cells. The expression of immune-related mucin and AMP genes varied in different organs, suggesting that different pathways are involved in each organ. However, the study found clear evidence that substituting 50 % of fish oil with algal oil resulted in increased expression in all mucosal surfaces, which may improve the health status of these mucosal barriers.

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Declaration of Competing interest

The authors have no personal relationship or financial interests that could influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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