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REVIEW

Immune and proteomic responses to the soybean meal diet in skin and intestine mucus of Atlantic salmon (*Salmo salar* L.)

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Abstract

The main objective of this study was to increase the knowledge about the mucosal immunity of *Salmo salar*, using soybean meal-induced enteritis as a model of inflammation. A control fish meal (FM) and a diet containing 20% soybean meal (SBM) were fed to salmon for seven weeks in seawater. There was no growth difference between groups. However, histology of distal intestine (DI) showed a mild inflammation in the fish fed SBM. Proteomic results revealed differences between the diets. Among the proteins detected uniquely in DI mucus of SBM group, complement C5, Galectin and Glutathione synthetase are involved in innate and adaptive immunity, inflammation, redox signalling and detoxification of xenobiotics in mammals, and similar roles are hypothesized in salmon. Adenylosuccinate synthetase and putative aminopeptidase were uniquely detected in the skin mucus of SBM group. Trypsin enzymatic activity was significantly decreased in the DI of SBM group. Significantly higher production of immunoglobulin M and Mucin-like protein in DI mucus in SBM group was observed, while an increase in immunoglobulin D and lysozyme but decrease in chymotrypsin was detected in the skin mucus of the same group. We propose mucosal immunoglobulins as diagnostic biomarkers for assessment of novel feed ingredients and aquafeeds.

KEYWORDS
ELISA, enzymatic activity, histology, proteomics, *Salmo salar*, SBMIE

1 | INTRODUCTION

Atlantic salmon (*Salmo salar* L.) production is under constant health management pressure, which is critical for further sustainable growth of the aquaculture industry (Lekang et al., 2016). Increased incidence of infectious diseases causes substantial economic losses; therefore, there is a need for better preventive measures (Gudding & Van Muiswinkel, 2013; Murray & Peeler, 2005). Fish are continually interacting with aquatic microbiota that affects mucosal surfaces of gills, skin, and intestinal mucus, which are part of the first lines of defence (Salinas, 2015). Fish are also exposed to many different stressors, including mechanical, environmental and nutritional, that together can damage fish mucosal barriers and facilitate pathogen entry into the host (Cabillon & Lazado, 2019). Nutritional stressors arise from various alternative feed ingredients, which may contain antinutritional factors, imbalanced amino acid profile, indigestible sugars and other chemicals that can cause reduced feed intake, reduced nutrient digestibility and adversely affect growth

performance and health (Francis et al., 2001). Soybean meal (SBM) is the major alternative to fish meal (FM), due to its high protein content and its favourable amino acid profile. However, the use of SBM in diets for salmon has disadvantages due to its high content of fibre and antinutritive factors, such as trypsin and protease inhibitors, lectin, antigen proteins and alkaloids, which affect digestive and nutrient absorption processes (Chikwati et al., 2013). The gut pathology known as SBM-induced enteritis (SBMIE) is a well-described inflammatory response in salmonids (Baeverfjord & Krogdahl, 1996; Krogdahl et al., 2010), but the specific antigens inducing this pathology are still unidentified and the mechanisms underlying SBMIE are not fully understood. In this regard, saponins have been pointed out as the main compound associated with gut inflammation in SBM fed fish (Knudsen et al., 2007; Krogdahl et al., 2015). However, recent studies demonstrated that not only saponins but also the combination of soyasaponins and other antinutritional factors present in other plant ingredients such as pea protein concentrate could induce enteritis (Chikwati et al., 2012; Kortner et al., 2012). Even when pea or bean protein concentrate are used alone, without SBM, it is possible to observe symptoms of intestinal enteritis such as seen in SMBIE (De Santis et al., 2015; Penn et al., 2011). There are numerous approaches that evaluated the effects of SBM in salmonids; for example, growth performance, nutrient digestibility, enzyme activity, gut transcriptome, gut microbiota and immunity, histology and histopathology (Bakke-McKellep et al., 2007; Heikkinen et al., 2006; Marjara et al., 2012; Merrifield et al., 2011; Zhou et al., 2018). Nevertheless, studies that evaluated the effect of SBM on the responses of skin and intestinal mucus on the protein level are scarce. Most of the studies evaluated the changes and the impact of SBM on the transcriptome level (Król et al., 2016), which also provides valuable evidence. Still, the translation from gene to protein is a complex process, and the conclusions based on the transcriptome level may vary enormously from the proteome data (Manzoni et al., 2016). The uncovering of functional proteomics changes of intestinal and skin mucus in FM and SBM fed fish will add significant insight into the understanding of the mechanisms of SBMIE. The technique also provides opportunities to search for biomarkers to be used as preventive methods against diseases in the aquaculture industry.

The main objective of this study was to increase the knowledge about the mucosal immunity of *Salmo salar* using SBMIE as a model of inflammation. The gained knowledge and standardized phenotypical tools will help us to assess the impact of other novel feed ingredients that are constantly emerging on the market and to reveal the mechanism of dietary immunomodulation.

2 | MATERIALS AND METHODS

2.1 | Animals, feeding and sampling

The experiment was carried out with post-smolts Atlantic salmon (*Salmo salar* L), provided by AquaGen As (Trondheim, Norway). Before the experiment, fish were kept at the fish laboratory at the Norwegian University of Life Sciences, Ås, Norway. When fish showed the morphological signs of smoltification (silvery colour, less visible parr marks, loose scales, blackfin margins of dorsal, caudal and pectoral fins, and almost transparent fins colour), they were transferred to the seawater (SW) facility at the Norwegian Institute for Water Research (NIVA). Further, fish were randomly distributed into 6 tanks (22 fish per tank), each one with 250 L capacity and a water flow of 8–10 L minutes $^{-1}$, resulting in three tanks per diet. The experimental fish had an average body weight of 107 g (n = 132), on the transfer day and a final average body weight of 158 g (n = 132) at the end of the experiment. The water salinity was gradually increased from 5 ppt at the transfer until full salinity (35 ppt) within three weeks. Continuous 24 hours light was provided during the experimental period. The average water temperature during the experiment was 11.5 ˚C, and the oxygen saturation was between 85 and 97%. The two experimental diets fed to fish for seven weeks were a control diet based on high-quality fishmeal (FM) and a challenge diet containing 20% soybean meal (SBM) (Table 1).

Automatic belt feeders distributed feed 3 hours per day with a feeding level of 2% of the body weight, adjusted by the average feed consumption in each tank over the last seven days with 10% excess per day. The diets were produced by extrusion and subsequent vacuum coating with fish oil at the Centre for Feed Technology, Norwegian University of Life Sciences, Ås, Norway. The uneaten feed was collected during the whole experiment. The recovery values for each feed (pellets) were measured to ensure correct calculations of uneaten /eaten feed (dry matter (DM), g), according to Helland et al. (1996)). After seven weeks in SW, five fish per tank (15 fish per diet) were randomly taken out, anaesthetized (80 mg L^{-1} tricaine methanesulfonate (MS 222)) and weighed individually. The skin mucus was gently scraped using a cell scraper (VWR) along the lateral line, collected into 2 ml cryotubes, and immediately frozen for further protein extraction. Thereafter, the distal intestine (DI) was eviscerated by sterilized scissors and divided into two pieces. From the first DI piece, the tissue was cut transversally, intestinal content was removed, then rinsed with phosphate-buffered saline (PBS) and intestinal mucus was gently scraped by using a sterile cell scraper, collected into 2-ml cryotubes and immediately frozen for further protein extraction. The other DI piece was placed in 10% formalin for 48 hours at room temperature, and subsequently transferred to 70% alcohol and stored at 4°C until further histology processing. The total weight of fish biomass was recorded at the transfer day and on the sampling day. The experiment was performed according to the guidelines established by the Norwegian Animal Research Authority. All animals were treated according to laws and regulations for experiments on live animals in EU (Directive 2010/637EU) and Norway (FOR-2015-06-18-761).

2.2 | Diets and ingredients

The diets and ingredients were ground and analysed for DM, crude protein (CP), crude lipid (CL) and ash (Table 1). Dry matter was

TABLE 1 Diet composition

Ingredient	FM	SBM
Formulation %		
Fish meal[a]	30	15
Soybean meal[b]	0	20
Corn gluten[c]	8	1
Wheat flour[d]	12.47	11
Wheat gluten[e]	8.75	21.62
Sunflower meal[f]	5	0
Soy protein concentrate[g]	16.39	10.05
Fish oil[h]	17.42	18.2
Threonine[i]	0.29	0.25
Lysine[j]	0.25	0.8
Rhodimet[k]	0.1	0.28
Choline chloride[l]	0.15	0.15
MCP[m]	0.39	0.86
Carophyll pink[n]	0.05	0.05
Yttrium oxide[o]	0.01	0.01
Vitamin C[p]	0.1	0.1
Vitamins/minerals premix[q]	0.63	0.63
Total Sum	100	100
Analysed content, g kg^{-1}		
Dry matter	921	916
Crude protein	429	456
Crude lipid	173	156
Ash	79	57

[a]LT fishmeal, Norsildmel, Egersund, Norway.
[b]Soybean meal, Denofa, Fredrikstad, Norway.
[c]Corn gluten, Heinz & Co. AG, Zurich, Switzerland.
[d]Wheat flour, Norgesmøllene AS, Norway.
[e]Wheat gluten, Amilina AB, Panevezys, Lithuania.
[f]Sunflower meal, Norgesfør, Norway.
[g]SPC, Lyckeby Culinar, Fjälkinge, Sweden.
[h]NorSalmOil, Norsildmel, Egersund, Norway.
[i]L-Threonine, CJ Biotech CO., Shenyang, China.
[j]L-Lysine CJ Biotech CO., Shenyang, China.
[k]Rhodimet NP99, Adisseo ASA, Antony, France.
[l]Choline chloride, 70% Vegetable, Indukern s.a., Spain.
[m]Monocalsium phosphate, Bolifor® MCP-F, Oslo, Norway.
[n]Carophyl pink. BASF, Germany.
[o]Yttrium, Metal Rare Earth Limited, Shenzhen, China.
[p]Vitamin C, BASF, Germany.
[q]Premix fish, Norsk Mineralnæring AS, Hønefoss, Norway. Per kg feed; Retinol 3150.0 IU, Cholecalciferol 1890.0 IU, α-tocopherol SD 250 mg, Menadione 12.6 mg, Thiamin 18.9 mg, Riboflavin 31.5 mg, d-Ca-Pantothenate 37.8 mg, Niacin 94.5 mg, Biotin 0.315 mg, Cyanocobalamin 0.025 mg, Folic acid 6.3 mg, Pyridoxine 37.8 mg, Ascorbate monophosphate 157.5 g, Cu: CuSulphate $5H_2O$ 6.3 mg, Zn: ZnSulphate 151.2 mg, Mn: Mn(Heikkinen et al.)Sulphate 18.9 mg, I: K-Iodide 3.78 mg, Ca 1.4 g.[d] Lygel F 60, Lyckeby Culinar, Fjälkinge, Sweden.

analysed by drying to constant weight at 104°C (Commission dir. 71/393/EEC), CP was evaluated using Kjeldahl nitrogen (Commission dir. 93/28/EEC) ×6.25), while CL was evaluated by HCl hydrolysis followed by diethyl ether extraction (Commission dir. 98/64/EC) and ash (Commission dir. 71/250/EEC).

2.3 | Histology

A histological assessment of DI sections was conducted at the histology laboratory, Veterinary Institute, Oslo. DI tissues sections (n = 30; 15 per diet) were routinely dehydrated in ethanol, equilibrated in xylene and embedded in paraffin. Longitudinal cuts of approximately 5 μm were stained with haematoxylin and eosin and examined under a light microscope. Tissue morphology evaluation was done by using a semi-quantitative scoring system as described by Penn et al. (2011). Selected tissue parameters and criteria for scoring were as following: 1) shortening of mucosal fold length, 2) increase in width and cellularity of the submucosa, 3) increase in width and cellularity of the lamina propria, and 4) reduction in enterocyte supranuclear vacuolization. The degree of histo-morphological changes was assessed and assigned to one of five categories, including scores ranging from 0 to 4, where 0 represented normal histology, 1—mild changes, 2—moderate changes, 3—marked changes and 4—severe changes. We had one intestinal section per fish, but the scoring criteria were quantified in the whole section area. The assignment of individual samples to the test diet groups was obtained after the evaluation was completed.

2.4 | Proteomics

Twenty-four randomly selected mucus samples (skin mucus (n = 12); DI mucus (n = 12)) from both dietary groups were thawed on ice and homogenized using beads and ice-cold lysis buffer (Tris 20 mM, NaCl 100 mM, Triton X-100 0.05%, EDTA 5 mM and protease inhibitor cocktail 1×). Then, the homogenate was centrifuged at 12000 ***g*** for 25 minutes at 4°C. The supernatant, containing soluble proteins, was then transferred to new tubes on ice. All protein samples were quantified by a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) following the manufacturer's instructions. For proteomic analysis, 20 μg of total protein in PBS were pH adjusted to 8 by adding ammonium bicarbonate (Sigma-Aldrich, Darmstadt, Germany). The samples were then digested with 10 μg trypsin (Promega, sequencing grade) overnight at 37°C. The tryptic peptides were analysed using an Ultimate 3000 RSLCnano-UHPLC system connected to a Q Exactive liquid chromatography-mass spectrometer (LC-MS/MS) (Thermo Fisher Scientific). LC-MS/MS was run at the Proteomics Core Facility (PCF) at Oslo University. The acquired raw data were analysed using MaxQuant version 1.4.1.2. and Perseus

version 1.6.0.7 based on MS1 intensity quantification. Proteins were quantified using the MaxLFQ algorithm. The data were searched against the salmon proteome (82390 sequences). Peptide identifications were filtered to achieve a protein false discovery rate (FDR) of 1% using the target-decoy strategy. The analysis was restricted to proteins reproducibly identified in at least three of the six replicates per diet. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2019) partner repository with the dataset identifier PXD017744.

2.5 | Detection of immunological markers by ELISA

Samples of skin and DI mucus (6 fish per diet) were resuspended in 200 µl of cold PBS. Total proteins of each sample were quantified using the BCA Protein Assay Kit (Pierce) following the manufacturer's instructions. Thereafter, the detection of immunoglobulin D (IgD), immunoglobulin M (IgM), lysozyme and Mucin-like (Muc-like) was performed by indirect ELISA (Morales-Lange et al., 2018). Briefly, each sample was diluted in carbonate buffer (60 mM $NaHCO_3$ pH 9.6) and seeded (by duplicate) in a 96-well plate (Nunc) at 50 ng μl^{-1} (100 µl) for overnight incubation at 4°C. Next, 200 µl of blocking solution (Bio-Rad) was incubated per well for 2 hours at 37°C and later the plates were incubated for 90 minutes at 37°C with 100 µl of the first antibody and for 60 minutes at 37°C with a secondary antibody (100 µl) diluted 1:5000 (goat anti-mouse IgG-HRP or mouse anti-rabbit IgG-HRP). Finally, chromagen substrate 3,3′,5,5′-tetramethylbenzidine single solution (TMB, Thermo Fisher) was added (100 µl) and incubated for 15 minutes at room temperature. The reaction was stopped with 50 µl of 1 N sulphuric acid and read at 450 nm on a SpectraMax microplate reader (Molecular Devices). The list of the antibodies used for ELISA detection is presented in Table 2.

Besides, the detection of trypsin and chymotrypsin was performed using the Fish Trypsin (TRY) ELISA kit (Cat no. MBS017140, MyBioSource) and Fish Chymotrypsin ELISA kit (MBS779023, MyBioSource), both according to the supplier instructions.

2.6 | Trypsin activity

Preparation of samples (6 fish per diet) and trypsin activity measurements in skin and intestine mucus samples were done by Trypsin Activity Assay kit (Colorimetric), following the manufacturer's instructions (Cat no. ab102531, Abcam).

2.7 | Statistical analysis

For group analysis of the growth, histo-morphological, immunological parameters and trypsin activity, statistical software GraphPad v7.03 was used. Shapiro–Wilk test was used to determine the normal distribution of the ELISA and enzyme activity data. From these results, significant differences between FM and SBM groups were established by Student's t-test (two-tailed). In addition, protein raw data were transferred to log normalization. Then, Volcano plot analysis, multivariate statistical analysis and data modelling were performed in R (https://www.r-project.org/). The results showed a Poisson distribution, and the common proteins detected in both diets are presented in the form of a heat map, with levels of protein expression across two dietary groups (FM and SBM). Hierarchical clustering was performed with the hclust function in R package. UniprotKB database was used for the functional annotation of the proteins. All differences were considered significant when the *p*-value was <.05.

3 | RESULTS

3.1 | Growth performance

The experiment was successful in terms of SW adaptation, as indicated by the animals' quick return to normal feeding routines after the transfer to the SW. Table 3 shows the growth parameters. No statistical differences were detected in any of the measured parameters between FM and SBM dietary treatments.

3.2 | Histology

Histological examination was performed on the DI samples taken at the end of the trial (49 days in SW). The main finding from the gut health assessment of the histology sections was a mild inflammation in the DI mucosa of the fish fed SBM (Figure 1). The inflammation observed correlates with the well-documented SBMIE known to occur in salmonids fed diets containing standard-processed (hexane-extracted) SBM. Four morphological tissue parameters were reported (Figure 1B) (mucosal fold height, submucosa width and cellularity, lamina propria width and cellularity and supranuclear vacuolization) to be different between diets. All fish fed FM presented normal DI histo-morphology, while fish fed SBM presented altered histo-morphological parameters with mild to moderate inflammation level (Figure 1B).

TABLE 2 Primary antibodies for indirect ELISA

Marker	Source	Type	Dilution	References
IgD	Mouse	Monoclonal	1:400	Ramirez-Gomez et al. (2012)
IgM	Mouse	Monoclonal	1:400	Cat. No FM–190AZ–5, Ango
Lysozyme	Rabbit	Polyclonal	1:800	Figure S1
Muc-like proteins	Mouse	Polyclonal	1:400	Figure S2

TABLE 3 Growth rate, feed intake (g/dry matter (DM)), feed conversion ratio, in Atlantic salmon fed fish meal (FM) and diet containing 20% soybean meal (SBM) diet for 49 days in seawater. Data are presented per dietary group (3 tanks per group) as means ± standard error mean

Growth parameters	FM	SBM	*p*-value
Initial weight (g)	2355 ± 11	2356 ± 2	.935
Final weight (g)	3539 ± 197	3437 ± 314	.797
Weight gain (g)	1183 ± 208	1081 ± 316	.799
Feed intake (DM, g)	1006 ± 125	960 ± 157	.829
Feed conversion ratio	0.87 ± 0.06	1.02 ± 0.22	.561
Specific growth rate rateraterate	0.82 ± 0.13	0.75 ± 0.20	.774

3.3 | Proteomics

We performed proteomic analysis on mucosal samples isolated from DI and skin after seven weeks in SW. In total, 723 and 1232 protein were detected in the mucosal samples from DI and skin, respectively (Data S1). From the detected proteins, 292 proteins were common in both groups in the DI mucus, while 1075 were detected in both groups in skin mucus. Venn diagrams show the number of common and unique proteins altered by FM and SBM diet (Figure 2a). We detected one unique protein in DI mucus in the FM group and nine unique proteins in the SBM group. In the skin mucus, we detected two unique proteins in each diet. The detected unique proteins are presented in Table 4.

To study the relative expression of the common proteins detected in both diets, we generated volcano plots comparing SBM to the FM diet. We observed one significant protein in the skin mucus of fish fed SBM diet (Figure 2c to the left), compared with the 30 significantly detected proteins from the DI mucus (Figure 2c to the right). The pattern of expression of the 30 significant proteins is shown as a heat map in Figure 2b. Among those, 11 proteins were significantly higher in DI mucus of the SBM group compared with 19 proteins in FM group (Data S1). Most of the proteins overexpressed in DI mucus in SBM group presented a catalytic/hydrolase activity involved in the metabolic and cellular process with more than a half that presented an extracellular region. A similar trend was observed in the DI mucus of the FM group, where most of the proteins presented a catalytic activity, involved in the metabolic process. Interestingly, we detected several peptides belonging to cathepsin proteins in the FM group (protein's id in red, Figure 2b).

3.4 | Immunological markers

The results of ELISA in skin mucus samples (Figure 3) showed that the production of IgD and lysozyme were higher ($p < .05$) in fish fed the SBM diet compared with fish fed the FM diet. The same trend was observed for IgM and Muc-like proteins on DI mucus samples (SBM group). On the other hand, chymotrypsin in skin mucus samples (SBM group) showed a significant decrease in its production compared with the FM diet. No significant differences in the production of trypsin were observed between the diets.

3.5 | Trypsin activity

In DI mucus, the enzymatic activity of trypsin (Figure 4) showed a significant decrease in the SBM group compared with the FM diet. In skin mucus, the activity of trypsin did not show significant differences between dietary groups.

4 | DISCUSSION

In the aquaculture industry, different factors such as the optimization of environmental parameters and dietary regimens have a stronger impact on the mucosal surfaces of farmed fish compared with their terrestrial agricultural counterparts (Beck & Peatman, 2015). Therefore, mucosal immunity has been proposed as a key component for maintaining optimal fish health during its productive stage (Lazado & Caipang, 2014). The mucosal surface is the physical interface between fish and its environment, acting against external aggressions, such as microbes and stressors, in coordination with the immune system (Salinas et al., 2011).

The main goal of the present study was to detect proteomic and phenotypic features of mucosal immunity in the mucus of skin and DI when SBMIE model was triggered. In line with other studies, in this experiment, SBM induced mild enteritis on DI with major changes observed in the degree of loss of supranuclear vacuolization. The degree of severity of the morphological changes and the effect of SBM on growth parameters depend on its inclusion levels in the diet formulation. Contrary to the results obtained by Marjara et al. (2012) where they used the same inclusion level of SBM as in the present study, more severity in SBMIE was observed after shorter feeding time in SW (21 days) compared with 49 days in our study. Study by Jacobsen et al. (2018) did not detect any sign of enteritis in Atlantic salmon fed 20% SBM inclusion for 93 days. The difference in the results could be due to the differences in fish genetic background, fish size, feeding intake, the level of adaptation to SW, the origin of SBM and the level of saponin's content in the diet (Jacobsen et al., 2018; Urán et al., 2009). In the study performed by Król et al. (2016), diet with 36% SBM inclusion was fed for eight weeks in freshwater (FW) and yet, they detected a moderate level of SBMIE. The responses to novel ingredients can be intensified in SW compared with FW. Thus, nutritional precaution is needed during this sensitive life stage of salmonids. In accordance with other studies (Romarheim et al., 2013), the inclusion of 20% SBM in diet did not affect growth performance, even though SBMIE was present.

Additionally, our proteomic results revealed differences between the diets, showing proteins detected exclusively in either FM or SBM group in DI and skin mucus. Among the proteins detected uniquely in DI mucus of fish fed SBM, we found complement C5, which is a crucial feature of the defensive complement in all vertebrates and has been recently characterized in Atlantic salmon (Johansen et al., 2019).

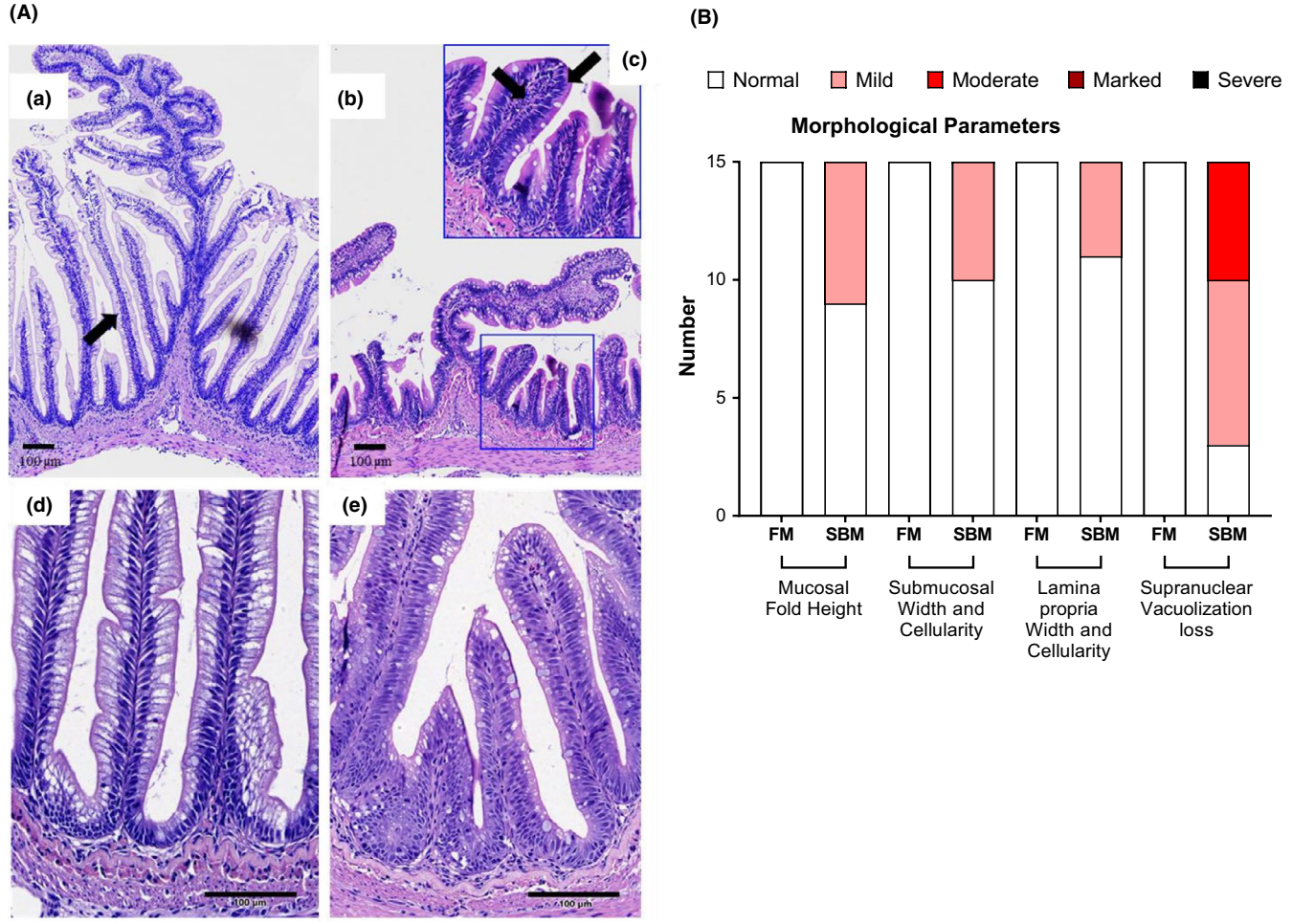

FIGURE 1 Histological evaluation of the distal intestine of Atlantic salmon. (A) Images (a) and (d) represent healthy morphology from fish fed FM diet (FM). Images (b) and (e) represent mild inflammatory changes from fish fed SBM diet with 20% soybean meal inclusion (SBM) after 7 weeks in seawater (c) a magnified view of the region marked by the blue square in image (b), moderate mucosal fold shortening, marked loss of supranuclear vacuoles (black arrow) in the enterocytes and mild infiltration of the lamina propria by inflammatory cells (black arrow). (B) Histo-morphological parameters assessed in fish fed FM or SBM diet (15 fish per diet). X-axis represents 4 selected tissue parameters (mucosal fold height, submucosa width and cellularity, lamina propria width and cellularity and supranuclear vacuolization); y-axis represents number of fish assigned to one of five categories including scores ranging from 0 to 4, where 0 represented normal histology, 1—mild changes, 2—moderate changes, 3—marked changes and 4—severe changes

Complement C5 is a key factor in a lytic cascade that can directly execute pathogens and plays an important part in host homeostasis and regulation of adaptive immunity and inflammation. These features of Complement C5 are in accordance with our histology results of SBMIE where inflammation occurred. SBM diet also affected Galectin-9 (Gal-9) in DI mucus. Galectins belong to highly conserved proteins that bind to different ligands with affinity to carbohydrates and can be involved in a wide range of immune-related processes (Johannes et al., 2018; Rabinovich & Gruppi, 2005; Rubinstein et al., 2004). Among those processes, Gal-9 regulates multiple biological functions by binding to T-cell Ig mucin-3 (Tim-3), regulating T-cell death and activating innate immune cells (Li et al., 2011). Modulation of the Gal-9/Tim-3 pathway affects the development of many diseases (Gleason et al., 2012; Monney et al., 2002). In humans, Gal-9, together with galectin-1, −3 and −4, play a role in intestinal homeostasis, and its expression is deregulated during the inflammation process such as inflammatory bowel disease. Therefore, these specific galectins have been suggested as markers for the severity of inflammation (Papa Gobbi et al., 2016). The functional role of galectins in fish is unclear. In salmon, galectins have been involved with a variety of roles in innate immune defence. As reported by Patel et al. (2020), galectin-3 is highly expressed in skin and gills and can agglutinate the Gram-negative bacterium *Moritella viscosa*. Further analysis is needed to elucidate the role of galectins on intestinal inflammation. In addition, glutathione synthetase was also exclusively detected in the SBM group. Glutathione synthetase is the second enzyme in the glutathione (GSH) biosynthesis pathway (Lu, 2013). In mammals, it is well established that GSH is a potent antioxidant, a key determinant of redox signalling, important in detoxification of xenobiotics, regulates cell proliferation, fibrogenesis and immune functions (Lu, 2013). The role of GSH is crucial for several functions of the immune system, both innate and adaptive, including the proliferation of T lymphocytes, neutrophils phagocytic activity and antigen

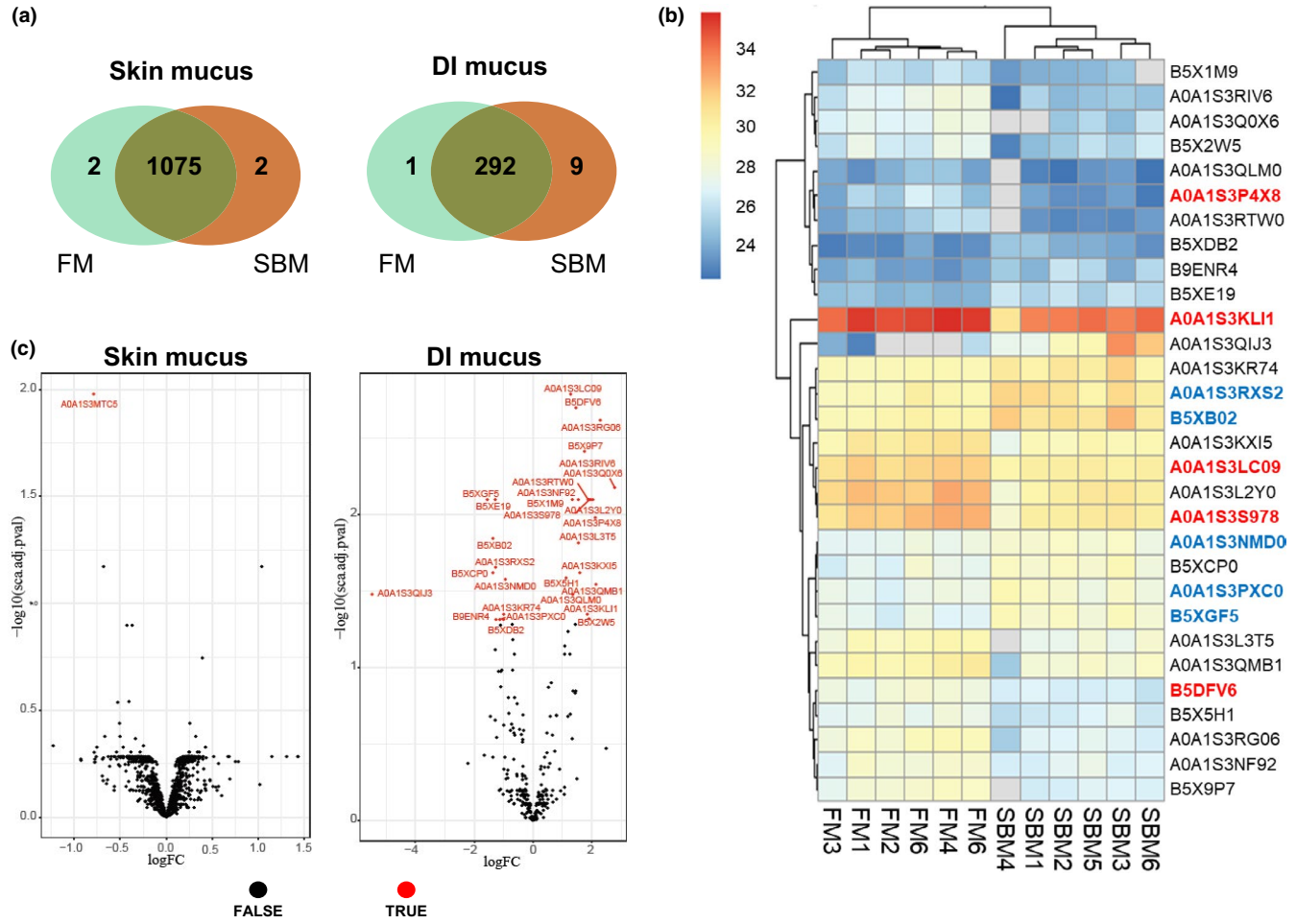

FIGURE 2 Common and unique proteins detected in skin and distal intestine mucus of Atlantic salmon. (a) Venn diagram shows proteins identified just in SBM (orange), FM (green) or detected in both diet groups in distal intestine and skin mucus. (b) Hierarchical clustering heat map shows the 30 significant proteins identified in FM (n = 6) and SBM (n = 6) group from DI mucus. The colours in the heat map indicate protein abundance and range from low abundance (blue; log2(LFQ) of 24 or lower) to high (red; log2(LFQ) of 34). Protein's Id belonging to cathepsin are marked in red. Protein's Id belonging to trypsin or chymotrypsin are marked in blue. (c) Volcano plot of significantly identified proteins in skin and distal intestine mucus (red colour). Proteins to the left were significantly higher in the SBM group, while proteins to the right were significantly higher in the FM group. Y-axis represents the p-value and x-axis fold of change

presentation since the first steps in antigen degradation require GSH (Ghezzi, 2011; Short et al., 1996). In the mice model that studied acute lung injury induced by lipopolysaccharide (LPS) (Ghezzi, 2011), GSH is shown to be a regulator of the balance between innate immunity (leukocyte infiltration at the site of infection to kill bacteria) and inflammation (leukocyte infiltration to the lung to failure the organ). In Atlantic salmon, changes in plasma and liver GSH were reported to be affected by infectious salmon anaemia (Hjeltnes et al., 1992), with increased levels of plasma GSH, but decreased levels of hepatic GSH in diseased fish compared with their healthy controls.

Among the proteins uniquely detected in the skin mucus of SBM fed salmon, we detected adenylosuccinate synthetase and putative aminopeptidase, which are involved in AMP biosynthesis and proteolysis, respectively. However, we did not find a relationship between SBMIE and protein profiles. Besides, among the common proteins, detected in both groups, just one protein was significantly different and corresponded to an uncharacterized protein with 72% confidence alignment to hydrolase. On the other hand, most of the proteins that were differentially detected in DI mucus of fish fed SBM corresponded to trypsin and chymotrypsin. In a study with humans suffering from Crohn's disease (CD), which presents symptoms similar to enteritis in salmonids, increased faecal and digestive proteases such as trypsin and chymotrypsin have been observed (Midtvedt et al., 2013). The authors suggest that the increased level of digestive protease might be due to the changes in microbiota, where patients with CD presented a reduction in the number of *Bacteroides*, the main group of bacteria known to be able to inactivate pancreatic trypsin (Midtvedt et al., 2013). Although we did not perform microbiota analysis in this study, a recent study of Booman et al. (2018) showed no differences in the microbiota composition of Atlantic salmon fed 20% SBM compared with the FM diet for three weeks feeding trial. On the contrary, Gajardo et al. (2017) detected a high

TABLE 4 List of unique proteins detected in the mucus of *S. salar*

Protein ID	Description	Sample	Diet group
A0A1S3SDZ4	Actin-related protein 2-like	Skin mucus	FM
A0A1S3STI9	Glyoxylate reductase/ hydroxypyruvate reductase-like	Skin mucus	FM
B5X373	Adenylosuccinate synthetase	Skin mucus	SBM
A0A1S3PFY6	Putative aminopeptidase W07G4.4	Skin mucus	SBM
A0A1S3M0Q1	Cathepsin Z-like	DI mucus	FM
A0A1S3PRG9	Complement C5	DI mucus	SBM
A0A1S3NCB3	Cytochrome b5-like	DI mucus	SBM
A0A1S3 N308	Erlin–1	DI mucus	SBM
B5XAH5	Galectin	DI mucus	SBM
A0A1S3MEJ0	Glutathione synthetase	DI mucus	SBM
A0A1S3N9 N3	N-acetyl-D-glucosamine kinase isoform X2	DI mucus	SBM
A0A1S3 N681	Trypsin-like	DI mucus	SBM
A0A1S3QGH7	Uncharacterized protein LOC106591807	DI mucus	SBM
A0A1S3LEQ8	kunitz-type serine protease inhibitor	DI mucus	SBM

abundance of lactic acid bacteria induced by SBM. Further experiments are needed to elucidate the effect of microbiota on the protein profile of enteritis induced by SBM. The proteins differentially detected in DI mucus of fish fed FM, mostly belong to the protease family of cathepsins. Interestingly, in mammals, cathepsin has been involved in inflammatory diseases (Liaudet-Coopman et al., 2006), but also the proteolytic activity of cathepsin B, D, H, and L plays a role in the generation of antigen peptides presented on the class-II molecules to T cells (Bryant et al., 2002). In fish, cathepsins are widely distributed in muscle and immunologically important organs, such as head kidney and spleen. This protein may play an important role in the protection of the host against microorganisms through the modulation of innate immunity (Tähtinen et al., 2002; Yamashita & Konagaya, 1990); however, more studies are needed to clarify the cathepsin roles in fish immunity under the effect of different feed ingredients.

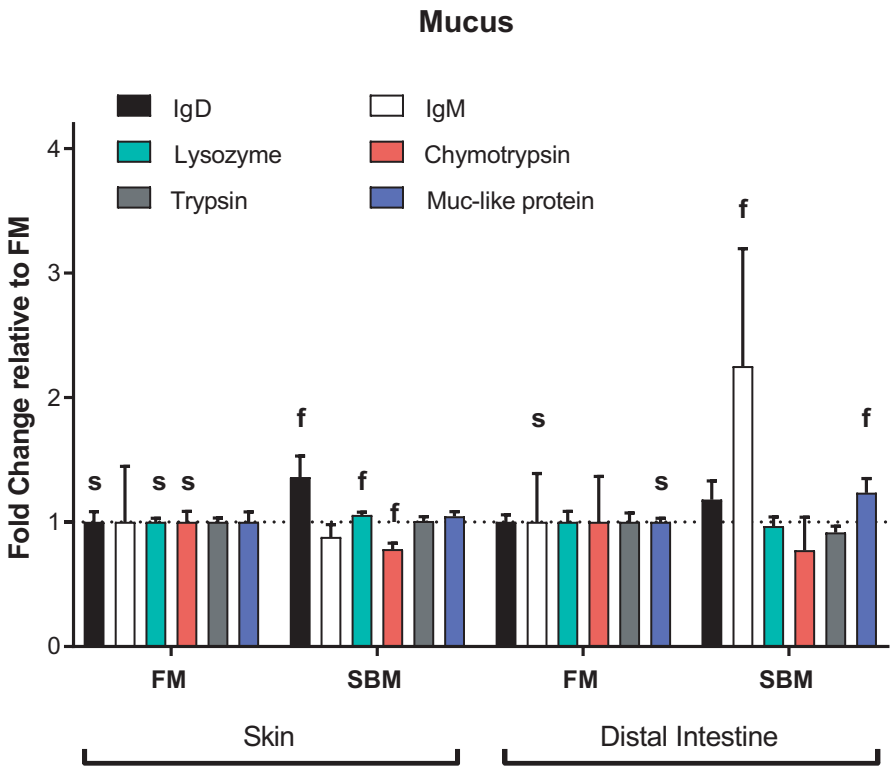

FIGURE 3 Phenotypical markers in skin and distal intestine mucus of Atlantic salmon detected by ELISA (in fold change relative to FM). IgD (black), IgM (white), Lysozyme (green), Chymotrypsin (red), Trypsin (grey) and Muc-like proteins (blue). Each bar: $n = 6$. f and s: significant difference ($p < .05$) compared with FM and SBM, respectively, by Student's t-test (two-tailed)

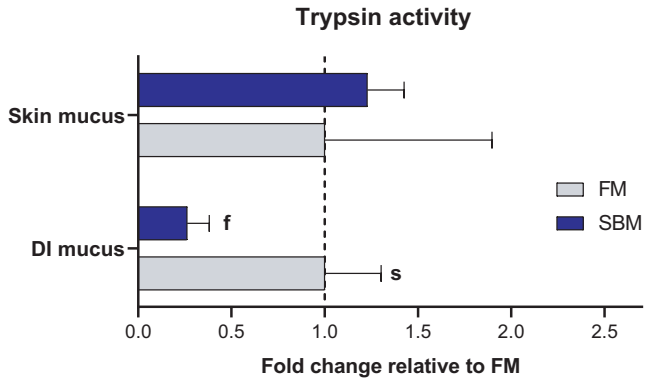

FIGURE 4 Trypsin activity in skin and distal intestine mucus of Atlantic salmon in fold change relative to FM. In grey: FM diet group (n = 6), in blue: SBM diet group (n = 6). f and s: significant difference (p < .05) compared with FM and SBM, respectively, by Student's t-test (two-tailed)

The use of antibodies for the detection of specific biomarkers from Atlantic salmon has been shown to be an efficient strategy to characterize the modulating effect of SBM diets on mucosal surfaces, such as skin and intestine, both important mucosa-associated lymphoid tissue (MALT), capable of controlling the homeostasis of the animal, through cellular and humoral components related to the immune response (Lazado & Caipang, 2014; Salinas et al., 2011).

Our results showed that the use of a SBM diet was able to induce a response by secretable molecules from both innate and adaptive immunity, since we detected an increase in IgD and lysozyme in the skin and IgM and Muc-like proteins in DI. We think that this may be related to the inflammatory condition produced by the SBM diet, which induces a response that tries to fight against SBM, as well as against infectious agents that can even be opportunistic. This proposal can be supported by the properties of the different biomarkers that we detected. Lysozyme is a protein with lithic activity and can act as an opsonin, promoting the phagocytosis process and contributing to the innate defence against bacterial infection (Esteban & Cerezuela, 2015). While IgD is an immunoglobulin with a higher structural plasticity and can be produced as a transmembrane or secreted protein in a species-specific manner (Chen & Cerutti, 2010). In fish, IgD has been described as a modulator between the innate and adaptive response, due to V-less region (Ramirez-Gomez et al., 2012). On the other hand, in higher vertebrates, secreted IgD can enhance mucosal homeostasis and immune surveillance by "arming" myeloid effector cells with antibodies against mucosal antigens (Gutzeit et al., 2018). However, this pathway can also cause an overreaction in the immune response by increasing inflammation and tissue damage (Chen & Cerutti, 2010). This is relevant if we relate the increase in IgD to the inflammatory condition caused by SBM in Atlantic salmon.

Continuing with the properties of the biomarkers that we detected in DI mucus, IgM is the most ancient antibody class and has the same function in all gnathostomes. The transmembrane form of IgM defines the B cell lineage, and in teleost fish, a secreted IgM form is produced as a tetramer in a different reduction or oxidation state that seems to modify the binding strength of the antibodies (Flajnik & Kasahara, 2010). It has been reported that the SBM diet causes significantly raised levels of IgM in the mid and DI mucosa (Krogdahl et al., 2000), which may be due to the different antigens present in SBM, such as glycinin and β-conglycinin (Wang et al., 2014) and as well, DI enteritis caused by SBM can lead to increased susceptibility to bacterial infections (Krogdahl et al., 2000). Finally, Muc-like proteins are involved in maintaining homeostasis between the local microbiota and the host. These proteins belong to a heterogeneous family of proteins composed of a long peptide chain with many tandem repeats (Koshio, 2016; Pérez-Sánchez et al., 2013). The same authors have proposed Muc-like proteins as prognostic markers of an intestinal phenotype susceptible to dietary changes and as diagnostic markers of the pathological effects of intestinal pathogens involving goblet cells depletion phenotype in gilt-head bream (Pérez-Sánchez et al., 2013).

Additionally, the results of enzymatic activity showed that it is also important to characterize the functionality of host proteins against compounds such as SBM. The lower trypsin activity detected in DI mucus of the SBM group can be due to peptidase inhibitors (PIs) that are present in SBM. PIs are involved in plant resistance to herbivorous insects (Souza et al., 2016) and one of those is Bowman-Birk inhibitor, which is difficult to inactivate by heating, producing a residual trypsin inhibitor activity and chymotrypsin inhibitor activity (He et al., 2017). On the contrary, Øverland et al. (2009) detected an increased trypsin activity in the faeces of Atlantic salmon fed 20% SBM inclusion in the diet. The explanation of lower trypsin activity in our study can be supported by Olli et al. (1994), who demonstrated that trypsin activity decreased at the highest level of trypsin inhibitor inclusion. It might be that the SBM used in our study has had a higher level of PIs than SBM used by Øverland et al. (2009), and therefore, the production of trypsin enzyme was depleted. Further studies with different levels of PIs are needed to confirm this statement and to understand the trypsin regulation and kinetics when high amounts of PIs are present in the ingredients that compose fish diets.

Characterization of the immune response of mucosal surfaces of Atlantic salmon fed with SBM diet, from a phenotypic point of view, that combine general (proteomic) and specific strategies, such as detection by ELISA, enzymatic assays and morphological parameters, allows us to determine biomarkers in more reliable manner than using transcriptional approaches, since proteins are the central final step dogma of molecular biology. In DI, a higher number of modulated proteins (compared with skin mucus) could be expected, mainly due to a possible local response of the fish against SBM. Here, the level of IgM and Muc-like proteins suggested a coordination of the adaptive immune response and the secretion of proteins that seek to protect the cell layer from both biological and mechanical damage. Following this idea, in order to increase the knowledge of the effect of SBM on mucosal surfaces, we believe that the production of antibodies against some of the proteins identified through proteomics such as Galectin, Glutathione synthetase and Complement

C5 could be produced, as they are also related to the modulation of the immune response. Finally, regarding the skin mucus, we believe that the production of IgD and lysozyme in fish fed with SBM is interesting, since they could be proposed as non-lethal and less invasive markers for the future investigations with fish, which could also integrate different phenotypic evaluation strategies to understand the interaction between nutrition and immunity at a deeper level.

5 | CONCLUSION

In this study, Atlantic salmon mucosal immunity parameters at the protein level were characterized towards a deeper understanding of the already established SBMIE model. Immunological markers IgD, IgM, Muc-like proteins and lysozymes detected at a phenotypical level in the skin and DI mucus demonstrated that both innate and adaptive parameters could be stimulated in SBMIE. Therefore, we propose that the use of phenotypic strategies can help to assess the impact of feed ingredients through potential diagnostic markers in aquaculture when new aquafeeds need to be evaluated. Extensive studies, with increased number of fish, are needed to build proteomic baseline data for healthy and diseased fish which will serve for comparison and correlation with other available diagnostic methods. New emerging knowledge will possibly help us to create diets that can assure good health and optimal growth.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest. All authors read and approved the final manuscript for submission. The content of the manuscript has not been published or submitted for publication elsewhere.

AUTHOR CONTRIBUTIONS

BD and MØ designed the feeding experiment. BD coordinated the execution of the experiment as well as sampling. BD and LL planned laboratory analysis. BML performed ELISA and enzyme assays. BD prepared samples for proteomics analysis and LL performed proteomics data analysis and proteomics statistics. BD, BML and LL were involved in manuscript writing and review, data analysis, producing figures and tables for the manuscript as well as statistical analysis and quality checking. MØ was involved in writing and reviewing the manuscript. LM designed antibodies and reviewed the manuscript.

DATA AVAILABILITY STATEMENT

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD017744. Other raw data that support the findings of this study are available from the corresponding author, upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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