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# Mucosal barrier status in Atlantic salmon fed marine or plant-based diets supplemented with probiotics

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ABSTRACT

Feed ingredients and additives significantly affect the mucosal health of fish. A 3 × 2 factorial experiment was conducted to investigate the mucosal health of Atlantic salmon (*Salmo salar*) fed three basal feeds (namely, BG1, BG5 and BG2; marine-, plant-, and soybean meal-based feeds, respectively) or the basal feeds with (+) or without (÷) probiotics, *Lactobacillus fermentum* and *Lactobacillus plantarum*. Six diets were fed to fish distributed into 12 tanks (approximately 43 fish/tank). The average start weight of the experimental fish was about 122.6 g. After 38-days of feeding, the dorsal skin, gills and distal intestine were obtained for analysing histomorphometry and mucus-related genes. Digesta were also collected to study short chain fatty acids (SCFAs).

Fish fed BG2 had significantly higher number of mucous cells/$\mu m^2$ skin epithelium (SNE) than those fed BG1 and BG5. Addition of probiotics significantly increased SNE in BG5+ and BG2+ group compared to BG1÷ group. Similarly, the area and number of mucous cells/$\mu m^2$ gill epithelium (GME and GNE) were significantly higher in BG2 group, followed by BG5 and BG1 fish groups. Probiotics significantly increased GME and GNE in all feed types. Concerning intestine, villi height (VH) and enterocyte height (EH) were significantly higher for BG1 group, followed by BG5 and BG2 groups. Compared to fish offered BG2, fish fed BG1 had significantly wider villi (VW) and narrower lamina propria (LPW). The number of mucous cells (NM) and intraepithelial lymphocytes (IEL) in the intestine were significantly higher in BG2 fed fish than those offered BG5 and BG1. The indices VH, VW, EH, and IEL were not affected by probiotics. Although higher NM and IEL were observed in BG2÷ fish compared to those fed the other two diets, probiotics reduced NM and IEL. Fish fed BG2÷ showed symptoms of inflammation, including disappearance of supranuclear vacuoles (SNV). Probiotics improved VH, significantly reduced LPW and aided in the reappearance of SNV in BG2+ fed fish. Some of the gene expression data supported histological findings; notably, levels of *muc5ac1* in the skin and *defensin3* and *cathelcidin1* in the intestine were correlated with histology data. Moreover, the total SCFA concentration was significantly affected by feed ingredients. Only acetoacetic acid was affected by both factors.

Our findings suggest that feed ingredients can significantly alter the mucosal protective barrier of the organs. Supplementation of probiotics alleviated the inflammatory responses and activated selected innate immune defence molecules, without affecting growth. The positive effect of the probiotics was similar regardless of the feed ingredients, suggesting that these probiotics can be utilized as immune regulators to evoke favourable responses on the skin, gills and intestine.

## 1. Introduction

Fish feed accounts for a significant part of the variable costs in the farming of Atlantic salmon (*Salmo salar*). Efficient utilization of feeds by the fish is the key to keep the production cost low and the economic turnover high (Iversen et al., 2020). Since 1990, salmon feed industry

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has gradually shifted its dependence from marine- to plant-derived ingredients (Aas et al., 2019); now soy protein concentrate is a key salmon feed ingredient, and pea protein concentrate, wheat gluten, corn gluten, fava beans, sunflower meal and sunflower protein are incorporated at lower levels (Aas et al., 2019; Øverland et al., 2009; Ytrestøyl et al., 2015). Regarding the fish oil, it has been replaced to a large extent by rapeseed oil in European salmon feeds (Aas et al., 2019; Sprague et al., 2016; Ytrestøyl et al., 2015).

Use of more plant-based ingredients has taken its toll on the health of fish (Sørensen et al., 2021). Less refined feed ingredients, in particular soybean meal (SBM) that contains various antinutritional factors such as saponins, cause enteritis in fish (Baeverfjord and Krogdahl, 1996; Booman et al., 2018; Knudsen et al., 2007; Krogdahl et al., 2015; Sanden et al., 2005; Sørensen et al., 2011; Vasanth et al., 2015). SBM derivatives-induced intestinal inflammation was characterized by abnormal intestinal villi and lamina propria, enterocytes lacking supranuclear vacuoles, immune cell-infiltrated lamina propria and submucosa, and intestinal ion and water transport disturbances (Baeverfjord and Krogdahl, 1996; Buttle et al., 2001; Kiron et al., 2020; Kortner et al., 2012; Krogdahl et al., 2000; Refstie et al., 2000; Urán et al., 2008a, 2008c, 2009; Van Den Ingh et al., 1996, 1991). These unfavourable conditions are known to affect the growth of the fish, shift the microbiota and weaken the local immune defences, thereby making the fish prone to diseases (Egerton et al., 2020; Gajardo et al., 2017; Krogdahl et al., 2000; Torrecillas et al., 2017).

Disease prevention and control coupled with reduced mortality during the grow-out phase of fishes is vital for sustainable development of aquaculture as well as to keep the production costs in check (Bang-Jensen et al., 2019; Iversen et al., 2020; Minniti et al., 2019). Fish body is exposed to various adverse environmental conditions, including many opportunistic pathogens that thrive in the rearing water. However, a rather complex immune system that encompasses innate and adaptive branches, exists to fight these threats and to maintain the health (Brunner et al., 2020; Magnadóttir, 2006). The first lines of defence include the mucosal barriers in the skin, gills and intestine (Cain and Swan, 2010; Kiron, 2012; Wang et al., 2011). The epithelial cells in these organs are covered by a mucus layer, which is mainly secreted by mucous cells. The mucus consists of several innate immune molecules such as mucins, protease, lysozyme, esterase, complement proteins, antibodies and antimicrobial peptides (AMPs) which could chemically inactivate the pathogens or arrest the formation of their colonies (Aranishi and Mano, 2000; Concha et al., 2003; Firth et al., 2000; Hatten et al., 2001; Johansson et al., 2008; Núñez-Acuña et al., 2018, 2016). In addition, the intestine mucus creates an optimum environment for the action of the digestive enzymes and aids in lubricating the digesta to ensure the integrity of intestinal mucosa during digestion (Kim and Ho, 2010). Therefore, a healthy mucosal surface with adequate number of mucous cells is essential to maintain the barrier functions and deploy appropriate molecules such as mucins and AMPs during defence (Pittman et al., 2011).

Commensal microbiota at the mucosal surfaces are also vital to maintain the mucosal barrier functions and to prevent colonization by potential pathogens (Lowrey et al., 2015). It is now known that microbiota can be modulated by feed ingredients (Hoseinifar et al., 2015; Nayak, 2010; Pérez-Sánchez et al., 2014) and additives such as probiotics (Gupta et al., 2019a) and pre-biotics (Gupta et al., 2019b). Lactic acid bacteria (LAB) such as *Lactobacillus* spp., *Lactococcus* spp., *Pediococcus* spp., *Carnobacterium* spp. and those belonging to the genus *Leuconostoc* can be considered as probiotics for aquaculture applications; for enhancement of gut health, to reduce the use of chemotherapeutics and to maintain fish welfare (Alonso et al., 2019; Andani et al., 2012; Hai, 2015; Merrifield et al., 2010; Ringø et al., 2018).

LABs have anti-inflammatory and antibacterial properties, and they are classified as GRAS (generally recognized as safe) (van Baarlen et al., 2013). Bacteria belonging to the genus, *Lactobacillus* are acid-tolerant facultative anaerobes, and they are either homo- or heterofermentative (Ringø et al., 2018). Kraus (1961) was the first to reveal the presence of lactobacilli in the gastrointestinal tract (GI) of a fish, herring (*Clupea harengus* L.). Since then, the existence of bacteria belonging to the genus *Lactobacillus* in the GI tract of several finfish species, including Atlantic salmon, has been reported by many authors (Gatesoupe, 2007; Hovda et al., 2007; Lauzon and Ringø, 2011; Merrifield et al., 2014; Ringø, 2004; Ringø et al., 2005; Ringø and Gatesoupe, 1998). Our group has reported the establishment of lactobacilli delivered through feeds in the intestinal mucus of Atlantic salmon (Gupta et al., 2019a).

It is now known that certain members of the microbiota can produce short chain fatty acids (SCFAs) by fermenting nondigestible carbohydrates in feeds (Adorian et al., 2020; Hoseinifar et al., 2017). The dominant SCFAs such as acetate, propionate and butyrate (Den Besten et al., 2013) act as energy providers, signalling molecules, gene expression regulators, inflammation suppressors and immune cell development regulators. Thus, they play a critical role in maintaining intestinal integrity and health (Koh et al., 2016; Louis et al., 2014; Morrison and Preston, 2016; Richards et al., 2016).

Important information on the fish mucosal health status can also be collected by studying the associated mucin and AMP related genes (Bridle et al., 2011; Broekman et al., 2013; Chang et al., 2006; Marcos-López et al., 2018). In Atlantic salmon, seven mucin secreting genes were reported previously; two *muc2* genes were mainly found in the intestine while five *muc5* were observed in other tissues such as pyloric caeca, gill or skin (Sveen et al., 2017). AMPs are a diverse group of defence molecules, and among them *cathelicidins* and *defensins* are powerful antimicrobials (Chang et al., 2006; Reyes-Becerril et al., 2013). Our previous study results indicated the ability of *muc2* to denote intestinal barrier status and the feed ingredient-induced alteration of AMP genes in the skin, gills and intestine (Sørensen et al., 2021). Furthermore, in mammals, SCFAs are suggested as biomarkers to assess the host health status (Farup et al., 2016). Hence, it is essential to gather more evidence on feed component-induced modulation of mucins and AMPs that are important gatekeepers of the mucosal barriers and SCFAs that support the health of the intestine.

The aim of the present short-term feeding study was to investigate the growth performance, the architecture of the mucosa of the first-line defence organs, expression of selected mucins and AMP genes in these organs and SCFAs in the digesta of Atlantic salmon post smolts fed plant-based or marine-based diets, with or without two lactic acid bacteria strains, *Lactobacillus fermentum* and *Lactobacillus plantarum* (1:1).

## 2. Materials and methods

The National Animal Research Authority (FDU: Forsøksdyrutvalget ID-5887) in Norway has approved the experiment, and the handling of the animals were in accordance with the approved protocols.

### 2.1. Experimental feed preparation

#### 2.1.1. Feed preparation

For this trial, three basal feeds were prepared at the Feed Technology Center, Nofima, Bergen, Norway (Table 1). Extruded feeds were formulated based on the following ingredients: fish meal and fish oil (BG1), a mixture of plant and marine ingredients at a ratio of 70:30 (BG5) and SBM with marine ingredients (BG2). The ingredients of the experimental feeds were first homogenized (30 min) using a horizontal ribbon mixer and then they were subjected to a preconditioning step. During this step, water and steam were added into an atmospheric double differential preconditioner (DDC). The preconditioning step was followed by extrusion through a TX-52 co-rotating, fully intermeshing twin-screw extruder (Wenger Manufacturing Inc., Sabetha, KS, USA). While the temperature of the feed mash that was fed into the extruder was 86–88 °C, temperatures of the extruded feeds were different; 120, 128, and 137 °C for BG1, BG2 and BG5, respectively. Two of the feeds,

**Table 1**
Ingredient composition (%) of the three basal feeds employed in the study.

| Ingredients | BG1 | BG5 | BG2 |
|---|---|---|---|
| Fishmeal | 50 | 10 | 30 |
| Wheat meal | 13.85 | 6.05 | 6.55 |
| Wheat gluten | 5 | 10 | 10 |
| Soy protein concentrate | 0 | 20 | 0 |
| Soybean meal | 0 | 0 | 20 |
| Corn gluten | 0 | 9 | 0 |
| Pea protein concentrate | 0 | 9 | 0 |
| Fish oil | 25 | 7.7 | 26.4 |
| Rapeseed oil | 0 | 19.8 | 0 |
| Mineral premix | 0.59 | 0.59 | 0.59 |
| Vitamin premix | 2 | 2 | 2 |
| Monosodium phosphate | 2.5 | 2.5 | 2.5 |
| Carop. Pink (10% Astax) | 0.05 | 0.05 | 0.05 |
| Yttrium oxide | 0.01 | 0.01 | 0.01 |
| Choline | 0.5 | 0.5 | 0.5 |
| Methionine | 0.3 | 0.9 | 0.6 |
| Lysine | 0 | 1.2 | 0.5 |
| Threonine | 0 | 0.4 | 0.1 |
| Histidine | 0.2 | 0.3 | 0.2 |

BG1, marine-based feed; BG5, plant-based feed; BG2, soybean meal-based feed. Three more experimental diets were prepared by coating two probiotic organisms to the three basal feeds.

BG2 and BG5 had lower wheat content; consequently, more moisture in the form of steam was added into the DDC to ensure good expansion of the feed pellets. The wet extrudates, expelled out of the 24 circular 2.5 mm dies at the extruder outlet, were cut with a rotating knife of the extruder. The extruded pellets were dried in a hot air dual layer carousel dryer (Paul Klockner, Nistertal, Germany) at constant air temperature (77 °C) to obtain pellets with approximately 7–8% moisture. Next, the feeds were coated with oil using a vacuum coater (Pegasus PG-10VC LAB, Dinnissen B.V., the Netherlands). Immediately after the oil coating, feeds were packed in sealed plastic buckets and shipped to the Research Station, Nord University, Bodø, Norway.

#### 2.1.2. Probiotics coating on feed pellets

Two species of probiotics, *L. plantarum* R2 Biocenol™ (CCM 8674) and *L. fermentum* R3 Biocenol™ (CCM 8675) were isolated from the intestinal content of rainbow trout (*Oncorhynchus mykiss*) obtained from a fish farm, Rybárstvo – Požehy s.r.o. Dubové in the Slovak Republic (Fečkaninová et al., 2019). Pure cultures of probiotics were grown on de Man, Rogosa and Sharpe (MRS) agar plates (HiMedia Laboratories, Mumbai, India) under anaerobic condition (Oxoid Gas Pack Anaerobic system) at 37 °C for 48 h before they were inoculated into 1000 mL of MRS broth and incubated for 18 h at 37 °C on a shaker. The culture was centrifuged at 4500 rpm for 20 min at 4 °C in a cooling centrifuge (Universal 320 R, Hettich, Germany). The resulting cell pellets were washed twice and resuspended in 30 mL of 0.9% (w/v) sterile saline. The feeds (batches of 1800 g) were thoroughly coated with the bacterial suspensions using a vacuum coater (Rotating Vacuum Coater F-6-RVC, Forberg International AS, Norway) at 70 kPa at the feed laboratory of Nord University, Bodø, Norway. Post coating, the bacterial counts on diets were &$10^8$ cells/g as determined by spread plating on MRS agar plates and incubating anaerobically (Oxoid Gas Pack Anaerobic system) for 48 h at 37 °C. The feeds without probiotics were coated with 0.9% of sterile saline. The coated diets were stored at 4 °C until they were fed to the experimental fish.

#### 2.1.3. Experimental feeds

In total, six experimental feeds were prepared for this study at the feed laboratory of Nord University, Bodø, Norway. The basal feeds without probiotics were named as BG1÷ (marine- based feed without probiotics), BG5÷ (plant-based feed without probiotics) and BG2÷ (SBM-based feed without probiotics). The basal feeds with probiotics were named as BG1+ (marine-based feed with probiotics), BG5+ (plant-based feed with probiotics) and BG2+ (SBM-based feed with probiotics). The nutrient and amino acid composition of the basal feeds is given in Table 2.

### 2.2. Fish, experimental design and feeding

Atlantic salmon post-smolts were obtained from Cermaq, Hopen, Bodø, Norway (Aquagen strain, Aquagen AS, Trondheim, Norway). The present experiment was the second phase of a large study (Sørensen et al., 2021) performed at the Research Station, Nord University, Bodø, Norway to test the effects of different combinations of plant and marine ingredients on the performance of Atlantic salmon. There were two replicate tanks for each treatment, and each tank contained 40–43 fish. The average initial weight of the fish was 122.6 ± 2.1 g (mean ± standard error of mean, SEM).

The feeding experiment was carried out in 12 circular fiberglass tanks (1100 L) that were connected to a flow-through system. Each tank was supplied with water pumped from Saltenfjorden, from a depth of 250 m. During the experiment, water flow rate was maintained at 1000 L per h, and the average temperature and salinity of the rearing water were 7.6 °C and 35 ‰, respectively. Oxygen saturation was always above 85%, measured at the water outlet. A 24 h photoperiod was maintained throughout the 38-day feeding trial. The fish were fed *ad libitum* using automatic feeders (Arvo Tech, Finland) during a 12-h period every day between 08:00 and 20:00 (7 feedings, 08:00–10:00, 10:00–12:00, 12:00–14:00, 14:00–16:00, 16:00–18:00, 18:00–19:00 and 19:00–20:00).

### 2.3. Sampling and data collection

At the beginning and end of the feeding experiment, all fish were individually weighed, and their fork lengths recorded. Fish were anesthetized using tricaine methanesulfonate (MS 222, 140 mg/L) before handling. Twelve fish per tank were sacrificed for obtaining the dorsal skin (left), gills (second arch) and intestine (approximately 2 cm of the anterior part of the distal intestine) (Sanden and Olsvik, 2009; Sundell

**Table 2**
Analyzed proximate composition (% as is) and amino acid composition (% as is) of the three experimental feeds.

| Composition | BG1 | BG5 | BG2 |
|---|---|---|---|
| Moisture | 5.3 | 6.3 | 4.9 |
| Protein | 42.5 | 42.8 | 42.2 |
| Lipid | 29.0 | 26.0 | 28.6 |
| Ash | 11.2 | 7.02 | 9.45 |
| Energy (KJ/100 g) | 2000 | 1994 | 2029 |
| Amino acids | | | |
| Alanine | 2.44 | 2.04 | 2.03 |
| Arginine | 2.35 | 2.35 | 2.33 |
| Aspartic acid | 3.50 | 3.51 | 3.43 |
| Glutamic acid | 6.92 | 9.04 | 8.03 |
| Glycine | 2.61 | 1.75 | 2.18 |
| Histidine | 1.01 | 1.17 | 1.02 |
| Hydroxyproline | 0.31 | 0.16 | 0.22 |
| Isoleucine | 1.66 | 1.66 | 1.64 |
| Leucine | 3.01 | 3.54 | 2.93 |
| Lysine | 2.89 | 3.05 | 2.85 |
| Phenylalanine | 1.67 | 2.10 | 1.79 |
| Proline | 2.19 | 2.88 | 2.47 |
| Serine | 1.81 | 2.04 | 1.91 |
| Threonine | 1.64 | 1.9 | 1.64 |
| Tyrosine | 1.25 | 1.50 | 1.35 |
| Valine | 1.96 | 1.88 | 1.86 |
| Tryptophan | 0.43 | 0.41 | 0.44 |
| Cysteine | 0.41 | 0.53 | 0.50 |
| Methionine | 1.37 | 1.68 | 1.67 |
| [a]Σ EPA/DHA | 5.90 | 1.7 | 5.8 |

BG1, marine-based feed; BG5, plant-based feed; BG2, soybean meal-based feed.
[a] Σ EPA/DHA was calculated based on the content in the fish oil.

and Sundh, 2012). Tissues from 6 fish were immediately placed in 10% neutral buffered formalin (NBF) for 24 h at room temperature for the histological evaluation, and tissues from remaining 6 fish were transferred to tubes filled with RNA later® (Ambion Inc., Austin, Texas, United States), and stored at −20 °C for gene expression analysis. Another 5 fish per tank were stripped for digesta and stored at −20 °C for analysing SCFA composition.

### 2.4. Growth performance calculations

Fish growth performance was analysed using the following equations.

$$\text{Weight gain (WG\%)} = ((\text{FW} - \text{IW})/\text{IW}) \times 100$$

$$\text{Specific growth rate (SGR)} = ((\text{Ln (FW)} - \text{Ln (IW)})/\text{D}) \times 100$$

$$\text{Thermal growth coefficient (TGC)} = \left(\left((\text{FW})^{(1/3)} - (\text{IW})^{(1/3)}\right)\Big/(\text{T} \times \text{D})\right) \times 1000$$

$$\text{Condition factor (CF)} = \left(\text{FW}/\text{FL}^3\right) \times 100$$

Where, FW = mean final body weight of fish (g), IW = mean initial body weight of fish (g), T is the water temperature in °C, D is feeding duration in days. IL and FL are the initial and final fork length (cm) of fish, respectively.

### 2.5. Histomorphometry

Standard histological procedures were adopted, and the analyses were performed at the histology laboratory of the Research station, Nord University, Bodø, Norway. Fixed tissues were dehydrated with increasing concentrations of ethanol, followed by immersion in xylene and paraffin (Sørensen et al., 2011). Next, tissue sections of 4 μm were prepared using microtome and mounted onto a glass slide, after which they were stained with Alcian blue - periodic acid–Schiff (pH 2.5). Stained slides (one section per fish) were covered with a coverslip after adding a drop of glue, Pertex® (Histolab Products AB, Askim, Sweden). Thereafter, microphotographs were captured at 40× magnification by a camera (Leica MC170HD, Heersbrugg, Switzerland) fitted on a light microscope (Leica DM1000, Wetzlar, Germany), and using a software, Leica Application Suite (LAS V4.12.INK, Heerbrugg, Switzerland). All the images were examined with ImageJ 1.52a (Schneider et al., 2012).

#### 2.5.1. Collection of skin samples from the dorsal area

Tissues (approximately 2 cm) were sliced transversely into 3 equal parts after removing most of the muscles that were attached to the skin and decalcified with 10% formic acid (25 blocks per L) for 5 h. The tissues were rinsed with phosphate-buffered saline (PBS) prior to standard histological procedure. Approximately 600–900 μm (length) skin microphotographs (9 per fish) were generated to investigate the skin mucous cells.

#### 2.5.2. Collection of gill samples

To measure the area or count the number of mucous cells in the gills, 10 secondary lamellae from 5 different filaments per fish were chosen. Thus, in this study 50 secondary lamellae per fish were examined to understand the effect of the diets.

#### 2.5.3. Histomorphometric analysis of the dorsal skin and gills

First, 'Freehand selections' tool of ImageJ was employed to demarcate the total area of skin epithelium (SE) and then 'Brightness and Hue' under 'Colour threshold' of the 'Image' menu was adjusted, while keeping 'Thresholding method' as 'Default', 'Threshold colour' set to red and 'Colour space' to HSB (hue, saturation and brightness). Next, using the measure option under the 'Analyze' menu SE was calculated (Gong et al., 2020). Thereafter, the 'Wand tool' was used to select individual mucous cells. Next, the background was cleared using 'Edit' and then the image was converted to 8 bits to retain only the mucous cells. The total area of skin mucous cells (SM) and number of skin mucous cells (SN) were determined by selecting 'Threshold' under 'Image' menu, and by setting 'Analyze particles' to '30 to infinity' under the 'Analyze' menu in ImageJ (Supplementary Fig. 1). SE, SM and SN were used to calculate 2 indices: SME (SM per SE) and SNE (SN per SE). The same image analysis procedure that is described for skin was employed for gills to examine the total area of gill epithelium (GE), the total area of gill mucous cells (GM) and number of gill mucous cells (GN). The obtained values were used to calculate 2 indices: GME (GM per GE) and GNE (GN per GE) (Supplementary Fig. 2).

#### 2.5.4. Collection of intestine samples

The intestine contents were first rinsed off with 10% NBF prior to fixation. After trimming off the excess tissues, the intestine segment was processed and embedded longitudinally. For the histomorphometric analysis, 10 simple, long, well-oriented and intact villi per fish were selected from 3 to 5 different locations. Approximately, 10 microphotographs per fish were generated.

#### 2.5.5. Histomorphometric analysis of distal intestine

The evaluation of the intestine histomorphology included a quantitative and a semi-quantitative assessment. For the quantitative assessment, height (VH) and width (VW) of villi, height of enterocytes (EH), and width of the associated lamina propria (LPW) were measured; these parameters helped us to evaluate the diet-induced alterations in the intestinal microscopic structure. Width of a villus varies along its height, and hence to measure VW, each villus was partitioned into 6 equal parts from the base to tip (Supplementary Fig. 3). From these 5 points, VW, EH and LPW were gauged employing the analysing tools ('straight' and 'segmented lines') of the ImageJ, and the average of the 5 values was registered. The semi-quantitative assessment included the evaluation of the number of intestinal mucous cells (NM), number of intraepithelial lymphocytes (IEL), and presence of supra nuclear vacuoles (SNV) in enterocytes of intestinal villi. A scoring system was developed (Supplementary Table 1) based on previous articles (Baeverfjord and Krogdahl, 1996; Bakke-McKellep et al., 2007; Knudsen et al., 2008; Silva et al., 2015; Urán et al., 2008a). Each index of interest received a score from 1 to 5, and these scores were used for the downstream analyses.

### 2.6. Gene expression analysis

For the present study, relative mRNA levels of mucin genes (*muc2*, *muc5ac1*, *muc5ac2,* and *muc5b*) in the skin, gills and distal intestine, and AMP genes (*defensin 1 - def1, defensin 2 - def2, defensin 3 - def3, defensin 4 - def4,* and *cathelicidin 1 - cathl1*) in the skin and distal intestine were studied. Primers were purchased from Eurofins Genomics (Luxembourg, Luxembourg) and the sequences and details of all target and reference genes are described in Sørensen et al. (2021). The RNA extraction, cDNA synthesis and qPCR were performed as described by Sørensen et al. (2021).

### 2.7. Quantification of short chain fatty acids by isotachophoresis

Approximately 1 g of digesta per fish was homogenized with deionized water (50 ml). The solution was filtered through normal filter paper. The filtrates (5 ml per fish) were kept in cryotubes at −20 °C until further analysis. The produced short chain fatty acids (formic, acetoacetic, lactic, succinic, acetic, propionic, valeric and butyric acids) were determined by capillary isotachophoresis (Electrophoretic analyzer EA 202 M, VILLA LABECO spol. s.r.o., Spisska Nova Ves, Slovakia) as described by Gancarcikova et al. (2020).

### 2.8. Statistical analysis

In the current study, tank was used as the experimental unit for growth performance calculations (Kiron et al., 2016). However, individual fish was considered as the experimental unit for histological evaluation (Bansemer et al., 2015; Cerezuela et al., 2013; Urán et al., 2008b), gene expression and SCFAs composition analyses. All statistical analyses were executed using R (version 3.6.3) and R studio (version 1.2.5033) for windows. Normality of the data was checked with Shapiro–Wilk test and the homogeneity of variance was assessed by Levene's test. In this experiment, feed type (based on ingredients composition) was taken as the first factor (FeedIn: BG1, BG5 and BG2) and probiotic treatment as the second factor (ProbTr: "÷" (without probiotics) and "+" (with probiotics)). The effects of the factors and their interaction (FeedIn×ProbTr) were assessed by analysing the data using two-way analysis of variance (two-way ANOVA). Hereafter, the term 'BG1' shows or represents the average value of both 'BG1÷' and 'BG1+' (marine-based feed with and without probiotics). We have adopted the same strategy for 'BG5' and 'BG2'. For the groups with (+) and without (÷) probiotics, the average values of the different FeedIn are shown in the tables. Parametric two-way ANOVA was employed for data (IW, IL, FW, FL, WG, SGR, TGC, CF, SME, SNE, GME, GNE, VH, VW, EH, LPW, *muc2*, *muc5ac1*, *muc5ac2*, *muc5b*, *def1*, *def3*, *def4*, and *cathl1*) that followed gaussian distribution and had equal variance. When necessary, data were log transformed (gene expression and SCFAs data). Significant differences among the means of the experimental groups were revealed by Tukey's honestly significant difference (HSD) test. Two-way aligned rank transform analysis of variance (ART ANOVA) from ARTool package (version 0.10.7) was used for non-parametric semi-quantitative data (NM, IEL and SNV). Here, post-hoc comparisons, based on estimated marginal means (emmeans), were performed on a linear model for the response aligned and ranked data (Feys, 2016; Wobbrock et al., 2011). Spearman correlations for all the combinations of histologically evaluated mucous cell indices and the selected mucus-related genes were evaluated using the function from the package "psych" in R software. Statistical differences are reported at a significance level of $p < 0.05$. Means ± SEM of parameters are presented in all tables and figures, except Table 4, Figs. 3 and 7.

## 3. Results

### 3.1. Growth performance

There were no mortalities during the experiment. The growth performance parameters are presented in Table 3. The fish weight increased from an average range of 116–127 g to a range of 186–200 g during the experiment. There were no significant differences in FW, FL, SGR, TGC and WG of the diet groups. On the other hand, CF was significantly affected by feed type (factor FeedIn, Table 3); fish fed BG2 had lower CF compared to BG1 and BG5. None of the growth performance parameters was affected by feeding the probiotics (factor ProbTr). Furthermore, we did not find any interaction (FeedIn×ProbTr) effect on the parameters.

### 3.2. Histomorphometry

The results of the two-way ANOVA for the main factors, FeedIn and ProbTr are presented in Table 4 and Figs. 1–3; employing the data on dorsal skin, gill and distal intestine morphometric indices from the histology study.

#### 3.2.1. Mucous cells in the dorsal skin

The results did not reveal any significant differences in SME of the diet groups (Fig. 1A). However, SNE was significantly influenced by both factors, feed type (FeedIn) and probiotics (ProbTr). Fish fed BG2 had significantly more SNE compared to the fish fed BG1 and BG5 (Table 4 and Fig. 1B). Note that the probiotic groups (+) had significantly more SNE compared to groups without probiotics (÷) (Table 4; the main factor effect). Diet groups BG2÷, BG2+ and BG5+ had significantly more SNE compared to the diet group BG1÷ (Fig. 1B). We did not find any significant interaction of the two factors (FeedIn×ProbTr). However, all the probiotic fed groups showed similar increasing tendency for SNE (Table 4 and Fig. 1B).

#### 3.2.2. Mucous cells in the gills

Histological evaluation of mucous cells in the gills revealed significant effects of feed type (factor FeedIn) and probiotics (factor ProbTr) on GME (Fig. 1C and Table 4) and GNE (Fig. 1D and Table 4). Fish fed BG2 had significantly more GME and GNE compared to BG5 followed by those fed BG1. Addition of probiotics to all the feed type significantly increased GME and GNE (Fig. 1C and D). We did not detect a significant

**Table 3**
Growth performance indicators of Atlantic salmon offered feeds with different combination of marine and plant ingredients, and with or without probiotic supplementation.

| Parameters: | | IW (g/fish) | IL (cm) | FW (g/fish) | FL (cm) | CF (g/cm$^3$) | SGR | TGC | WG (%) |
|---|---|---|---|---|---|---|---|---|---|
| Means of main effect: | | | | | | | | | |
| FeedIn | BG1 | 126.91 ± 1.56 | 21.51 ± 0.10 | 196.92 ± 5.45 | 24.67 ± 0.25 | 1.31 ± 0.01$^B$ | 1.15 ± 0.05 | 2.74 ± 0.14 | 55.11 ± 2.98 |
| | BG5 | 124.58 ± 2.22 | 21.33 ± 0.09 | 199.90 ± 7.51 | 24.64 ± 0.19 | 1.34 ± 0.02$^B$ | 1.24 ± 0.09 | 2.94 ± 0.23 | 60.47 ± 5.29 |
| | BG2 | 116.34 ± 2.59 | 21.16 ± 0.11 | 186.36 ± 3.37 | 24.78 ± 0.15 | 1.22 ± 0.00$^A$ | 1.24 ± 0.03 | 2.88 ± 0.06 | 60.25 ± 1.71 |
| ProbTr | ÷ | 121.83 ± 2.85 | 21.30 ± 0.01 | 195.78 ± 5.01 | 24.74 ± 0.15 | 1.29 ± 0.02 | 1.25 ± 0.05 | 2.94 ± 0.13 | 60.82 ± 3.13 |
| | + | 123.38 ± 2.35 | 21.37 ± 0.01 | 193.01 ± 5.16 | 24.65 ± 0.17 | 1.29 ± 0.03 | 1.18 ± 0.04 | 2.77 ± 0.11 | 56.41 ± 2.57 |
| Means of interaction effect: | | | | | | | | | |
| BG1 | ÷ | 126.99 ± 2.03 | 21.44 ± 0.14 | 194.30 ± 11.47 | 24.58 ± 0.47 | 1.31 ± 0.01 | 1.12 ± 0.11 | 2.64 ± 0.30 | 52.90 ± 6.58 |
| | + | 126.82 ± 3.23 | 21.57 ± 0.19 | 199.54 ± 5.74 | 24.76 ± 0.36 | 1.32 ± 0.02 | 1.19 ± 0.01 | 2.84 ± 0.05 | 57.33 ± 0.51 |
| BG5 | ÷ | 123.43 ± 4.85 | 21.34 ± 0.22 | 205.56 ± 7.12 | 24.85 ± 0.15 | 1.34 ± 0.02 | 1.34 ± 0.01 | 3.20 ± 0.01 | 66.58 ± 0.78 |
| | + | 125.72 ± 1.86 | 21.31 ± 0.08 | 194.24 ± 14.96 | 24.43 ± 0.34 | 1.33 ± 0.05 | 1.14 ± 0.16 | 2.69 ± 0.43 | 54.40 ± 9.61 |
| BG2 | ÷ | 115.08 ± 4.36 | 21.10 ± 0.16 | 187.48 ± 5.30 | 24.80 ± 0.19 | 1.23 ± 0.01 | 1.29 ± 0.03 | 2.98 ± 0.03 | 62.97 ± 1.57 |
| | + | 117.61 ± 4.26 | 21.22 ± 0.20 | 185.25 ± 6.15 | 24.76 ± 0.33 | 1.22 ± 0.01 | 1.20 ± 0.01 | 2.77 ± 0.01 | 57.53 ± 0.48 |
| *p*-values | FeedIn (F) | 0.286 | 0.207 | 0.364 | 0.900 | 0.008 | 0.518 | 0.648 | 0.495 |
| | ProbTr (P) | 0.977 | 0.619 | 0.724 | 0.726 | 0.882 | 0.323 | 0.371 | 0.305 |
| | F × P | 0.948 | 0.849 | 0.681 | 0.665 | 0.913 | 0.298 | 0.338 | 0.295 |

BG1, marine-based feed; BG5, plant-based feed; BG2, soybean meal-based feed; FeedIn, factor feed ingredients; ProbTr, factor probiotics; ÷, without probiotics; +, with probiotics. F × P, Interaction between feed type and probiotics. IW, initial weight; IL, initial length; FW, final weight; FL, final length; CF, condition factor; SGR, specific growth rate; TGC, thermal growth coefficient; WG%, weight gain in percentage. Values are expressed as means ± SEM of two replicates. The uppercase letters A, B and C (based on post-hoc results) represent significant differences ($p < 0.05$) among feed groups (BG1, BG5 and BG2). Interaction effect was not detected for any of the growth parameters.

**Table 4**
Atlantic salmon skin, gills and intestinal histomorphometric indices.

| Tissues: | | Skin | | Gills | | Intestine | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Parameters: | | SME (ratio) | SNE (number /μm) | GME (ratio) | GNE (number /μm) | VH (μm) | VW (μm) | EH (μm) | LPW (μm) | NM (score) | IEL (score) | SNV (score) |
| Means of main effect: | | | | | | | | | | | | |
| FeedIn | BG1 | 0.1573 ± 0.01 | 0.0010 ± 0.00$^{A}$ | 0.0327 ± 0.00$^{A}$ | 0.0005 ± 0.00$^{A}$ | 1144.59 ± 46.97$^{C}$ | 116.03 ± 3.73$^{B}$ | 57.35 ± 1.93$^{C}$ | 8.10 ± 0.46$^{A}$ | 3 (1.8)$^{B}$ | 5 (1.0)$^{B}$ | 5 (0.0)$^{B}$ |
| | BG5 | 0.1774 ± 0.01 | 0.0012 ± 0.00$^{AB}$ | 0.0481 ± 0.00$^{B}$ | 0.0007 ± 0.00$^{B}$ | 999.19 ± 31.57$^{B}$ | 103.52 ± 2.61$^{A}$ | 48.40 ± 1.23$^{B}$ | 6.64 ± 0.38$^{A}$ | 4 (1.3)$^{C}$ | 4 (1.0)$^{B}$ | 5 (0.0)$^{B}$ |
| | BG2 | 0.1712 ± 0.01 | 0.0013 ± 0.00$^{B}$ | 0.0612 ± 0.00$^{C}$ | 0.0010 ± 0.00$^{C}$ | 877.21 ± 50.65$^{A}$ | 105.03 ± 4.18$^{A}$ | 43.87 ± 1.79$^{A}$ | 18.31 ± 1.22$^{B}$ | 2 (1.0)$^{A}$ | 2 (2.0)$^{A}$ | 1 (1.0)$^{A}$ |
| ProbTr | ÷ | 0.1660 ± 0.01 | 0.0011 ± 0.00$^{X}$ | 0.0375 ± 0.00$^{X}$ | 0.0006 ± 0.00$^{X}$ | 979.98 ± 37.83 | 107.96 ± 3.32 | 49.22 ± 1.43 | 12.38 ± 0.74$^{Y}$ | 4 (2.3)$^{Y}$ | 4 (3.0) | 5 (1.8)$^{X}$ |
| | + | 0.1713 ± 0.01 | 0.0012 ± 0.00$^{Y}$ | 0.0571 ± 0.00$^{Y}$ | 0.0009 ± 0.00$^{Y}$ | 1034.02 ± 48.30 | 108.43 ± 3.69 | 50.52 ± 1.87 | 9.66 ± 0.64$^{X}$ | 2 (1.0)$^{X}$ | 3 (1.0) | 5 (3.0)$^{Y}$ |
| *p*-values | FeedIn (F) | 0.149 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| | ProbTr (P) | 0.534 | 0.008 | <0.001 | <0.001 | 0.249 | 0.752 | 0.27 | <0.001 | <0.001 | 0.888 | 0.002 |
| | F × P | 0.789 | 0.452 | 0.366 | 0.263 | 0.083 | 0.603 | 0.448 | 0.003 | 0.008 | <0.001 | <0.001 |

BG1, marine-based feed; BG5, plant-based feed; BG2, soybean meal-based feed; FeedIn, factor feed ingredients; ProbTr, factor probiotics; ÷, without probiotics; +, with probiotics. SME, total area of skin mucous cells per total area of skin epithelium; SNE, number of skin mucous cells per total area of skin epithelium; GME, total area of gill mucous cells per total area of gill epithelium; GNE, number of gill mucous cells per total area of gill epithelium; VH, height of villi; VW, width of villi; EH, height of enterocyte; LPW, width of lamina propria; NM, number of intestinal mucous cells; IEL, number of intraepithelial lymphocytes; SNV, supra nuclear vacuoles. Significant differences ($p < 0.05$) among feed groups (BG1, BG5 and BG2) are indicated by uppercase superscripts A, B and C (based on the post-hoc tests for the group) and those between probiotic groups (without, ÷ and with, +) are indicated by X and Y in each column (based on the probiotic main effect). For interaction effects (F × P) and post-hoc results for each feed type, please refer to corresponding figures. Values for SME, SNE, GME, GNE, VH, VW, EH and LPW are presented as means ± SEM, n = 12 per treatment group. Parametric data were analysed by two-way ANOVA followed by Tukey's HSD test. The nonparametric score data (NM, IEL and SNV) were analysed with functions from ARTool package (nonparametric two-way ANOVA) followed by post-hoc tests using functions from emmeans package. Median, interquartile range (IQR) is reported for score data (NM, IEL, SNV).

interaction effect between feed ingredients and probiotics for the indices, GME or GNE.

### 3.2.3. Distal intestine histomorphometry

The morphological indices of the distal intestine, VH, VW, EH, LPW, NM, IEL and SNV, were differently affected by feed type (factor FeedIn) and probiotic treatment (factor ProbTr). In addition, the interaction (FeedIn×ProbTr) effects also depended on the indices. The marine-based feed group (BG1) had higher value for most of the indices except LPW (Table 4). The values for fish fed the plant-based feed (BG5) showed the same trend but ranked in between the values of the other two feed groups. Fish fed the feed with SBM (BG2) had the lowest values for most indices and had all the signs of enteritis (Table 4).

*3.2.3.1. Height of villi (VH).* The VHs of the feed groups were significantly different (Fig. 2A). Fish fed BG1 had longest villi, followed by fish fed BG5 and shortest villi was observed for fish fed BG2. Addition of probiotics did not alter the VH in fish fed BG1 and BG5, but VH tended to increase in the diet group, BG2+ (Fig. 2A). The interaction between feed ingredients and probiotics was not significant.

*3.2.3.2. Width of villi (VW).* Average VW for the fish fed BG1 was significantly higher compared to BG5 and BG2 (Table 4). Addition of probiotics did not alter the average VW in any of the feed groups (Fig. 2B). However, the probiotics tended to increase the VW in fish fed diets BG1+ and BG5+. The interaction FeedIn×ProbTr was not significant.

*3.2.3.3. Height of enterocyte (EH).* Feed type had a significant effect on the EH. Significantly shortest enterocytes were observed in fish fed BG2. The average EH in the fish fed BG1 was significantly 1.18- and 1.31-fold higher than BG5 and BG2, respectively (Fig. 2C). The probiotic treatment or interaction (FeedIn×ProbTr) did not have a significant effect on EH.

*3.2.3.4. Width of lamina propria (LPW).* The LPW was significantly affected by feed type and supplementation of probiotics. In addition, the two factors were found to interact with each other. Significantly wider lamina propria was observed in fish fed BG2 compared to BG1 and BG5. The LPW was significantly reduced in fish fed BG1+ and BG2+ while no changes were observed for fish fed the BG5+ (Fig. 2D).

*3.2.3.5. Number of distal intestinal mucous cells (NM).* The score for NM was significantly affected by feed ingredients (factor FeedIn) and probiotics (factor ProbTr) (Table 4 and Fig. 3A). Furthermore, the two factors were found to interact significantly (Table 4 and Fig. 3A). The scores for NM were significantly lower (more mucous cells, Supplementary Table 1) for fish fed BG2 compared to the other two feed groups. The NM per villus of fish fed diet groups BG1+ and BG5+ were significantly increased compared to the respective groups (BG1÷ and BG5÷) without probiotics. However, fish fed the BG2+ showed a decreased NM (higher score; less mucous cells). The percentage of the score for NM is shown in Fig. 3A.

*3.2.3.6. Number of intraepithelial lymphocytes (IEL).* The IELs were also significantly affected by feed ingredient composition (Table 4 and Fig. 3B). Although the factor ProbTr did not have an effect on IEL, the interaction of the factors was significantly different. The score for IEL was significantly lower (more IEL per simple villi, Supplementary table 1) for fish fed BG2 compared to those fed the other two feeds. The IEL score for fish fed diet groups BG1+ and BG5+ were significantly reduced compared to those without probiotics. However, the BG2+ group had a higher score (less IELs) compared to BG2÷ (Fig. 3B).

*3.2.3.7. Supranuclear vacuoles (SNV).* Fish fed BG1 and BG5 feeds had

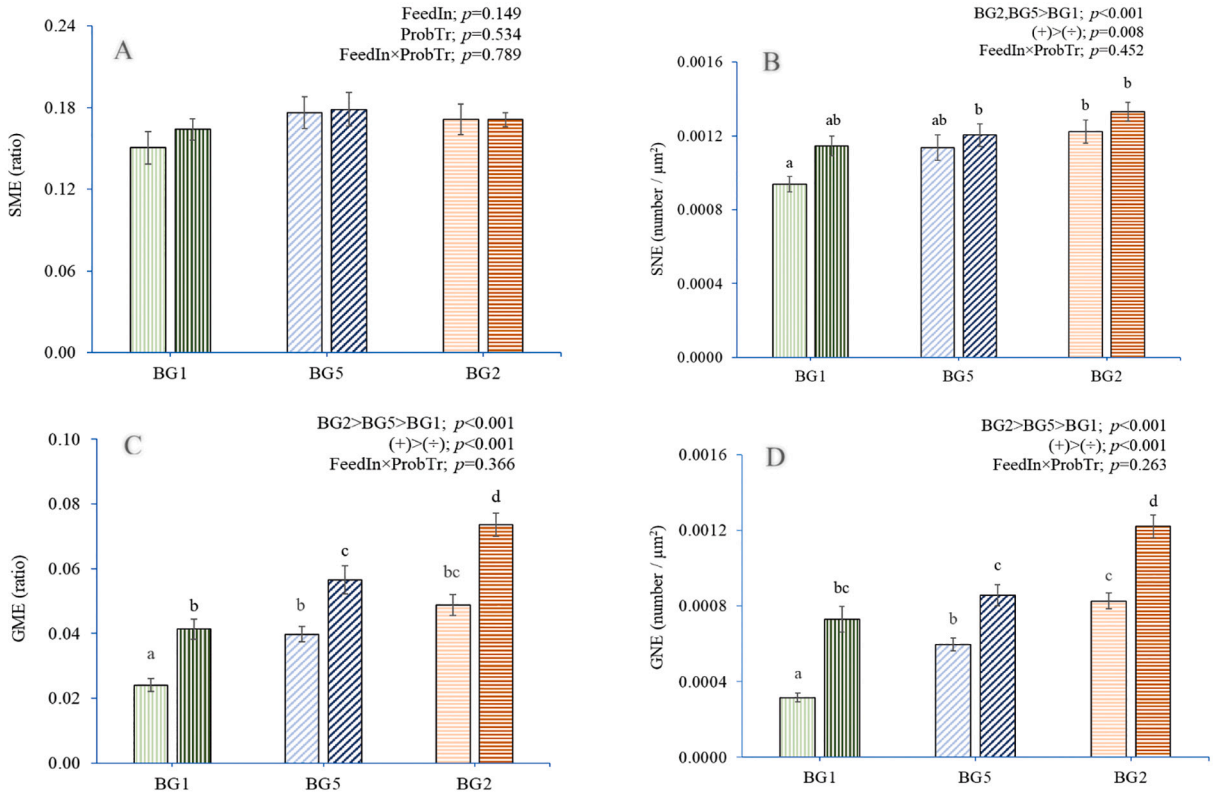


**Fig. 1.** Mucous cell-based indices associated with the skin and gills of Atlantic salmon offered different experimental feeds. (A) SME - the total area of mucous cells per total area of epithelium in the dorsal skin, (B) SNE - the number of mucous cells per total area of epithelium in the dorsal skin, (C) GME - the total area of mucous cells per total area of epithelium in the gills and (D) GNE - the number of mucous cells per total area of epithelium in the gills. BG1, BG5 and BG2 are marine-, plant- and soybean meal-based feeds, respectively. For each feed, light colour bar (left side) represents diet without probiotics (÷) and dark colour (right side) represents diet with probiotics (+). The effects of main factors (FeedIn and ProbTr) and their interaction (FeedIn×ProbTr) were determined by two-way ANOVA and *p* values are indicated in the upper right corner. Different lowercase letters denote significant difference ($p < 0.05$) among all diet groups; based on post-hoc (Tukey's HSD) tests. Values are presented as mean ± SEM.

larger SNVs along the entire apical part of the enterocytes in the villi. The SNV score (almost 5) of these two groups were not affected by probiotics (Table 4). On the other hand, the BG2÷ fed fish had the lowest score of 1 (almost no SNV in the enterocytes) and when the fish were fed probiotics we observed a significant increase in SNV. Scattered small SNV seemed to reappear in some enterocytes of the fish fed the diet BG2+ and it had received an average score of 1.7 out of 5 (Table 4 and Fig. 3C).

### 3.3. Gene expression

Relative expression of mucin genes in the skin, gills and intestine were found to be tissue specific (Table 5). The skin expressed *muc5ac1*, *muc5ac2* and *muc5b*. The gills expressed *muc5ac2* and *muc5b*. The distal intestine expressed only *muc2*. Relative expression of AMP genes in the skin and distal intestine of Atlantic salmon were also tissue specific. The skin expressed *def1* and *cathl1*. The distal intestine expressed *def3, def4* and *cathl1*. Results showed that feed ingredients (factor FeedIn), probiotics (factor ProbTr) and their interaction (FeedIn×ProbTr) affected the expression patterns of the genes in the 3 tissues differently. The results of the two-way ANOVA are presented in Table 5, and the effect of the interaction could be deciphered from Figs. 4–6.

#### 3.3.1. Dorsal skin

The transcription of mucin and AMP genes in the dorsal skin were significantly affected either by feed ingredients or probiotics (either with or without an interaction effect), the exception was the expression of *def1* (Fig. 4D). Feed ingredient composition (main effect of the factor FeedIn) significantly altered the transcription of *muc5ac2* (Fig. 4B) and *cathl1* (Fig. 4E), but not those of the other two mucin genes. Fish fed BG1 and BG5 had significantly higher expression of *muc5ac2* and *cathl1*, respectively (Table 5, Fig. 4B and E). Probiotics significantly upregulated the expression of mucin genes in BG5 and BG2; *muc5ac1* (Fig. 4A, factor ProbTr) and *muc5b* (Fig. 4C, factor ProbTr). On the other hand, the expression of these two genes were downregulated in BG1+ fed fish. As for *cathl1*, fish fed diet BG2+ showed upregulation, while other diets tended to downregulate the expression of the AMP gene. The interaction (FeedIn×ProbTr) was significant for *muc5ac1* and *muc5b* as well as for the AMP gene, *cathl1*.

#### 3.3.2. Gills

Expression of the mucin gene, *muc5ac2* in the gills was not significantly altered by either feed ingredient composition or probiotics (Fig. 5A). The mucin gene, *muc5b*, by contrast, was significantly affected by feed composition, and significantly higher expression was observed for the fish fed BG5 compared to BG2 (Table 5, factor FeedIn). When compared to fish fed plant-based feed (BG5), expression of *muc5b* was downregulated by 1.8-fold in fish fed SBM-based feed (BG2). Addition of probiotics to feed did not significantly affect the expression of *muc5b*. However, there was an upregulation (1.3-fold) and downregulation (1.4-fold) tendency in fish fed BG5+ and BG2+, respectively compared to BG5÷ and BG2÷ (Fig. 5B).

#### 3.3.3. Distal intestine

Expression of the mucin gene, *muc2* was affected only by the feed ingredient composition (factor FeedIn). Fish fed BG2 had significantly lower mucin mRNA levels compared to the other two feed groups. Probiotics did not influence the mucin expression in any of the feed

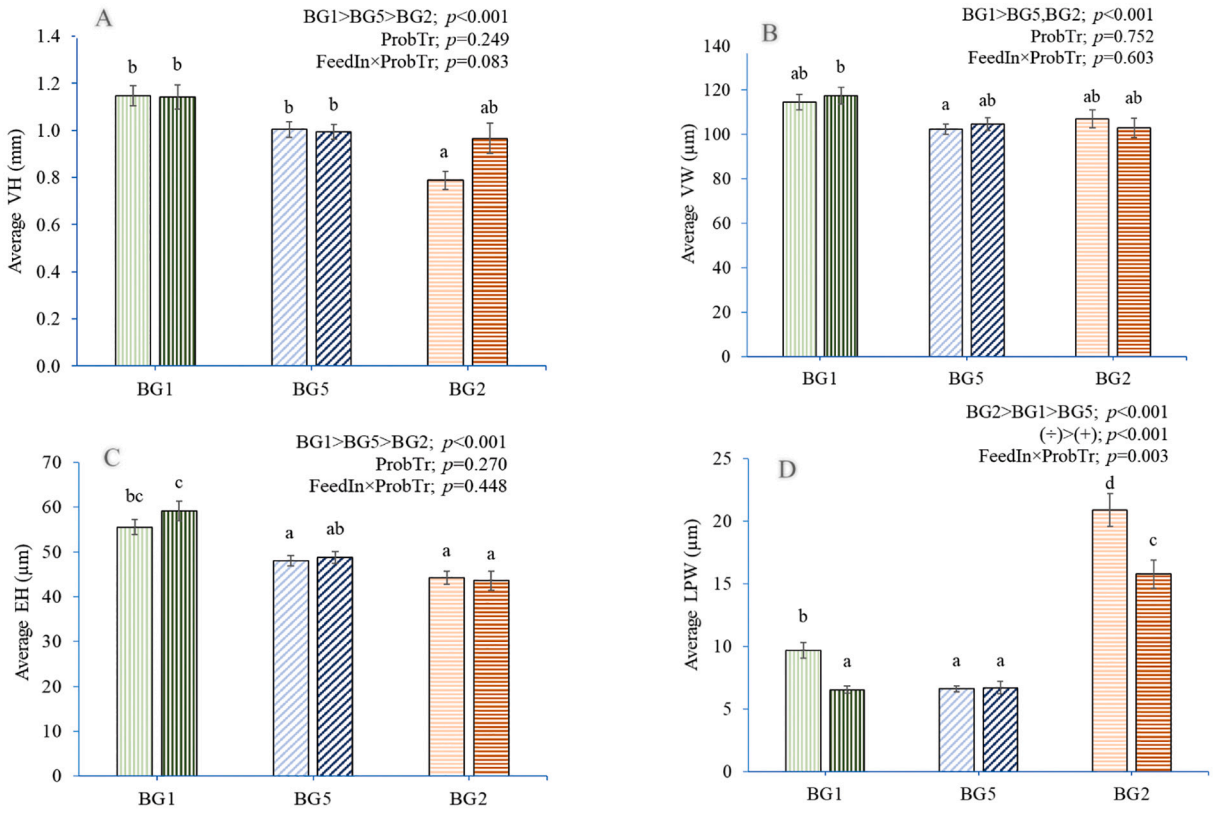


**Fig. 2.** Mucosa-based indices associated with the distal intestine of Atlantic salmon offered different feeds. (A) VH - height of villi, (B) VW - width of villi, (C) EH - height of enterocytes and (D) LPW - width of lamina propria. BG1, BG5 and BG2 are marine-, plant- and soybean meal-based feeds, respectively. For each feed, light colour bar (left side) represents diet without probiotics (÷) and dark colour (right side) represents diet with probiotics (+). The effects of main factors (FeedIn and ProbTr) and their interaction (FeedIn×ProbTr) were determined by two-way ANOVA and *p* values are indicated in the upper right corner. Different lowercase letters denote significant difference ($p < 0.05$) among all diet groups; based on post-hoc (Tukey's HSD) tests. Values are presented as mean ± SEM.

groups (Fig. 6A). Feed ingredient composition affected the expression of all AMP genes. Compared to other feed groups, fish fed BG2 had lower mRNA levels of *def3* (Fig. 6B) and *def4* (Fig. 6C, factor FeedIn). We observed an increasing trend in *def4* expression in the BG5+ fed fish (factor ProbTr; $p = 0.052$). However, fish fed BG1 had lower mRNA levels of *cathl1* (Fig. 6D). Supplementation of probiotics to the diet groups significantly influenced the AMP genes, especially *cathl1*. All probiotics-incorporated diet groups had significantly increased the expression of *cathl1* compared to their respective groups without probiotics. A significant interaction ($p = 0.056$) between feed ingredients and probiotics was observed for *def3*. The relative mRNA level of *def3* was upregulated in fish fed BG1+ and BG5+ compared to BG1÷ and BG5÷, while such a change was not observed for BG2. The mRNA level of *def4* was downregulated in fish fed BG1, while the mRNA levels in fish fed BG5 and BG2 were upregulated (not significantly; after probiotic feeding). The interaction between feed ingredients and probiotics (FeedIn×ProbTr) was not statistically significant.

### 3.4. Correlation between mucous cell indices and mucus-related gene expression data

Analysis of the data using Spearman correlation test revealed significant correlation between most of the histologically analysed mucous cell indices (Fig. 7). Significant positive correlations were observed for the following pairs: between SME and SNE ($r = 0.45$, $p < 0.001$), between GME and GNE ($r = 0.90$, $p < 0.001$). NM was positively correlated with GME ($r = 0.43$, $p < 0.001$) and GNE ($r = 0.50$, $p < 0.001$). Likewise, SNE was positively correlated with GME ($r = 0.43$, $p < 0.001$) and GNE ($r = 0.52$, $p < 0.001$). The correlations or the interactions among mucus-related genes from the skin, gills and intestine are also reported in Fig. 7. Significant correlation was also detected between histologically analysed mucous cells indices and most of the mucus-related gene data. SNE was positively correlated with skin *cathl1* ($r = 0.32$, $p = 0.007$) and negatively with skin *muc5ac2* ($r = -0.30$, $p = 0.015$). NM was positively correlated with intestinal *cathl1* ($r = 0.45$, $p = 0.001$) and negatively with intestinal *muc2* ($r = -0.38$, $p = 0.004$).

### 3.5. Short chain fatty acid composition

In total 7 short chain fatty acids were detected in the digesta and the sum of these SCFAs varied from 31 to 60 mmol/L, based on values from fish fed the different diets (Table 6). The total SCFAs were significantly affected by feed ingredient composition. Fish fed BG1 had significantly higher total SCFAs, followed by BG5 and BG2. Fish fed feeds without probiotics had significantly more total SCFAs than those with probiotics. The interaction between the two main factors (FeedIn×ProbTr) was not significantly different for the total SCFAs. Most of the individual SCFAs, except acetoacetic acids were significantly affected only by feed ingredients. Feeding with probiotics resulted in a significant reduction in acetoacetic acid and a tendency towards reduction in succinic acid ($p = 0.051$, Table 6) in digesta compared to those of fish fed diets without probiotics. Fish fed BG1 had more lactic acids, while BG5 feed groups had more acetoacetic acids in the digesta. Irrespective of feed groups, the concentration of butyric acid was the lowest among the determined SCFAs.

## 4. Discussion

Mucosal surfaces of the skin, gills and intestine with their inherent protecting capacity and arsenal of immune molecules are vital for the

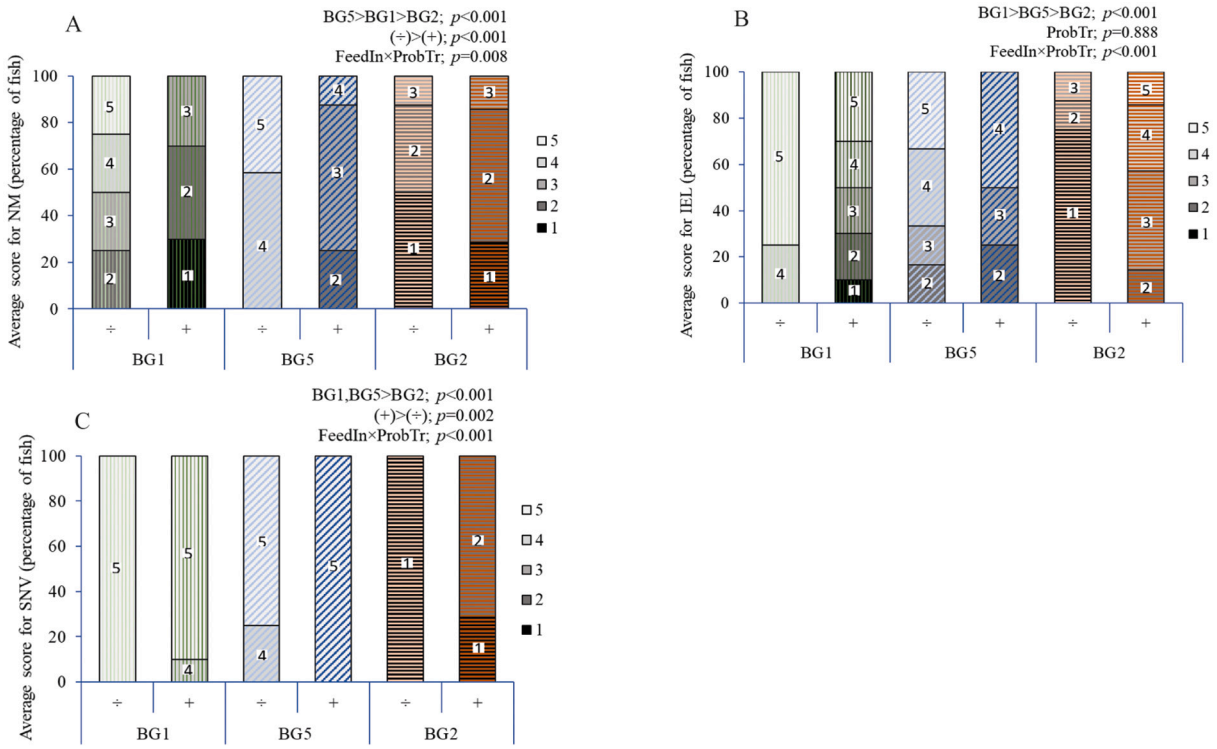


**Fig. 3.** Scores associated with the cells and cell feature of the distal intestine of Atlantic salmon offered different feeds. (A) NM - number of mucous cells, (B) IEL - number of intraepithelial lymphocytes and (C) SNV - supranuclear vacuoles per villi. BG1, BG5 and BG2 are marine-, plant- and soybean meal-based feeds, respectively. For each feed, light colour bar (left side) represents diet without probiotics (÷) and dark colour (right side) represents diet with probiotics (+). The effects of main factors (FeedIn and ProbTr) and their interaction (FeedIn×ProbTr) were determined by non-parametric two-way ANOVA and *p* values are indicated in the upper right corner. Scores are presented as percentage and legends indicate the scores. The labels on the stacked bar plots are the scores of a particular group.

health of fishes. A damaged mucosal surface in fish fails to effectively carry out its function, thereby making the fish susceptible to infectious diseases. The present study was designed to reveal the efficacy of both plant- or marine-based feeds and probiotics, by assessing the growth performance, morphology of the mucosal surfaces of the skin, gills and distal intestine, mucus-related gene expression in the aforementioned organs and SCFA composition in the digesta of Atlantic salmon. Overall, the present study showed that feed ingredient composition (FeedIn), probiotic treatment (ProbTr) and interaction between feed ingredients and probiotics (FeedIn×ProbTr) significantly affected the parameters of interest.

Use of probiotics isolated either from the GI (Ramesh et al., 2015) or mucus (Tapia-Paniagua et al., 2012) of aquatic animals could be considered as an efficient strategy to ensure sustainable aquaculture. In the present study, a mix of *Lactobacillus plantarum* R2 Biocenol™ (CCM 8674) and *Lactobacillus fermentum* R3 Biocenol™ (CCM 8675) were coated on the feeds. Earlier studies have indicated that a combination of two or more probiotic bacteria, including species from *Lactobacillus,* may improve growth and immune performance of the host aquatic animals (Alishahi et al., 2018; Beck et al., 2015; Foysal et al., 2020; Wang and Gu, 2010; Xu et al., 2012).

The LAB strains used in the present study were isolated from the intestinal content of rainbow trout (*Oncorhynchus mykiss*) and the bacteria were considered as probiotics based on the features, namely tolerance to different pH values, bile, temperature, antagonistic activity against salmonid pathogens such as *Aeromonas salmonicida* subsp. *salmonicida* CCM 1307 and *Yersinia ruckeri* CCM 6093 and the best growth properties *in vitro* (Fečkaninová et al., 2019). These probiotic strains have the potential for use in prevention, intervention or therapy of infections in aquaculture. Our previous study indicated that dietary supplementation with the two LAB strains modulated the composition and interaction of the intestinal microbiota of Atlantic salmon. *L. fermentum* feeding increased the bacterial diversity in the intestinal mucus of the fish (Gupta et al., 2019a). Among the LAB strains isolated from Chinese pickles, *L. fermentum* showed the most effective antibacterial activity against *Staphylococcus aureus* (Song et al., 2021). In a study with common carp, a diet supplemented with *L. fermentum* URLP18 at $2 \times 10^8$ CFU/g improved growth performance, non-specific immunity and health status and survival rate during a *Aeromonas hydrophila* challenge (Krishnaveni et al., 2021). Improved disease resistance was also demonstrated in tilapia fed *L. plantarum* prior to infecting the fish with the bacterial fish pathogen *Edwardsiella tarda* (Sherif et al., 2021). In the latter study, there was no difference in mortality between groups fed *L. plantarum* for 2 and 4 weeks prior to the infection with *E. tarda*. *L. plantarum* has also demonstrated a protective role in tilapia exposed to waterborne aluminum (Al) (Yu et al., 2017); the bacteria significantly increased feed utilization and growth performance, decreased the mortality of Al-exposed fish, reduced pathological conditions as well as Al accumulation in tissues. We did not include a challenge experiment as part of this study because our design was intended to investigate if there was any effect of supplementation of the two probiotics *L. fermentum and L. plantarum* in marine- or plant- derived feeds.

### 4.1. Effect of feed ingredients and probiotics on the growth performance

The 38-day long feeding study did not reveal any significant differences in most of the performance indices of the study groups. The plant-derived ingredients are approximately 3–6 times cheaper than fishmeal (The World Bank, 2021). Hence, our nonsignificant differences in the growth data indicate that cheaper non-marine source derived ingredients can impart the same growth in Atlantic salmon compared to marine-based ingredients. The lower condition factor of BG2 fed fish after 38 days of feeding can be in line with other studies that employed SBM in the diets of Atlantic salmon (Baeverfjord and Krogdahl, 1996;

**Table 5**
Gene expression in the skin, gills and intestine of Atlantic salmon.

| Tissues: | | Skin | | | | | Gills | | Intestine | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Gene type: | | AMPs | | Mucins | | | Mucins | | AMPs | | | Mucins |
| Parameters: | | *def1* | *cath1* | *muc5ac1* | *muc5ac2* | *muc5b* | *muc5ac2* | *muc5b* | *def3* | *def4* | *cath1* | *muc2* |
| Means of main effect: | | | | | | | | | | | | |
| FeedIn | BG1 | 0.63 ± 0.07 | 0.45 ± 0.06$^{A}$ | 0.43 ± 0.05 | 0.53 ± 0.07$^{B}$ | 0.30 ± 0.04 | 1.08 ± 0.09 | 0.12 ± 0.02$^{AB}$ | 1.05 ± 0.25$^{AB}$ | 1.10 ± 0.11$^{B}$ | 0.08 ± 0.03$^{A}$ | 2.75 ± 0.20$^{B}$ |
| | BG5 | 0.79 ± 0.09 | 0.65 ± 0.06$^{B}$ | 0.48 ± 0.06 | 0.35 ± 0.06$^{A}$ | 0.38 ± 0.04 | 1.02 ± 0.08 | 0.18 ± 0.03$^{B}$ | 1.38 ± 0.34$^{B}$ | 0.87 ± 0.14$^{A}$ | 0.10 ± 0.04$^{AB}$ | 2.53 ± 0.24$^{B}$ |
| | BG2 | 0.75 ± 0.06 | 0.44 ± 0.04$^{A}$ | 0.58 ± 0.10 | 0.35 ± 0.05$^{A}$ | 0.32 ± 0.05 | 0.91 ± 0.09 | 0.10 ± 0.02$^{A}$ | 0.40 ± 0.11$^{A}$ | 0.77 ± 0.08$^{A}$ | 0.12 ± 0.03$^{B}$ | 0.71 ± 0.05$^{A}$ |
| ProbTr | ÷ | 0.73 ± 0.07 | 0.50 ± 0.05 | 0.42 ± 0.06$^{X}$ | 0.37 ± 0.05 | 0.29 ± 0.04$^{X}$ | 1.01 ± 0.09 | 0.13 ± 0.02 | 0.59 ± 0.12 | 0.85 ± 0.11 | 0.04 ± 0.01$^{X}$ | 1.99 ± 0.15 |
| | + | 0.71 ± 0.07 | 0.53 ± 0.05 | 0.58 ± 0.08$^{Y}$ | 0.44 ± 0.06 | 0.37 ± 0.04$^{Y}$ | 0.99 ± 0.09 | 0.13 ± 0.02 | 1.30 ± 0.34 | 0.98 ± 0.12 | 0.16 ± 0.06$^{Y}$ | 2.00 ± 0.18 |
| *p*-values | FeedIn (F) | 0.089 | <0.001 | 0.627 | 0.006 | 0.169 | 0.165 | 0.005 | 0.001 | 0.002 | 0.028 | <0.001 |
| | ProbTr (P) | 0.803 | 0.283 | 0.014 | 0.159 | 0.021 | 0.927 | 0.458 | 0.065 | 0.052 | <0.001 | 0.939 |
| | F × P | 0.827 | 0.005 | <0.001 | 0.636 | 0.001 | 0.342 | 0.353 | 0.056 | 0.201 | 0.311 | 0.907 |

BG1, marine-based feed; BG5, plant-based feed; BG2, soybean meal-based feed; FeedIn, factor feed ingredients; ProbTr, factor probiotics; ÷, without probiotics; +, with probiotics. AMPs, Antimicrobial peptides; *def1, defensin1; def3, defensin3; def4, defensin4; cath1, cathelicidin1;* F × P, Interaction between feed type and probiotics. Significant differences ($p < 0.05$) among feed groups (BG1, BG5 and BG2) are indicated by uppercase superscripts A, B and C (based on post-hoc tests for the group), and between probiotic groups (without, ÷ and with, +) are indicated by the uppercase letters X and Y in each column (based on the probiotic main effect). For interaction effects and post-hoc results for each feed type, please refer to the corresponding figure. Values are presented as means ± SEM, n = 12 per treatment group. Data were analysed by two-way ANOVA followed by Tukey's HSD test.

Knudsen et al., 2007; Krogdahl et al., 2015; Sørensen et al., 2021; Urán et al., 2008b). A previous study showed that fish fed BG2 had morphological changes consistent with soybean meal-induced enteritis (Sørensen et al., 2021). This condition is associated with saponins in full fat soybean meal (Knudsen et al., 2007; Krogdahl et al., 2015). Heat treatment can dampen the action of heat stable antinutritional factors (ANFs) such as saponins, phytate, tannins, oligosaccharides, phytoestrogens (Drew et al., 2007; Liener, 1994). On the other hand, heat labile ANFs, typical proteins such as lectins and protease inhibitors are easily inactivated to safe levels during extrusion (Romarheim et al., 2006). All the experimental feeds were extruded prior to the LAB coating. Hence, we expect that heat labile ANFs in soybean meal were inactivated during extrusion, while the process did not remove the heat stable ANFs in the BG2 diet. Phytate is usually reduced through enzymatic treatment (Storebakken et al., 1998), and was most likely present in BG5. The duration of the experiment was too short to reveal effects of feed composition or probiotics on growth performance, in contrast to other studies that reported the ability of probiotics to improve growth performance, survival rate and health status of fish (Ramos et al., 2017; Wuertz et al., 2021; Xia et al., 2020).

### 4.2. *Effect of feed ingredients and probiotics on histology and gene expression*

#### 4.2.1. *Dorsal skin mucous cells, mucin and AMP genes*

Histological evaluation of salmon skin indicated that the feed ingredients and probiotics evoked changes in the microscopic structure of the epidermis. We observed an increase in the number of skin mucous cells per unit skin epithelium area (SNE) when the fish were fed plant- (BG5) and SBM-based (BG2) feeds. The fish fed marine-based feeds (BG1) had a significantly lower SNE. Based on the findings from the study of the intestine, antinutritional factors present in the plant and SBM-based feeds can cause intestinal inflammation (Krogdahl et al., 2015), which in turn can activate the mucosal immune system of skin, as described in Sørensen et al. (2021). Such a connection between local immune systems has been shown in many cases. In humans, for example, intestinal bowel disease is known to cause disturbances in the host defence system and overstimulate certain immune pathways, and this response can lead to cutaneous disorders such as sub-epidermal blisters (Huang et al., 2012). The increased SNE by probiotics indicate an activation of skin mucosal response as described in Hernandez et al. (2010).

The area of skin mucous cells per unit area of skin epithelium (SME) was not significantly affected by feed ingredients or the probiotic treatment. Nevertheless, we observed a positive correlation between SNE and SME. Marine-based feed groups had less SNE, but apparently larger mucous cells. On the other hand, SBM-based feed groups had higher SNE, so apparently, smaller mucous cells. Mucous cells in the epidermis are essential for the production of mucus (Pittman et al., 2013), and mucus contains mainly mucins, which either bind to outer layer of epidermis and provide additional layer of defence to protect epithelial cells, or create viscous gel that prevents microbial penetration (Dang et al., 2020; Dash et al., 2018). Hyperplasia of skin mucous cells seems to be a general response to unfavourable physiological factors, like stress and low pH (Zuchelkowski et al., 1985, 1981), chemical factors, like high water aluminium levels (Ledy et al., 2003), high water nitrate levels and low dissolved oxygen (Vatsos et al., 2010), or biological factors, like pathogens (van der Marel et al., 2010).

Fishes are constantly in contact with pathogens (opportunistic or obligatory), and when pathogens invade the skin, the mucous cells that are located in the epidermis, will continuously secrete mucus to physically remove the pathogens from the surface (Karlsen et al., 2018; Peatman et al., 2015). In addition, AMPs present in fish mucus kill pathogens by interacting directly and disturbing the osmotic pressure in microbial cells (Mahlapuu et al., 2016; Raju et al., 2020). Administration of probiotics to the plant-based feed tended to downregulate the mRNA levels of AMPs while the SBM-fed group had higher expression of

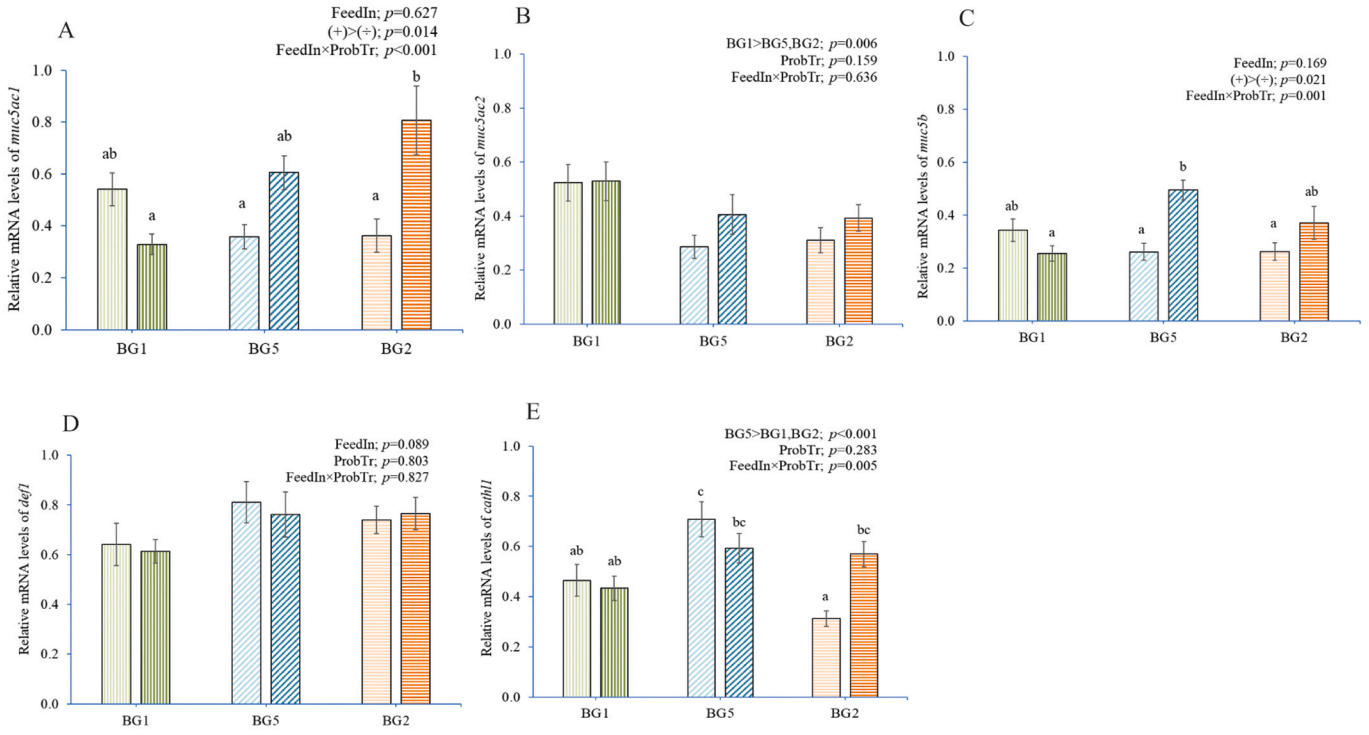


**Fig. 4.** Relative mRNA levels of mucin and antimicrobial peptide genes in the skin of Atlantic salmon fed different diets. (A) *muc5ac1*, (B) *muc5ac2*, (C) *muc5b*, (D) *def1* (*defensin1*) and (E) *cathl1* (*cathelicidin1*). BG1, BG5 and BG2 are marine-, plant- and soybean meal-based feeds, respectively. For each feed, light colour bar (left side) represents diet without probiotics (÷) and dark colour (right side) represents diet with probiotics (+). The effects of main factors (FeedIn and ProbTr) and their interaction (FeedIn×ProbTr) were determined by two-way ANOVA and *p* values are indicated in the upper right corner. Different lowercase letters denote significant difference ($p < 0.05$) among all diet groups; based on post-hoc (Tukey's HSD) tests. Values are presented as mean ± SEM.

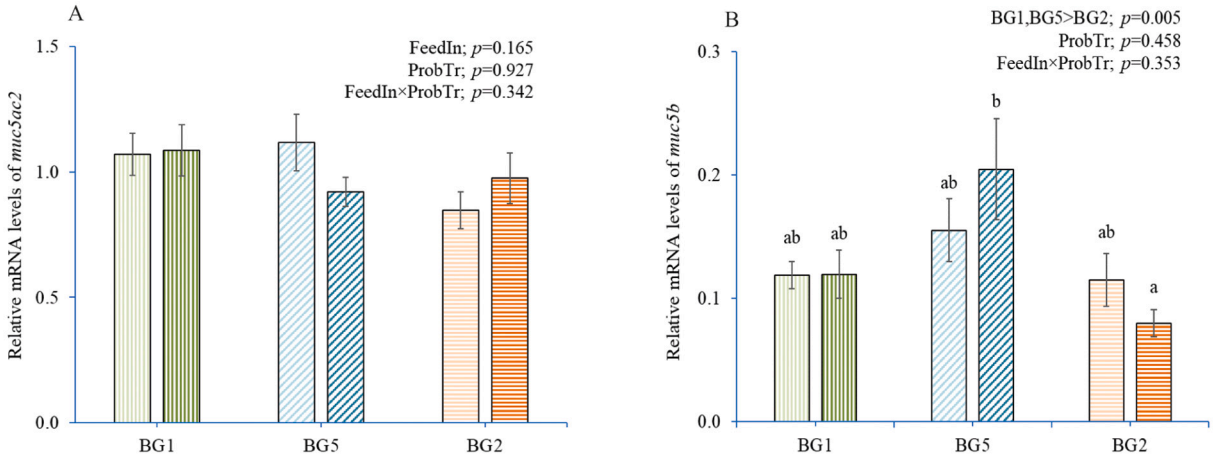


**Fig. 5.** Relative mRNA levels of mucin genes in the gills of Atlantic salmon fed different diets. (A) *muc5ac2* and (B) *muc5b*. BG1, BG5 and BG2 are marine-, plant- and soybean meal-based feeds, respectively. For each feed, light colour bar (left side) represents diet without probiotics (÷) and dark colour (right side) represents diet with probiotics (+). The effects of main factors (FeedIn and ProbTr) and their interaction (FeedIn×ProbTr) were determined by two-way ANOVA and *p* values are indicated in the upper right corner. Different lowercase letters denote significant difference ($p < 0.05$) among all diet groups; based on post-hoc (Tukey's HSD) tests. Values are presented as mean ± SEM.

AMP genes. Furthermore, the positive correlation between SNE and the skin *cathl1* suggests that probiotics might have influenced both the AMP gene expression and the number of skin mucous cells.

As for the mucin gene, *muc5ac2* it was downregulated in fish fed plant- and SBM-based feeds; this result is not in line with the histological observation of increased number of mucous cells in the skin. Although the probiotic supplementation did not influence the expression of a gel-forming mucin gene, *muc5ac2* in any of the diet groups, the combination of plant-based, or SBM-based feeds and probiotics caused an upregulation of two other gel-forming mucin genes, namely *muc5ac1* and *muc5b*. This observation agrees with the result on increased number of mucous cells observed in skin epidermis.

### 4.2.2. Gills mucous cells and mucin genes

Pathogens can increase the gill mucous cell number and mucus production (Andrews et al., 2010; Lødemel et al., 2001). Hyperplasia and hypertrophy of gill mucous cells are general responses to external stimuli (Dang et al., 2020, 2019; Haddeland et al., 2020). A correlation

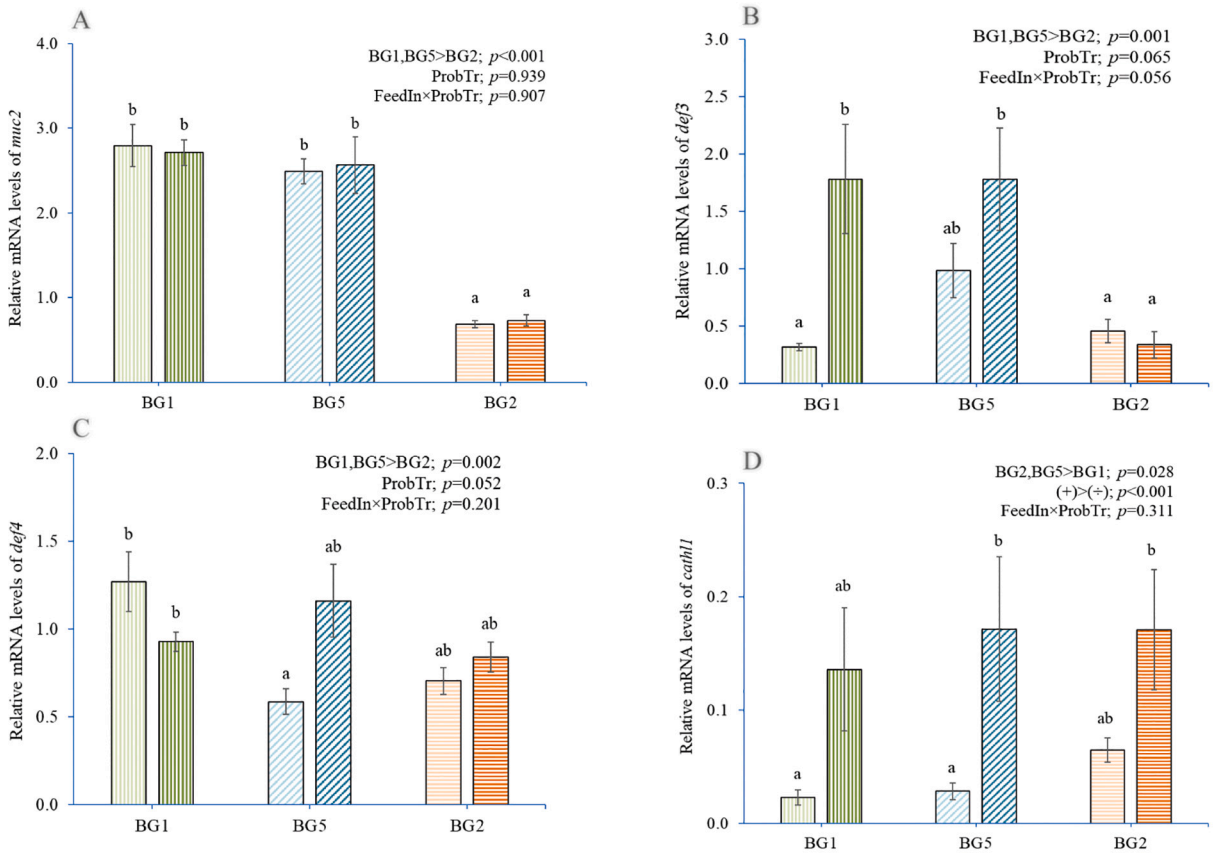


**Fig. 6.** Relative mRNA levels of mucin and antimicrobial peptides genes in the distal intestine of Atlantic salmon fed different diets. (A) *muc2*, (B) *def3* (*defensin3*), (C) *def4* (*defensin4*) and (D) *cathl1* (*cathelicidin1*). BG1, BG5 and BG2 are marine-, plant- and soybean meal-based feeds, respectively. For each feed, light colour bar (left side) represents diet without probiotics (÷) and dark colour (right side) represents diet with probiotics (+). The effects of main factors (FeedIn and ProbTr) and their interaction (FeedIn×ProbTr) were determined by two-way ANOVA and *p* values are indicated in the upper right corner. Different lowercase letters denote significant difference ($p < 0.05$) among all diet groups; based on post-hoc (Tukey's HSD) tests. Values are presented as mean ± SEM.

between number of mucous cells and the mucus secretion was documented by Bosi et al. (2005).

In the present study, our analyses detected significant effects of both feed ingredients and probiotics on the gill mucous cells. The parameter GME was used to assess the total area of gill mucous cells that cover unit area of gill epithelium. For fish fed marine-based feed without probiotics (BG1÷), it was 0.024, indicating that 100 $\mu m^2$ of gill epithelium is covered by 2.4 $\mu m^2$ of mucous cells. For the plant-based (BG5÷) and SBM (BG2÷)- based groups, GME values were 0.039 and 0.049, respectively. Probiotic incorporation in all the three feeds - marine-, plant- and SBM-based - significantly increased the GME by 1.7, 1.4 and 1.5 times than their corresponding groups without probiotics (BG1÷, BG5÷ and BG2÷), respectively. Having more mucous cells is linked to better disease resistance in the case of amoebic gill disease (Roberts and Powell, 2005). Therefore, probiotics used in our study have shown their potential to be included among the candidates that can be utilized in aquaculture disease prevention.

Histomorphometric analysis of fish gills showed a similar trend for GNE and GME. Furthermore, there was a positive correlation between GME and GNE, indicating that the GME might have increased due to increased GNE. GNE indicates the number of mucous cells per unit area of gill epithelium. Fish fed feeds without probiotics (÷) had lower value for GNE compared to feed groups with probiotics (+). Fish fed the marine-based feed (BG1÷) had on average 300 mucous cells per $mm^2$. For fish fed the plant (BG5÷)- and SBM-based feed (BG2÷), the GNE were 2 and 3 times higher compared to marine-based feed (BG1÷) groups, respectively. The feeds BG1+, BG5+ and BG2+ increased the number of gill mucous cells per unit area of gill epithelium (GNE) by 2.3, 1.4 and 1.5 times, respectively, compared to the respective fish groups fed feeds without probiotics. The dietary administration of probiotics might have altered the metabolism in the intestine and the metabolites (bile acids, lipoproteins, amino acids and SCFAs) might have translocated through blood to the gills, thus the increased response (Martin et al., 2007).

The relative mRNA levels of *muc5ac2* in the gills were unaffected by feed ingredients and probiotics. The lower expression of *muc5b* in fish fed BG2 indicates the gill health marker potential of the gene. Dietary administration of probiotics to BG2÷ feed groups further downregulated the expression of the mucin gene *muc5b*. However, an upregulation pattern was observed for fish fed BG5. A study has revealed that the number of goblet cells in the airway epithelium of rats increased and there was a subsequent increase in the expression of the mucin genes *muc5a* and *muc5b* (Kim et al., 2019). The significant positive correlation between gill mucous cell indices (GME and GNE) and other two mucous cell indices in the skin (SNE and SME) indicates the relationship between mucosal tissues in different organs and their response to different feed ingredients and probiotics. However, gill mucin gene expression results did not significantly correlate with histological observations related to gills (Fig. 7).

#### 4.2.3. Distal intestinal morphology, mucin and AMP genes

The height of the simple villi differed among the feed groups and this observation is in line with earlier studies (Baeverfjord and Krogdahl, 1996; Krogdahl et al., 2015; Moldal et al., 2014; Sohrabnezhad et al., 2017; Urán et al., 2009). Intact and longer villi are associated with more enterocytes, higher enzyme production, and improved absorption of

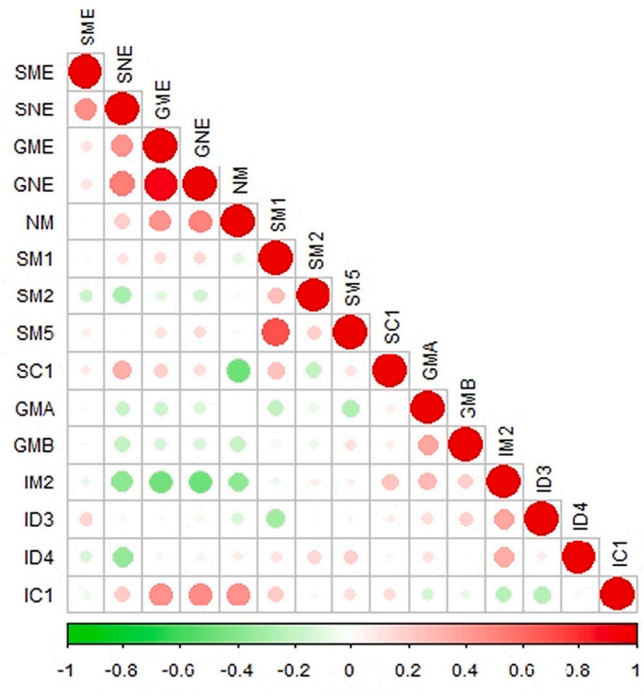


**Fig. 7.** Plot showing the correlation for all the combinations of histologically evaluated mucous cell parameters and the selected mucus-related genes. Skin *defensin1* is not shown because the correlation is not significant. Significant correlations ($p < 0.05$; Spearman rank correlation test) are shown using circles. Positive correlations are indicated by shades of red, and negative correlations are shown by shades of green. Blank cells indicate non-significant correlations between the variables. SME, the ratio between total area of mucous cells and total area of epithelium in the dorsal skin. SNE, the ratio between number of mucous cells and total area of epithelium in the dorsal skin. GME, the ratio between total area of mucous cells and total area of epithelium in the gills. GNE, the ratio between number of mucous cells and total area of epithelium in the gills. NM, number of intestinal mucous cells. SM1, skin *muc5ac1*. SM2, skin *muc5ac2*. SM5, skin *muc5b*. SC1, skin *cathelicidin1*. GMA, gill *muc5ac2*. GMB, gills *muc5b*. IM2, intestine *muc2*. ID3, intestine *defensin3*. ID4, intestine *defensin4*. IC1, intestine *cathelicidin1*. *(For interpretation of the references to colour in this figure legend, the reader* should*referthe web version of this article.)*

nutrients (Caspary, 1992). Wild caught post-smolt Atlantic salmon weighing 120 g was reported to have villi of height 0.7 mm (Løkka et al., 2013). In the present study, fish fed the marine-based diet had the longest villi (on average 1.1 mm), but SBM-based feed reduced the height to 0.87, due to inflammation. Feeding the fish with probiotics increased the villi height in the SBM fed group. This result is in line with earlier studies that reported improved intestinal structure and immunity in tilapia fed lactic acid bacteria (Pirarat et al., 2011) and increased villi height in rainbow trout fingerlings fed probiotic-supplemented (*Bacillus cereus*) diet (Gisbert et al., 2013). Even in piglets, probiotic feeding increased villi height (Gāliņa et al., 2020). The lactic acid bacteria, *Pediococcus acidilactici* in combination with short chain fructooligosaccharides increased villi height in the anterior intestine of Atlantic salmon reared in sea cages (Abid et al., 2013). The two probiotic strains used in the present experiment might have colonized the intestine, as noted in a previous study (Gupta et al., 2019a) and fermented the oligosaccharides to produce more total SCFAs, thus improving the villi structure. The fish fed marine-based feed had significantly wider villi compared to the other two feed groups, and the administered probiotics did not affect the villi width in Atlantic salmon, as observed in rainbow trout fed probiotic-supplemented (*Bacillus cereus*) feed (Gisbert et al., 2013). Average width of villus was calculated based on measurements taken at 5 different locations of a villus (Supplementary Fig. 3). Although the fish fed SBM-based feed had widened villi, quantitative measurements revealed that the villi width of plant-based feed group was similar to that of SBM-based feed group. Width of the villi was calculated considering both height of enterocytes (two sides) and width of lamina propria. It should be noted that in the fish fed the SBM-based feed, the height of enterocytes decreased while the width of the lamina propria increased. Therefore, we did not observe any significant differences in the overall width in fish fed the plant- and the SBM-based feeds. Nonetheless, studies that assessed SBM-induced enteritis reported widening of villi width (Moldal et al., 2014); based on semi-quantitative scoring. Our findings suggest that width of villi cannot solely be used as an index to quantify the morphological changes in the distal intestine. Other indices like height of the enterocytes and width of the lamina propria should also be included.

The present study has also evaluated the height of the enterocytes (columnar epithelium) in the distal intestine of Atlantic salmon. Marine-based feed in the present study provided essential nutrients including amino acids to the fish so that the columnar epithelium can develop

**Table 6**
Short-chain fatty acid concentration (mmol/L) in the digesta of the study groups.

| Parameters: | | Formic acids | Acetoacetic acids | Lactic acids | Succinic acids | Acetic acids | Propionic acids | Butyric acids | Total acids |
|---|---|---|---|---|---|---|---|---|---|
| Means of main effect: | | | | | | | | | |
| FeedIn | BG1 | $4.83 \pm 0.27^{C}$ | $10.99 \pm 0.59^{A}$ | $16.73 \pm 0.65^{C}$ | $9.63 \pm 0.52^{B}$ | $12.50 \pm 0.72^{B}$ | $3.83 \pm 0.42^{B}$ | $2.56 \pm 0.60^{B}$ | $59.93 \pm 1.55^{C}$ |
| | BG5 | $3.14 \pm 0.31^{B}$ | $15.04 \pm 0.78^{B}$ | $6.73 \pm 0.45^{B}$ | $5.99 \pm 0.34^{A}$ | $8.09 \pm 0.64^{A}$ | $2.56 \pm 0.26^{A}$ | $0.86 \pm 0.07^{A}$ | $41.92 \pm 1.14^{B}$ |
| | BG2 | $2.42 \pm 0.20^{A}$ | $10.04 \pm 0.46^{A}$ | $4.59 \pm 0.36^{A}$ | $5.52 \pm 0.24^{A}$ | $9.40 \pm 0.90^{A}$ | $2.32 \pm 0.27^{A}$ | $1.12 \pm 0.14^{A}$ | $34.56 \pm 1.95^{A}$ |
| ProbTr | ÷ | $3.58 \pm 0.30$ | $12.45 \pm 0.79^{Y}$ | $9.09 \pm 0.43$ | $7.39 \pm 0.38$ | $10.52 \pm 0.86$ | $3.06 \pm 0.36$ | $1.94 \pm 0.40$ | $47.21 \pm 1.56^{Y}$ |
| | + | $3.35 \pm 0.22$ | $11.59 \pm 0.43^{X}$ | $9.75 \pm 0.55$ | $6.71 \pm 0.35$ | $9.48 \pm 0.65$ | $2.75 \pm 0.27$ | $1.08 \pm 0.15$ | $43.73 \pm 1.53^{X}$ |
| Means of interaction effect: | | | | | | | | | |
| BG1 | ÷ | $5.14 \pm 0.43^{d}$ | $10.23 \pm 0.62^{b}$ | $16.12 \pm 0.68^{d}$ | $10.00 \pm 0.40^{b}$ | $12.70 \pm 0.87^{b}$ | $4.24 \pm 0.52^{b}$ | $3.01 \pm 0.89$ | $60.24 \pm 1.35^{d}$ |
| | + | $4.51 \pm 0.11^{cd}$ | $11.75 \pm 0.55^{b}$ | $17.78 \pm 0.63^{d}$ | $9.27 \pm 0.64^{b}$ | $12.30 \pm 0.57^{b}$ | $3.42 \pm 0.32^{ab}$ | $2.10 \pm 0.32$ | $59.61 \pm 1.75^{d}$ |
| BG5 | ÷ | $3.53 \pm 0.36^{bc}$ | $14.90 \pm 1.1^{cd}$ | $6.65 \pm 0.36^{bc}$ | $6.19 \pm 0.40^{a}$ | $9.03 \pm 0.69^{a}$ | $2.59 \pm 0.30^{a}$ | $1.72 \pm 0.14$ | $44.10 \pm 1.41^{c}$ |
| | + | $2.76 \pm 0.27^{ab}$ | $15.18 \pm 0.47^{d}$ | $6.82 \pm 0.53^{c}$ | $5.80 \pm 0.27^{a}$ | $7.15 \pm 0.58^{a}$ | $2.54 \pm 0.22^{a}$ | NA | $39.74 \pm 0.86^{bc}$ |
| BG2 | ÷ | $2.08 \pm 0.12^{a}$ | $12.23 \pm 0.66^{bc}$ | $4.51 \pm 0.24^{a}$ | $5.99 \pm 0.33^{a}$ | $9.81 \pm 1.02^{ab}$ | $2.36 \pm 0.26^{a}$ | $1.11 \pm 0.16$ | $37.29 \pm 1.92^{ab}$ |
| | + | $2.77 \pm 0.28^{ab}$ | $7.85 \pm 0.26^{a}$ | $4.66 \pm 0.48^{ab}$ | $5.06 \pm 0.15^{a}$ | $8.99 \pm 0.79^{a}$ | $2.29 \pm 0.28^{a}$ | $1.14 \pm 0.12$ | $31.83 \pm 1.98^{a}$ |
| *p*-values | FeedIn(F) | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.002 |
| | ProbTr (P) | 0.472 | 0.048 | 0.240 | 0.051 | 0.106 | 0.276 | 0.492 | 0.010 |
| | F × P | 0.013 | <0.001 | 0.460 | 0.214 | 0.616 | 0.458 | 0.383 | 0.291 |

BG1, marine-based feed; BG5, plant-based feed; BG2, soybean meal-based feed; FeedIn, factor feed ingredients; ProbTr, factor probiotics; ÷, without probiotics; +, with probiotics. F × P, Interaction between feed type and probiotics. NA, No data available. Valeric acid was tested, but not detected in any of the diet groups. The uppercase letters A, B and C (based on post-hoc tests) represent significant differences ($p < 0.05$) among feed groups (BG1, BG5 and BG2); and the uppercase letters X and Y (based on the probiotic main effect) represent significant differences between the probiotic groups (without, ÷ and with, +). Significant differences ($p < 0.05$) among all groups are indicated by different superscripts (a, b, c, or d; post hoc results for each feed type) in each column. Values are mean ± SEM, n = 10 per treatment group. Data were analysed by two-way ANOVA followed by Tukey's HSD test.

properly without any height-associated defects. When compared to marine-based feed, on average, a nine and 13.5 μm reduction in the height of intestinal epithelium was observed in the fish fed plant- and SBM-based feed, respectively. Indeed, enterocytes of the distal intestine of Atlantic salmon are the first cells that are affected when the fish are fed SBM-based diets (Urán et al., 2008a, 2008c). The present study quantitatively confirmed that the intestinal epithelium height was reduced in fish fed plant- and SBM-based ingredients.

Previous studies have assessed the width of the lamina propria by semi-quantitative scoring (Knudsen et al., 2007). The present study has used a quantitative approach and found a widening of lamina propria in fish fed SBM-incorporated feed. SBM-induced enteritis causes widening of the central stroma of the mucosal folds (Baeverfjord and Krogdahl, 1996). The present study showed that administration of probiotics significantly reduced the width of the lamina propria in fish fed BG1+ and BG2+. Probiotics also reduced the number of intraepithelial lymphocytes in the group fed BG2+ that had intestinal inflammation. In contrast, feeding *P. acidilactici* was found to increase the number of intraepithelial lymphocytes in Atlantic salmon (Vasanth et al., 2015). The present study also suggests that probiotics alleviate the progression of inflammation caused by SBM. A possible mechanism could be that probiotics reduce the lamina propria width possibly by suppressing the influx of the inflammatory cells. Other studies with mammals have also shown improved intestinal tight-junction and barrier function via modulation of protein components (Sultana et al., 2013; Yang et al., 2016). However, further research is needed to assess the inflammatory response markers.

In the present study, acid and neutral goblet cells were found scattered among the intestinal epithelial cells of fish fed BG1 and BG5. Administration of probiotics to these groups further increased the number of mucous cells. This is in line with other studies in fish that reported increased proliferation and differentiation of goblet cells and a consequent increase in mucus secretion in seabream fed *L. fermentum* (Dawood et al., 2015). Furthermore, *L. rhamnosus* or *P. acidilactici* feeding was found to increase the number of mucous cells in tilapia intestine (Pirarat et al., 2011; Standen et al., 2013). Moreover, higher goblet cell density was reported in rainbow trout fed *Spirulina platensis* (Sheikhzadeh et al., 2019). Dietary and oral administration of probiotic was also found to increase the number of goblet cells in the intestine of mice (El Aidy et al., 2013), piglets (Gāliņa et al., 2020; Zhang et al., 2017) and pigs (Desantis et al., 2019).

A possible mode of action of probiotics is that they colonize the mucus and make use of the mucin molecules as carbon, nitrogen and energy sources (Meslin et al., 1999). They release end-products of mucus fermentation, different secretory metabolites, and bioactive factors, which activate diverse signalling cascades and secretory elements that affect goblet cells. Members of microbiota can release proteolytic enzymes like meprin β from the apical membrane of enterocyte. Meprin β helps in the detachment of mucus from goblet cells and the metalloprotease cleaves the N-terminal region of the MUC2 mucin (Derrien et al., 2010; Schütte et al., 2014). Moreover, probiotic structural elements, such as lipopolysaccharides, flagellin A, and lipoteichoic acids or several metabolites (adenosine triphosphate) can regulate mucin gene expression by affecting the host immune responses (Dharmani et al., 2009).

The increased number and aggregated mucous cells in fish fed SBM-based feed (BG2) may be a general response to inflammation. Interestingly and in contrast to the observations in the skin and gill histomorphometry, the group fed the SBM-based diet supplemented with the probiotics (BG2+), did not show a further increase in the number of mucous cells, compared to BG2÷ but a decrease. A possible explanation for this observation, could be that feeding fish with SBM and probiotics, both factors that tend to increase the production of mucus, for a prolonged period of time, could have led to a depletion of the mucous cells. This has been observed in many cases of chronic intestinal inflammation, wherein the initial increased mucus production was markedly decreased after a while (Dharmani et al., 2009; Kim and Ho, 2010). However, both BG5+ and BG2+ had almost similar scores linked to mucous cells. This indicates a potential interaction of the probiotics and the different feed ingredients on the number of mucous cells.

In the present study, the *muc2* expression in the distal intestine was not altered by the administration of probiotics. The upregulation of the AMP genes *def3*, *def4* and *cathl1* with the administration of probiotics suggests increased immune responses (Rakers et al., 2013). Moreover, significant positive correlation between *cathl1* and NM indicates that feed ingredients or probiotics influenced the AMP gene, *cathl1* and increased the mucous cells number in the intestine of Atlantic salmon. Intestinal mucin gene *muc2* was positively correlated with skin *cathl1* ($r = 0.24$, $p = 0.051$) and gill *muc5ac2* ($r = 0.28$, $p = 0.018$), indicating the association of the mucosal areas in different mucosal tissues (Fig. 7). In colonic murine mucosa, *cathelicidin* gene was upregulated by bacterial DNA through Toll-like receptor-mediated pathway (Koon et al., 2011). Intestinal inflammation breaks the mucosal barrier, which in turn gives way for opportunistic bacteria to translocate into the intestinal layers (Vrakas et al., 2017). *Cathelicidins* were upregulated in the inflamed intestine of Atlantic salmon fed soy saponin (Kiron et al., 2020). It is also stated that gastrointestinal tract disorders can be treated through supplementation of *cathelicidin* peptides (Chow et al., 2013). Hence, probiotic-induced *cathl1* can be considered as a strategy to counteract intestinal inflammation.

Supranuclear vacuoles in the distal intestinal enterocytes of Atlantic salmon appear approximately 54 days post hatch (Sahlmann et al., 2015). Macromolecules like proteins are taken up via pinocytosis in epithelial cells, and some intracellular proteins like ferritin ends up in supranuclear vacuoles (Elbal et al., 2004; He et al., 2012; Rombout et al., 1985). Endocytic vesicles and lysosomes fuse, and subsequently ferritin digestion occurs in the SNVs. Accumulation of SNV in distal intestinal enterocytes of Atlantic salmon has also been reported previously (Baeverfjord and Krogdahl, 1996; Krogdahl et al., 2003; Sanden et al., 2005; Urán et al., 2008a). The marine-based feed group in the present study had large SNVs along the entire apical part of the distal intestinal enterocytes. In the present study, fish fed the plant-based feed also had similar characteristics. However, the fish fed SBM-based feed developed enteritis and lacked SNVs. Such anomalies have also been reported by other authors (Bakke-McKellep et al., 2000; Krogdahl et al., 2003; Krogdahl and Bakke-McKellep, 2005; Nordrum et al., 2000; Urán et al., 2009). Disappearance of SNVs is associated with reduced endocytosis or uptake block (Urán et al., 2008c), corroborating with the results on the absence of small SNVs. Probiotic feeding in the present study helped in the reappearance of SNVs in the enterocytes of the fish. Thus, we suggest new modes of action of probiotics on the host health; enhancing endocytosis and aiding in subsequent reappearance of the SNVs.

### 4.3. Effect of feed ingredients and probiotics on short chain fatty acids

Intestinal microbiota utilizes dietary ingredients especially certain fibres, and by fermenting them they produce SCFAs which are absorbed by the intestinal epithelium of fish. Of these SCFAs, butyrate is utilized by the intestinal epithelial cells as an energy source, propionate is taken up by liver and high levels of acetate can be detected in blood (Louis and Flint, 2017). The SCFAs improve growth and health of the fish because they reduce the luminal pH, avoid infections, strengthen immune system and maintain mucosal integrity (Adorian et al., 2020; Guillon and Champ, 2000; Hoseinifar et al., 2017; Park and Floch, 2007). Supplementation of dietary fibres, which can be utilized by microbiota to produce SCFAs (e.g. acetic and butyric acid) in the digesta, was shown to influence the intestinal mucous cells as well as skin mucus production in fish (Adorian et al., 2020).

In the present study, plant- and SBM-based feeds were expected to provide more fibres than marine-based feed but did not result in more SCFAs. It has been reported that there is no linear correlation between dietary fibre and SCFA concentration in rat cecum (Den Besten et al.,

2013; Levrat et al., 1991). Moreover, administration of probiotics was also expected to influence the microbiota because one of the probiotics (*Lactobacillus*) was found to be a core member in Atlantic salmon (Gupta et al., 2019a). Thus, feed ingredient composition and probiotics were expected to alter the SCFA profile. The level of SCFAs in salmon faeces was in the same range as reported for rats (Campbell et al., 1997). In the rat study, the SCFAs ranged from 36 to 61 mmol/L depending on the intake of fibre, but the ratio between the acetate:propionate:butyrate remained the same. We also observed a significant reduction in total SCFAs in the plant- (BG5) and SBM (BG2)-based feed consumed fish compared to the marine-based feed group. The ratio between acetic: propionic acids was in the range 3 to 4.16; lower in the BG1 fed fish and higher for the BG2 fed group. The ratio between propionic:butyric acids was between 1.41 and 2.1. Marine-based and plant or SBM-based diet can shape the SCFA profile differently; we observed a shift in dominant SCFA (from lactic acid to acetoacetic acid). The plant or SBM-based feeds, BG5 and BG2 contained more long fibres rather than oligosaccharides and type of fibre is known to affect the formation and profile of SCFAs. Fish fed plant or SBM-based ingredients had lower faecal dry matter content (Sørensen et al., 2021), which could explain the lower concentration of SCFAs.

In humans, acetate, propionate and butyrate account for 85–95% of the SCFAs and acetic acid alone accounts for more than 50% (Markowiak-Kopeć and Śliżewska, 2020). Studies of human microbiota have revealed the relationship between SCFAs and microbiota, and intestinal microbiome balance maintenance and microbial metabolite production stimulation by probiotic microorganisms (Markowiak-Kopeć and Śliżewska, 2020; Tsukuda et al., 2021). Acetic acid was a dominant SCFA in the present experiment also, but lactic acid and acetoacetic acid were higher in fish fed BG1 and BG5, respectively. Research with rats has also shown that SCFAs are involved in MUC gene transcription and thickness of mucous layer; feeds that provide more SCFAs, but low proportion of butyrate, help in forming thicker mucous layer in the colon (Hedemann et al., 2009). In line with this, our experiment showed the best gut health in fish fed BG1, producing the highest concentration of total SCFAs, with a rather high concentration of acetic acid. The SCFAs provide energy to the intestinal epithelium cells and stimulates the release of gastrointestinal peptide or growth factors which may affect cell proliferation, thereby increasing villi height (Blottiere et al., 2003; Pelicano et al., 2005). Although we observed only lower concentration of butyric acid in the digesta, the SCFA may still have an important role for intestinal health in Atlantic salmon. In the present study, administration of probiotics had significantly reduced the total SCFAs as well as the acetoacetic acid concentration in digesta. Campbell et al. (1997) observed the lowest concentration of SCFAs in the intestine of rats fed cellulose instead of short chain fibres. A noteworthy observation in the present study was that the probiotic administration tended to reduce the total SCFAs ($p = 0.010$), mainly because of a significant reduction in acetoacetic acids in fish fed the BG2 diet. However, further research should confirm how probiotics could shift the microbiota profile in the host intestine.

## 5. Conclusion

The present study has shown that number of mucous cells in the dorsal skin, gills and distal intestine were affected by feed ingredient composition and probiotics. Appearance of many mucous cells can be interpreted as an overall immune response to intestinal inflammation. The distal intestinal histomorphology of fish was influenced by the feed ingredient composition. Intestinal indices of fish fed plant-based feed was almost similar to that of fish fed marine-based feed. However, fish fed SBM-based feed developed enteritis. Addition of probiotics to SBM-based feed groups did not completely prevent the development of enteritis. However, positive responses like increased villi height, reduced width of lamina propria, reduced number of intraepithelial lymphocytes and reappearance of supra nuclear vacuoles were observed in Atlantic salmon post-smolts. Expression of mucin and AMP genes were tissue specific and the mRNA levels were affected by feed ingredient composition and probiotics. Correlation between mucous cell histomorphometric indices and gene expression data suggests that feed ingredients or probiotics influence both the mucus cell counts and mucus-related gene expression. Moreover, short chain fatty acid composition was also altered. In order to boost innate immune response and enhance intestinal health, the probiotics employed in the present study can be incorporated in marine- and plant-based feed without compromising fish growth. Although probiotics tended to alleviate the feed induced inflammation, further knowledge should be acquired if these probiotics are to be used as supplements in SBM-based salmon feed.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

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