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Supplementation of lactic acid bacteria has positive effects on the mucosal health of Atlantic salmon (*Salmo salar*) fed soybean meal

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ABSTRACT

We investigated the ability of lactic acid bacteria, when added individually or in combination in feeds, to prevent soybean meal-induced enteritis in Atlantic salmon. A control diet, designed to induce enteritis, was formulated with marine ingredients and 20% soybean meal. Three more diets were produced by coating the control diet with two bacteria, either singly (*Lactobacillus plantarum*; *L. fermentum*) or in combination. The fish were fed the abovementioned diets for 38 days. We performed histological assessments and evaluated the expression of selected mucin and antimicrobial peptide genes in the dorsal skin, gills, and distal intestine. Digesta were also collected to study the short chain fatty acids. Feeding bacteria, individually or in combination, altered the short chain fatty acids−acetoacetic acid, lactic acid, succinic acid, propionic acid−and the total fatty acids in the digesta significantly. Of all the determined short chain fatty acids, the concentration of acetoacetic acid was the highest, and the fish fed the combination of the two bacteria had the significantly highest value. Succinic acid was also significantly higher in fish fed the combination compared to the control group and the *L. fermentum* group. Total fatty acids were significantly higher in fish fed the combination than those fed *L. fermentum*. Compared to the control and probiotic combination-fed fish, those fed *L. plantarum* had higher *defensin1* expression in the skin. We also observed significantly higher number of gill mucous cells in the fish fed the blend compared to the control group. Lamina propria width was significantly reduced in fish fed the blend. Supra nuclear vacuoles were higher in fish fed the single species or the blend, compared to the control group. Thus, adding the probiotics to a soybean meal diet can elevate the digesta short chain fatty acids and intestine supranuclear vacuoles, and reduce the lamina propria width, which probably indicate prevention of enteritis.

Abbreviations[1,2]: AB, Alcian blue; AMPs, Antimicrobial peptides; ANOVA, Analysis of variance; CF, Condition factor; CFU, Colony forming unit; CT, Control diet; DNA, Deoxyribonucleic acid; FDU, Forsøksdyrutvalget; FL, Final fork length; FW, Final body weight; GE, Total area of gill epithelium; GM, Total area of gill mucous cells; GM2, Gills *muc5ac2*; GM5, Gill *muc5b*,; GME, Total area of gill mucous cells (GM) / Total area of gill epithelium (GE); GN, Number of gill mucous cells; GNE, Number of gill mucous cells (GN) / Total area of gill epithelium (GE); HOE, Height of enterocytes; HOV, Height of villi; HSB, Hue, saturation and brightness; HSD, Honestly significant difference; IC1, Intestine *cathelicidin1*,; ID3, Intestine *defensin3*; ID4, Intestine *defensin4*; IEL, Number of intraepithelial lymphocytes; IL, Initial fork length; IM2, Intestine *muc2*; IW, Initial body weight; LAB, Lactic acid bacteria; LAS, Leica Application Suite; LF, Diet with probiotic bacteria *Lactobacillus fermentum*; LP, Diet with probiotic bacteria *Lactobacillus plantarum*; LP&LF, Diet with both probiotic bacteria *L. fermentum* & *L. plantarum*; MRS, Man, Rogosa and Sharpe; NBF, Neutral buffered formalin; NOM, Number of mucous cells (in distal intestine); PAS, Periodic acid–Schiff; PC, Principal component; PCA, Principal component analysis; PCR, Polymerase chain reaction; RNA, Ribonucleic acid; RVC, Rotating vacuum coater; SBM, Soybean meal; SBMIE, Soybean meal induced enteritis; SC1, Skin *cathelicidin1*; SCFAs, Short chain fatty acids; SD1, Skin *defensin1*; SE, Total area of skin epithelium; SEM, Standard error of mean; SGR, Specific growth rate; SM, Total area of skin mucous cells; SM1, Skin *muc5ac1*; SM2, Skin *muc5ac2*; SM5, Skin *muca5b*; SME, Total area of skin mucous cells (SM) / Total area of skin epithelium (SE); SN, Number of skin mucous cells; SNE, Number of skin mucous cells (SN) / Total area of skin epithelium (SE); SNV, Supra nuclear vacuoles; TGC, Thermal growth coefficient; TJ, Tight junction; WG, Weight gain; WLP, Width of lamina propria; WOV, Width of villi.

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1. Introduction

Feeds are formulated with various ingredients to deliver appropriate nutrients to farmed fish. Feed ingredients may affect the barrier status and health of mucosal surfaces (Nimalan et al., 2022; Sørensen et al., 2021). Barrier functions are mediated by microbiota, which is greatly affected by ingredients and additives such as prebiotics and probiotics in feeds (Ringø et al., 2016; Gupta et al., 2019; Wang et al., 2020). Administration of feeds with probiotics can be considered an environment-friendly disease management tool that targets the host innate immune system (Andani et al., 2012; Beck et al., 2015; Jahangiri and Esteban, 2018; Ringø et al., 2020, 2018; Zorriehzahra et al., 2016). Oligosaccharides (prebiotics) in feeds are fermented by the administered probiotics and certain resident microbes. The fermentation products including short chain fatty acids (SCFAs) such as acetate, propionate and butyrate (Asaduzzaman et al., 2018; Hills et al., 2019; Markowiak-Kopeć and Śliżewska, 2020; Rimoldi et al., 2018) display pleiotropic functions to maintain microbial homeostasis and host health (Louis et al., 2014; Rivière et al., 2016). In a human colon carcinoma (Caco-2) epithelial cell model, butyrate promoted epithelial barrier function by suppressing the expression of certain barrier proteins that increase gut permeability, facilitating assembly of tight junction (TJ) complexes, and modulating the epigenetic landscape in host cells (Chang et al., 2014; Kelly et al., 2015; Peng et al., 2009; Zheng et al., 2017). Notably, in salmonids too, dietary butyrate has been shown to upregulate the expression of TJ molecules and innate immune parameters in vivo (Hoseinifar et al., 2017; Mirghaed et al., 2019). Such studies are beginning to unravel the complex relationship between fish and microbiota and how microbial metabolites can promote intestinal integrity, though more research is warranted to identify strains that produce butyrate in situ in the salmonid gut. Evidence from in vivo studies substantiates the concept that the intestinal barrier is a key determinant of host health. Therefore, if probiotic strains for salmonids can increase nutrient uptake, improve barrier function, and reduce overall mortality, such products have great potential for application in aquaculture.

The mode of action of probiotics includes antimicrobial activity (by decreasing luminal pH, competitive exclusion of pathogens, production of antimicrobial substances), barrier function enhancement, and immune system modulation (Angahar, 2016; Cordero et al., 2015; Jahangiri and Esteban, 2018; Moriarty, 1997; Nayak, 2010; Ng et al., 2009; Ringø, 1999; Ringø et al., 2020). Among the innate immune responses, pro- or anti-inflammatory genes have key roles in regulating intestinal homeostasis (Bäuerl et al., 2013; Krishnaveni et al., 2021). An in vitro study with rainbow trout (*Oncorhynchus mykiss*) intestinal cells in primoculture reported the anti-inflammatory and pro-inflammatory responses evoked by the probiotic strain, *Lactobacillus plantarum* R2, depending on the type of bacterial infection (Cingeľová Maruščáková et al., 2021). The authors of the aforementioned study have demonstrated the ability of probiotics to appropriately regulate inflammation based on the immune status and demand of an organism. However, the effectiveness of probiotics depends on several factors such as mode of administration (through diet or directly into the water or as a vaccine), type and number of species (single or combination of species), source (terrestrial or aquatic host), duration (feeding days) and viability in the gastrointestinal tract of the host (Asaduzzaman et al., 2018; Beck et al., 2015; Ringø et al., 2020).

Some of the commercially available non-fish-derived probiotics may have limited viability in fish gut (Lazado et al., 2015). Therefore, viable candidates with proven ability to adhere to the intestine mucus and eventually exert positive effects on hosts are vital for applications in aquaculture. Several bacterial candidates including lactic acid bacteria (LAB), pseudomonads, and yeast have been recognized as viable and efficient probiotics in aquaculture (Fečkaninová et al., 2019; Gildberg et al., 1995; Lobo et al., 2014; Suzer et al., 2008; Tapia-Paniagua et al., 2010). Among the LAB species, *L. plantarum* was known to have an anti-inflammatory property (Duary et al., 2012; Sherif et al., 2021) and *L. fermentum* was found to have antibacterial activity (Song et al., 2021). Zheng et al. (2020) reported that whiteleg shrimp (*Penaeus vannamei*) intestine harboured specific beneficial bacteria (the genera *Demequina*, *Rubritalea*, *Tenacibaculum*, *Marinicella* and *Phaeobacter*) when the shrimp received *L. plantarum* supplemented (cell-free extract) diet. Their study also indicated the highest abundance of *Acidobacteria*, with a 70-fold increase compared to those fed the control diet. Krishnaveni et al. (2021) reported that dietary supplementation *of L. fermentum* URLP18 increased the LAB population and improved the growth performance and feed utilization in *Cyprinus carpio*.

There are also studies on the application of two or more probiotic bacterial species, including those belonging to the genus *Lactobacillus*; the combined effect was found to enhance the growth and immune performance of the host aquatic animals (Alishahi et al., 2018; Foysal et al., 2020; Wang and Gu, 2010). Moreover, a mixture of LAB (*L. plantarum* and *Lactococcus lactis*), both isolated from the hindgut of olive flounder (*Paralichthys olivaceus*) are effective against *Streptococcus iniae* (Beck et al., 2015). Probiotics for aquaculture should have an excellent ability to strengthen mucosal health not only to control pathogen-caused diseases but also to endure inflammatory reactions associated with new/novel plant ingredients in aquafeeds.

Soybean meal (SBM) (defatted and dehulled product of soybeans), or saponins isolated from soy are commonly used to create enteritis models in Atlantic salmon (*Salmo salar*) (Baeverfjord and Krogdahl, 1996; Kiron et al., 2022; Sørensen et al., 2011, 2021; Urán et al., 2008a). SBM contains a range of antinutritional factors, and a 20% inclusion can adversely affect the growth and gut health of salmonids, mainly due to saponins (Booman et al., 2018; Knudsen et al., 2008, 2007; Krogdahl et al., 2015, 2010). SBM-induced enteritis (SBMIE) model is an ideal tool to study the protective effect of probiotics on fish gut. Previous studies have documented the properties of the two strains *Lactobacillus plantarum* BiocenolTM (CCM 8674) and *Lactobacillus fermentum* BiocenolTM (CCM 8675) as potential probiotic strains (Fečkaninová et al., 2019). The ability of these two strains to adhere to the enterocytes was documented by Gupta et al. (2019) and the capacity to modulate intestinal health was reported by Nimalan et al. (2022). In our two previous studies, a mix of the two strains at a concentration of ~10^8 cells per g feed were used. In the study of Nimalan et al. (2022), we reported the effects of supplementing three different diets (marine-, plant- and soybean meal-based) with a mixture of *L. plantarum* R2 Biocenol™ (CCM 8674) and *L. fermentum* R3 Biocenol™ (CCM 8675); mucosal health was assessed by studying the histomorphology and expression of selected mucin and AMP genes in the dorsal skin, gills and distal intestine as well as changes in short chain fatty acids (Nimalan et al., 2022). The two LABs improved the mucosal health of Atlantic salmon but did not alleviate SBMIE signs (Nimalan et al., 2022).

There is only limited information regarding the ability of LABs to prevent SBMIE in Atlantic salmon. Also, there is a need to clarify if a single LAB is equally efficient as a mix of the two species. A feeding experiment was therefore designed to understand whether the individual effects of the two probiotic species (*L. plantarum* R2 Biocenol™ (CCM 8674) and *L. fermentum* R3 Biocenol™ (CCM 8675)) are as effective as their combined influence on the mucosal barriers to prevent SBMIE in Atlantic salmon. It should be noted that the probiotics were added in the feed, at the same concentration that we employed in previous studies. This study investigated the mucosal health of the skin, gills, and distal intestine by evaluating histomorphometric parameters and mucin and antimicrobial peptide (AMP) gene expression in post-smolt Atlantic salmon fed SBM-based feed. In addition, the concentration of SCFAs in the faeces was also evaluated to study the effect of probiotics on the

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metabolites.

2. Materials and methods

This study was approved by the National Animal Research Authority (FDU: Forsøksdyrutvalget ID-5887) in Norway. The experiment was the second phase of a study that assessed the effects of plant and marine feed ingredients on the performance of Atlantic salmon (Sørensen et al., 2021).

2.1. Basal feed formulation, probiotic culture and coating, and experimental diets

A feed based on fish meal, fish oil, soybean meal (SBM), wheat meal, wheat gluten and micronutrients was produced at the Feed Technology Center, Nofima, Bergen, Norway (Table 1). The nutrient and amino acid composition of the feed is given in Table 2. Details about the extrusion process and experimental feed production can be found in Nimalan et al. (2022).

Two lactic acid bacteria (LAB) species; *L. plantarum* R2 Biocenol™ (CCM 8674); and *L. fermentum* R3 Biocenol™ (CCM 8675) were obtained from the intestinal content of rainbow trout obtained from a fish farm, Rybárstvo – Požehy s.r.o. Dubové in the Slovak Republic (Fečkaninová et al., 2019). The strains are part of the culture collection of the Czech Republic (Czech Collection of Microorganisms, Brno). Pure cultures of LAB were grown anaerobically (Oxoid Gas Pack Anaerobic system) on De Man, Rogosa and Sharpe (MRS) agar plates (HiMedia Laboratories, Mumbai, India) at 37 °C for 48 h. Next, the culture was inoculated into 1000 ml of MRS broth and incubated at 37 °C for 18 h on a shaker, before they were 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 experimental diets (batches of 1800 g) were thoroughly coated with the LAB suspensions (single species and mixture of both species) using a vacuum coater (Rotating Vacuum Coater F-6-RVC, Forberg International AS, Oslo, Norway) at 70 kPa at the feed laboratory of Nord University, Bodø, Norway.

Table 1
Ingredient composition (%) of the four experimental diets.

	CT	LP	LF	LP&LF
Fishmeal[a]	30	30	30	30
Wheat meal[b]	6.55	6.55	6.55	6.55
Wheat gluten[c]	10	10	10	10
Soybean meal[d]	20	20	20	20
Fish oil[e]	26.4	26.4	26.4	26.4
Mineral premix[f]	0.59	0.59	0.59	0.59
Vitamin premix[f]	2	2	2	2
Monosodium Phosphate[g]	2.5	2.5	2.5	2.5
Choline chloride[g]	0.5	0.5	0.5	0.5
Methionine[g]	0.6	0.6	0.6	0.6
Lysine[g]	0.5	0.5	0.5	0.5
Threonine[g]	0.1	0.1	0.1	0.1
Histidine[g]	0.2	0.2	0.2	0.2
Sterile saline, %	0.9	0	0	0
Carop. Pink (10% Astax)[h]	0.05	0.05	0.05	0.05
Lactobacillus plantarum (cells/g)[i]	0	$\sim10^8$	0	$\sim10^4$
Lactobacillus fermentum (cells/g)[i]	0	0	$\sim10^8$	$\sim10^4$

CT – control diet without probiotics, LP – control diet with the probiotic species *Lactobacillus plantarum*, LF - control diet with the probiotic species *Lactobacillus fermentum*, and LP&LF – control diet with both *L. plantarum* and *L. fermentum*.

[a] Fishmeal - LT fiskemel – Pelagia Protein (Ryttervik, Egersund, Norway)

[b] Wheat – Norgesmøllene AS (Bergen, Norway)

[c] Wheat gluten – Tereos Syral (Nicaise, France)

[d] Soybean meal – Fiskå mølle (Etne, Norway)

[e] Fishoil – Vedde Sildoljefabrikk (Langevåg, Norway)

[f] Nofima mineral premix, 0.59% inclusion give per kg diet: Fe: 60 mg, Mn: 30 mg, Zn:130 mg, Cu: 6 mg, Mg: 750 mg, K: 800 mg, Se: 0.3 mg; Nofima vitamin premix, 2% inclusion give per kg diet: Vitamin A: 2 000IE, vitamin D3: 2 500IE, vitamin E: 200 mg, vitamin K3: 20 mg, vitamin B1: 20 mg, vitamin B2: 30 mg, vitamin B6: 25 mg, vitamin B12: 0.05 mg, niacin: 200 mg, Ca-D-pantonat: 60 mg, biotin: 1.0 mg, folic acid: 10 mg, vitamin C: 200 mg

[g] Monosodium Phosphate, Choline chloride, Methionine, Lysine, Histidin, Threonin – Vilomiks (Hønefoss, Norway)

[h] Carophyll Pink – DSM Nutritional Products (Village-Neuf, France)

[i] Autochtohonous *Lactobacillus plantarum* BiocenolTM (CCM 8674) and *Lactobacillus fermentum* BiocenolTM (CCM 8675) from the intestinal content of healthy rainbow trout.

Table 2
Analyzed proximate composition (% as is) and amino acid composition (% as is) of the basal feed.

Composition	Values
Moisture	4.9
Protein	42.2
Lipid	28.6
Ash	9.45
Energy (KJ/100 g)	2029
Amino acids	
Alanine	2.03
Arginine	2.33
Aspartic acid	3.43
Glutamic acid	8.03
Glycine	2.18
Histidine	1.02
Hydroxyproline	0.22
Isoleucine	1.64
Leucine	2.93
Lysine	2.85
Phenylalanine	1.79
Proline	2.47
Serine	1.91
Threonine	1.64
Tyrosine	1.35
Valine	1.86
Tryptophan	0.44
Cysteine	0.50
Methionine	1.67

The LAB were coated on to the pellets of the probiotic feeds. Experimental feed pellets were first transferred to the vacuum coater, then vacuum pump was started to create a vacuum inside the coater. Paddles were started before spraying the oil on to the pellets to ensure constant mixing for even distribution of the LAB suspension. In total, four experimental feeds were used in this study; a control diet without probiotics, CT; a diet with *L. plantarum*, LP; a diet with *L. fermentum*, LF and one diet with a combination of both LAB (*L. plantarum* and *L. fermentum*), LP&LF. After coating, the bacterial counts on each diet were $\sim10^8$ CFU/g as determined by spread plating on MRS agar plates and incubating at 37 °C for 48 h. In the probiotic combination diet, the bacterial counts ($\sim10^4$ CFU/g) of each species were kept similar to maintain identical counts in all the three diets (LP, LF, and LP&LF). The control diet (CT) was coated with 0.9% of sterile saline. The coated diets were stored at 4 °C until they were fed to the experimental fish.

The SBM-based feed (without LAB) was intended to induce enteritis and served as the negative control. A reader can clarify that the CT diet-caused enteritis, by considering a marine-based feed without LAB (BG1) from our previous publications (Nimalan et al., 2022; Sørensen et al., 2021) as the positive control. The BG1-based histomorphometric results are used in the discussion section to emphasize the ability of the negative control (CT in this study) to induce inflammation compared to the positive control (BG1).

2.2. Animal, rearing condition and feeding

Atlantic salmon post-smolts were purchased from Cermaq, Hopen, Bodø, Norway (Aquagen strain, Aquagen AS, Trondheim, Norway). These fish were reared in 8 circular fiberglass tanks (1100 L) connected to a flow-through system and each tank contained 40–43 fish, with an

average initial weight of 129.2 ± 2.2 g (mean ± standard error of mean, SEM). The water was pumped at 1000 L per h from Saltenfjorden, from a depth of 250 m. The average temperature and salinity of the 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 Tec, Huutokoski, Finland) for 12 h per 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). The experimental fish used in the current study were first offered feeds without the LAB for a period of 65 days before the start of LAB feeding (Sørensen et al., 2021).

2.3. Sampling strategy for assessing the growth, histology, gene expression and SCFAs

Prior to handling, fish were anesthetized using tricainemethanesulfonate (MS 222, 140 mg/L). The weight and fork length of fish were individually recorded, at the beginning and end of the experiment. The dorsal skin (left), gills (second arch) and intestinal (approximately 2 cm of the anterior part of the distal intestine) (Sanden and Olsvik, 2009; Sundell and Sundh, 2012) tissues were obtained from 12 fish per tank, of which tissues from 6 fish were immediately placed in 10% neutral buffered formalin (NBF) for 24 h at room temperature, for the histological evaluation. Tissues from the 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. For SCFA analysis, 5 fish per tank were stripped to collect the digesta and the samples were stored at − 20 °C.

2.4. Growth performance

Fish growth performance indicators were calculated as follows: Weight gain (WG%) = ((FW - IW) / IW) × 100. Specific Growth Rate (SGR) = ((Ln (FW) - Ln (IW)) / D) × 100. Thermal growth coefficient (TGC) = (((FW)^(1/3) - (IW)^(1/3)) / ((T × D))) × 1000. Condition factor (CF) = (FW / FL^3) × 100. Where, FW = final body weight of fish (g), IW = 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. Short chain fatty acid composition analysis

The digesta (approximately 1 g per fish) were first thoroughly mixed with deionized water (50 ml). Then membrane filter paper with 0.45 μm pore size (Supor®−450, PALL Life Sciences, Emiliano Zapata, Mexico) was used to filter the solution. Until further analysis, the filtrates (5 ml per fish) were kept in cryotubes and stored at − 20 °C. The produced SCFAs (acetic, acetoacetic, butyric, formic, lactic, propionic, valeric and succinic acids) were quantified 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).

2.6. Mucin and AMP gene expression analysis

Primers were purchased from Eurofins Genomics (Luxembourg, Luxembourg) and the sequences and details of the primers of all target and reference genes are described in Sørensen et al. (2021). For this experiment, relative mRNA levels of mucin genes (*muc2*, *muc5ac1*, *muc5ac2* and *muc5b*) in the skin, gills and distal intestine, and AMP genes (*defensin1*, *defensin2*, *defensin3*, *defensin4*, and *cathelicidin1*) in the skin and distal intestine were studied. The RNA extraction, cDNA synthesis and qPCR were performed as described elsewhere (Sørensen et al., 2021).

2.7. Histomorphometric evaluations

Tissues were processed and embedded in paraffin following standard histological procedures (Øverland et al., 2009; Sørensen et al., 2011) at the histology laboratory of Nord University, Bodø, Norway. The skin tissues (approximately 2 cm) were sliced transversely into 3 equal parts and decalcified with 10% formic acid (25 blocks per L) for 5 h, prior to processing. As for the gill samples, the arches were trimmed before processing. Regarding the intestine samples, the contents were first rinsed off with 10% NBF prior to fixation, and the tissues were embedded longitudinally. The Leica microtome was used to cut tissue sections of 4 μm and the prepared slides (one per fish) were stained with Alcian blue - periodic acid–Schiff (AB-PAS) at pH 2.5. A camera (Leica MC170HD, Heersbrugg, Switzerland) fitted on a light microscope (Leica DM1000, Wetzlar, Germany) was used to generate microphotographs at 40 × magnification by using a software, Leica Application Suite (LAS V4.12. INK, Heerbrugg, Switzerland). ImageJ 1.52a (Schneider et al., 2012) software was used to assess all the images.

2.7.1. Dorsal skin mucous cell image analyses

Approximately 600–900 μm (length) of skin microphotographs (9 per fish) were generated to evaluate the mucous cells. 'Freehand selections' tool of ImageJ was selected 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). The next step was to select the 'Analyze' menu to measure the SE (Gong et al., 2020). The 'Wand tool' was used to select individual mucous cells. 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 the skin mucous cells (SM) and the 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 (Nimalan et al., 2022). SE, SM and SN were used to calculate 2 indices: SME (SM ÷ SE) and SNE (SN ÷ SE).

2.7.2. Gill mucous cell image analyses

To evaluate the area and 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. The same image analysis procedure that is described for the skin, was employed for the gills also to examine the total area of gill epithelium (GE), the total area of gill mucous cells (GM), and the number of gill mucous cells (GN). The obtained values were used to calculate 2 indices: GME (GM ÷ GE) and GNE (GN ÷ GE) (Nimalan et al., 2022).

2.7.3. Distal intestine image analyses

For the morphometric 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. Height of villi (HOV), the width of villi (WOV), the height of enterocytes (HOE), and the width of the associated lamina propria (WLP) were quantitatively measured to understand the diet-induced aberrations in intestinal structure. The width of the villus varies along its height, and hence, to measure WOV, each villus was partitioned into 6 equal parts from the base to the tip (Nimalan et al., 2022). From these 5 points, WOV, HOE and WLP were gauged by employing the analysing tools ('straight' and 'segmented lines') of the ImageJ, and the average of the 5 values were registered. Semi-quantitative assessment was adopted to study the morphological changes in the following indices: number of mucous cells (NOM), number of intraepithelial lymphocytes (IEL), and supra nuclear vacuoles (SNV) of intestinal villi. An ordinal scoring strategy (from 1 to 5) for each index was developed (Supplementary Figure 1–3) based on Baeverfjord and Krogdahl (1996); Bakke-McKellep et al. (2007); Knudsen et al. (2008); Silva et al. (2015); Urán et al. (2008a, 2008b, 2009).

2.8. Statistics

All statistical analyses were executed in R studio (version 1.2.5042) for windows. In this experiment, the probiotic treatment effect was analysed by one-way analysis of variance (one-way ANOVA). The Shapiro–Wilk test was used to check the normality of data. Levene's test was used to assess the homogeneity of variance. One-way ANOVA was performed on SCFAs, most of the histology and gene expression data. Significant differences were revealed by carrying out Tukey's honestly significant difference (HSD) post-hoc test. When necessary, data were log-transformed (GNE, WOV, HOE, *defensin3,* and *cathelicidin1* in the intestine, *muc5b* in the gills, *muc5ac2* and *muc5b* in the skin and all the SCFAs data except formic and acetoacetic acids). Welch's ANOVA was performed for data that showed heteroscedasticity (growth performance, skin *muc5b*, and *muc2*). Kruskal-Wallis was performed for the gill data (GME) and semi-quantitively assessed ordinal data (NOM, IEL and SNV). Significant differences were revealed by performing Dunn's multiple comparison test. The function from the package "corrplot" in R was used to run Spearman correlations for all the combinations of histologically evaluated mucous cell indices and the selected mucin and AMP genes. To assess the ability of CT to induce enteritis, the histomorphometric indices, HOV, HOE, WOV, WLP, NOM, IEL, and SNV were compared with those of the BG1 diet reported in our previous publication (Nimalan et al., 2022); employing either parametric unpaired two-samples t-test or non-parametric Wilcoxon test (Supplementary file 1). The statistical significance is reported when $p < 0.05$. The gene expression data (missing values were replaced with group average) and distal intestinal histomorphometric indices were subjected to principal component analysis (PCA). In this 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., 2008a), gene expression and SCFAs analyses (Bansemer et al., 2015). Means ± SEM of parameters are presented in all tables and figures.

3. Results

3.1. Growth performance indicators

During the course of the experiment, the mean weight of the fish increased from 129.2 g to 198.8 g. There were no significant differences in FW, FL, CF, SGR, TGC and WG% among diet groups. The growth parameters are presented in Table 3.

3.2. Effects of probiotics on the short chain fatty acids in the digesta

The concentration of faecal SCFAs is presented in Table 4. Formic, acetoacetic, lactic, succinic, acetic, propionic and butyric acids were detected in all the samples. The concentration of acetoacetic was high and butyric acid was low in all the groups, regardless of the probiotic treatment. Among all SCFAs, formic, acetic and butyric acids were not significantly affected by the probiotics, when applied singly or in combination. However, other SCFAs such as acetoacetic, lactic, succinic and propionic acids as well as total SCFAs were altered by probiotics. Fish fed a combination of the two probiotics (LP&LF) had a significantly higher concentration of acetoacetic (compared to CT, LP and LF), succinic (compared to CT and LF) and total SCFAs (compared to LF). In addition, fish fed LF had more propionic acid, compared to LP, which had a higher content of acetoacetic acid compared to fish fed LF and CT. Fish fed LP had more lactic acid, compared to LF (Table 4).

3.3. Effects of probiotics on mucin and AMP gene expression

The uncorrelated variables (principal components) of the expressed AMPs and mucin-related genes and their loadings are shown in Fig. 1, with principal component (PC) 1 and PC 2 explaining 23.3% and 14.6% of the variance in the data, respectively. The cumulative proportion of variance explained by 5 PCs was 70% (Supplementary Fig. 4). The variation in skin *muc5ac1* (SM1), skin *muc5ac2* (SM2), skin *muca5b* (SM5), and intestine *muc2* (IM2), were mainly explained by PC1, while the skin *defensin1* (SD1), skin *cathelicidin1* (SC1), intestine *defensin3* (ID3), and intestine *defensin4* (ID4) were explained by PC2. All the genes, except SC1, had positive loading on PC1, while only SM2, SM5, GM2, and IC1 had positive loading on PC2 (Supplementary Fig. 5). Mucin genes had the highest loadings on PC1 (e.g.: SM1, SM2, SM5 and

Table 3
Growth performance of Atlantic salmon fed soybean meal-based diet and the three diets coated with a single probiotic species or a combination of two probiotic species.

Growth parameters	Diet groups				*p*-value
	CT	LP	LF	LP&LF	
IW (g/fish)	135.0 ± 2.57	124.7 ± 7.14	129.6 ± 3.97	127.4 ± 2.35	0.497
IL (cm)	21.9 ± 0.25	21.5 ± 0.23	21.6 ± 0.28	21.6 ± 0.07	0.802
FW (g/fish)	214.0 ± 2.54	200.7 ± 11.53	197.7 ± 9.38	194.5 ± 5.65	0.311
FL (cm)	25.7 ± 0.15	25.1 ± 0.47	25.1 ± 0.48	25.0 ± 0.25	0.469
CF (g/cm^3)	1.27 ± 0.01	1.27 ± 0.00	1.25 ± 0.01	1.25 ± 0.00	0.149
SGR	1.21 ± 0.02	1.25 ± 0.00	1.11 ± 0.04	1.11 ± 0.12	0.300
TGC	2.95 ± 0.03	2.97 ± 0.06	2.65 ± 0.14	2.63 ± 0.30	0.505
WG (%)	58.52 ± 1.14	60.89 ± 0.03	52.51 ± 2.56	52.72 ± 7.24	0.293

CT – control diet without probiotics, LP – control diet with the probiotic species *Lactobacillus plantarum*, LF - control diet with the probiotic species *Lactobacillus fermentum*, and LP&LF – control diet with both *L. plantarum* and *L. fermentum*. 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 presented as means ± SEM of two replicates. Significant difference ($p < 0.05$) among diet groups on each row was revealed by a one-way Welch test or Kruskal-Wallis test.

Table 4
Short-chain fatty acid concentration (mmol/L) in the digesta of the study groups.

SCFAs	Diet groups				*p*-value
	CT	LP	LF	LP&LF	
Formic acid	2.60 ± 0.30	2.20 ± 0.25	1.92 ± 0.24	2.30 ± 0.17	0.287
Acetoacetic acid	9.18 ± 0.55^{a}	12.84 ± 0.36^{b}	9.85 ± 0.26^{a}	14.64 ± 0.42^{c}	< 0.001
Lactic acid	5.03 ± 0.42^{b}	5.13 ± 0.25^{b}	3.98 ± 0.18^{a}	4.93 ± 0.17^{ab}	0.008
Succinic acid	5.63 ± 0.15^{ab}	6.46 ± 0.39^{bc}	5.41 ± 0.14^{a}	6.89 ± 0.19^{c}	< 0.001
Acetic acid	12.72 ± 1.49	9.52 ± 0.49	9.79 ± 0.48	11.73 ± 1.25	0.866
Propionic acid	2.76 ± 0.37^{ab}	2.27 ± 0.32^{a}	3.59 ± 0.32^{b}	2.23 ± 0.16^{a}	0.005
Butyric acid	1.25 ± 0.35	1.85 ± 0.21	1.93 ± 0.29	1.42 ± 0.22	0.235
Total acids	37.47 ± 2.08^{ab}	39.16 ± 1.03^{ab}	36.09 ± 0.87^{a}	43.43 ± 1.99^{b}	0.007

CT – control diet without probiotics, LP – control diet with the probiotic species *Lactobacillus plantarum*, LF - control diet with the probiotic species *Lactobacillus fermentum*, and LP&LF – control diet both *L. plantarum* and *L. fermentum*. SCFAs; short chain fatty acids. Values are presented as means ± SEM, n = 10 per diet group. Significant difference ($p < 0.05$) among diet groups, indicated on each row, was revealed by a one-way ANOVA followed by Tukey's honestly significant difference (HSD) post-hoc test.

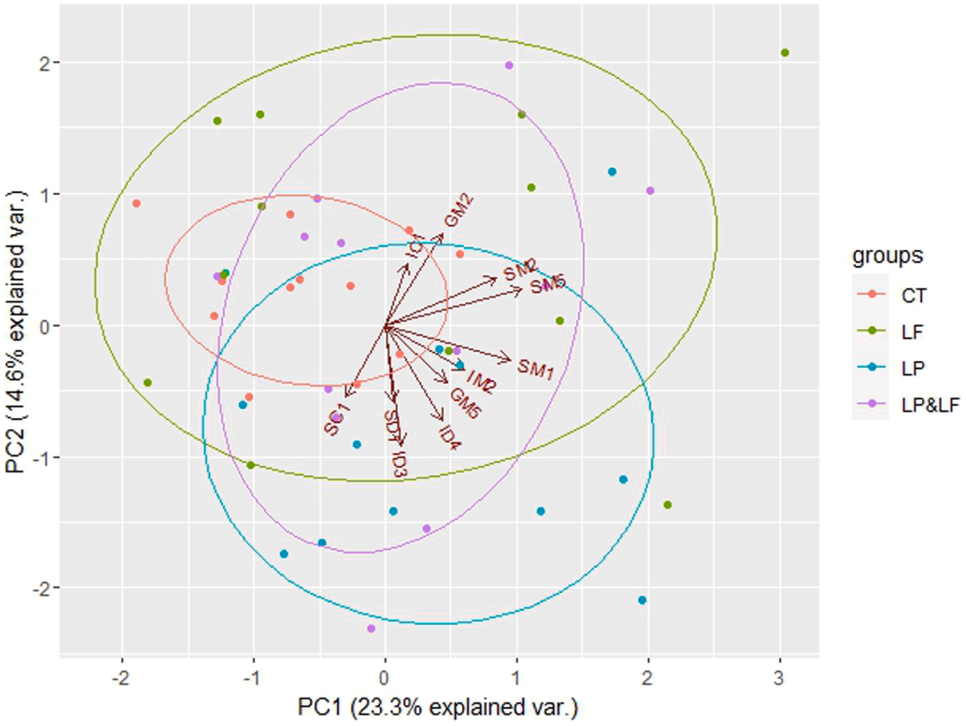

Fig. 1. Principal component analysis (PCA) biplot showing the skin, gill and intestine samples and loading vectors. Expression of AMP and mucin genes were used for this dimensionality reduction. CT, control diet without probiotics; LF, control diet with the probiotics *Lactobacillus fermentum*; LP, control diet with the probiotics *Lactobacillus plantarum*; and LP&LF, control diet with a mixture of *L. plantarum* and *L. fermentum*. SM1, skin *muc5ac1*; SM2, skin *muc5ac2*; SM5, skin *muca5b*; SD1, skin *defensin1*; SC1, skin *cathelicidin1*, GM2, gills *muc5ac2*; GM5, gill *muc5b*; IM2, intestine *muc2*; IC1, intestine *cathelicidin1*; ID3; intestine *defensin3*; ID4, intestine *defensin4*.

IM2), and are therefore the "mucin gene component" while the AMP component was PC2, with the highest loadings of AMP genes (e.g.: SD1 and SC1). SM1, SM2 and SM5 are positively related to each other, likewise the following pairs have shown positive correlation: IC1 and GM2; SD1 and ID3.

3.3.1. Mucin and AMP genes in the dorsal skin

Among the studied mucin and AMP genes, *muc5ac1*, *muc5ac2*, *muc5b, defensin1* and *cathelicidin1* were expressed in the skin. Almost all the mucin and AMP genes were not affected by the individual or combined administration of probiotics. The gene *defensin1* was an exception; fish fed LP had significantly higher *defensin1* mRNA level compared to CT and LP&LF (Table 5). A trend towards statistical significance was noted in the case of *muc5ac1*, with the highest value for the LP fed fish and the lowest for the CT group.

3.3.2. Mucin genes in the gills

The gills expressed *muc5ac2* and *muc5b*, among the studied mucin genes. The *muc5ac2* mRNA levels tended to be higher in fish fed LP&LF (Table 5), while no other mucin genes were significantly altered by the LAB supplementation in the diets.

3.3.3. Mucin and AMP genes in the distal intestine

The distal intestine expressed the *muc2*, *defensin3*, *defensin4* and *cathelicidin1*, among the assessed genes. The single or combined administration of probiotics did not affect any of the mucin or AMP genes. However, a trend towards significance was observed for *muc2* mRNA levels in fish fed LP and LP&LF compared to CT (Table 5).

3.4. Effects of probiotics on the architecture of the skin, gills and distal intestine

3.4.1. Mucous cell indices in the dorsal skin

The results revealed that approximately 100 μm^2 of skin epidermis in Atlantic salmon was covered by mucous cells of a total average area 14 μm^2, corresponding to an average of 1148 mucous cells in 1 mm^2 of

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

Tissues	Type of genes	Name of genes	Diet groups				*p*-value
			CT	LP	LF	LP&LF	
Skin	**AMP**	*defensin1*	0.58 ± 0.06^{a}	0.93 ± 0.08^{b}	0.71 ± 0.07^{ab}	0.67 ± 0.06^{a}	0.005
		cathelicidin1	0.47 ± 0.07	0.60 ± 0.07	0.48 ± 0.05	0.43 ± 0.07	0.318
	Mucin	*muc5ac1*	0.31 ± 0.04	0.65 ± 0.12	0.42 ± 0.07	0.35 ± 0.06	0.076
		muc5ac2	0.25 ± 0.05	0.41 ± 0.10	0.69 ± 0.19	0.52 ± 0.10	0.164
		muc5b	0.28 ± 0.03	0.34 ± 0.03	0.36 ± 0.07	0.30 ± 0.04	0.527
Gills	**Mucin**	*muc5ac2*	0.95 ± 0.06	0.90 ± 0.04	1.04 ± 0.10	1.18 ± 0.10	0.080
		muc5b	0.13 ± 0.02	0.18 ± 0.03	0.17 ± 0.04	0.21 ± 0.04	0.701
Intestine	**AMP**	*defensin3*	0.38 ± 0.10	0.70 ± 0.14	0.48 ± 0.18	0.64 ± 0.18	0.512
		defensin4	0.72 ± 0.06	0.74 ± 0.08	0.56 ± 0.07	0.72 ± 0.08	0.310
		cathelicidin1	0.11 ± 0.03	0.13 ± 0.04	0.15 ± 0.05	0.07 ± 0.01	0.901
	Mucin	*muc2*	0.75 ± 0.06	0.93 ± 0.05	0.78 ± 0.04	0.89 ± 0.10	0.096

CT – control diet without probiotics, LP – control diet with the probiotic species *Lactobacillus plantarum*, LF - control diet with the probiotic species *Lactobacillus fermentum*, and LP&LF – control diet with both *L. plantarum* and *L. fermentum*. AMP; Antimicrobial peptide. Values are presented as means ± SEM, n = 12 per diet group. Significant difference ($p < 0.05$) among diet groups, indicated on each row, was revealed by a one-way ANOVA followed by Tukey's honestly significant difference (HSD) post-hoc test or Welch test.

epidermis. The mucous cell indices, SME and SNE were not significantly affected by the LAB supplementation. However, SME values revealed a decreasing trend in LP fed fish compared to the other diet groups (Table 6). Representative histological images of skin epidermis and dermis regions for each diet group are presented in Fig. 2.

3.4.2. Mucous cell indices in the gills

Histological evaluation of gill mucous cells revealed the probiotic supplementation-induced changes in both GME and GNE. Fish fed LP&LF had significantly more GME (compared to CT) and GNE (compared to CT, LP and LF) (Table 6). Representative histological micro-photographs of primary and secondary gill filaments for each diet group are presented in Fig. 3.

3.4.3. Distal intestine histomorphometry

Effects of supplementing a single or a mixture of the two probiotics on the distal intestine indices of Atlantic salmon were examined. For all diet groups, representative histological images of the distal intestine are presented in Fig. 4. The PCA biplot shows the intestinal health indicators and the different experimental groups (Fig. 5), with PC1 explaining 38.7% of the variance, and PC2 corresponding for 27.8%. Approximately 80% of the cumulative proportion of variance could be explained when a 3rd. PC was included (Supplementary Figure 6). The indices HOE, NOM and WOV are explained by PC1, while the HOV, WLP, IEL and SNV are explained by PC2. All the indices, except NOM, SNV and IEL had positive loading on PC1, while HOV and SNV had negative loading on PC2 (Supplementary Figure 7). The indices WOV and HOE are positively related to each other. Likewise, the following pairs are positively correlated: HOV and SNV; NOM and IEL. The indices WOV and NOM are negatively correlated. The other pairs that are negatively correlated are: HOV and IEL, WLP and SNV. We observed differential clustering of the groups LP&LF and CT. However, the clusters of LP and LF cannot be differentiated.

The examined histomorphometric indices such as HOV, WOV, WLP and SNV were significantly but differently altered by the single or combined administration of probiotics. Fish fed the combination of probiotics (LP&LF) had significantly higher villi compared to CT which is SBM-based feed without probiotics (CT). While the WOV value of LP fed fish showed a decreasing tendency, LF fed fish had significantly reduced WOV compared to CT fed fish. Fish fed the CT had significantly wider lamina propria compared to fish fed the LP or LP&LF. Fish fed LP&LF had significantly narrower lamina propria than LF. The fish fed LP, LF and LP&LF had significantly higher SNV scores than those fed CT. Other indices (HOE, NOM and IEL) were not significantly affected by probiotics. The HOE varied between 43 and 48 μm. A tendency towards significance was noted for the NOM values, with the highest value for LF and the lowest for the LP&LF (Table 6).

Table 6
Histomorphometric indices in the skin, gills and intestine of Atlantic salmon.

Indices	Diet groups				*p*-value
	CT	LP	LF	LP&LF	
SME (ratio)	0.1563 ± 0.009	0.1248 ± 0.008	0.1317 ± 0.007	0.1382 ± 0.010	0.059
SNE (number / μm^2)	1.1099 ± 0.051	1.1710 ± 0.045	1.1336 ± 0.042	1.1768 ± 0.060	0.751
GME (ratio)	0.0399 ± 0.005^a	0.0514 ± 0.004^{ab}	0.0493 ± 0.003^{ab}	0.0657 ± 0.005^b	< 0.001
GNE (number / μm^2)	0.0006 ± 8e-05^a	0.0007 ± 3e-05^a	0.0007 ± 5e-05^a	0.0008 ± 4e-05^b	0.008
NOM (score)	3.00 ± 0.30	3.42 ± 0.23	3.67 ± 0.22	2.75 ± 0.25	0.073
HOV (μm)	897.65 ± 30.23^a	976.84 ± 26.08^{ab}	979.53 ± 29.40^{ab}	1021.44 ± 30.12^b	0.033
WOV (μm)	126.73 ± 5.28^b	113.19 ± 2.40^{ab}	112.13 ± 3.51^a	116.61 ± 2.73^{ab}	0.044
HOE (μm)	48.26 ± 2.13	43.35 ± 1.28	43.23 ± 1.34	46.95 ± 1.14	0.063
WLP (μm)	27.62 ± 1.62^c	22.07 ± 0.98^{ab}	24.61 ± 1.71^{bc}	18.39 ± 1.08^a	< 0.001
IEL (score)	3.67 ± 0.19	3.00 ± 0.33	3.25 ± 0.18	2.92 ± 0.23	0.127
SNV (score)	1.00 ± 0.00^a	2.08 ± 0.08^b	1.75 ± 0.18^b	2.17 ± 0.11^b	< 0.001

CT, control diet without probiotics; LP, control diet with the probiotic species *Lactobacillus plantarum*; LF, control diet with the probiotic species *Lactobacillus fermentum*; and LP&LF, control diet with both *L. plantarum* and *L. fermentum*. 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; NOM, number of intestinal mucous cells; HOV, height of villi; WOV, width of villi; HOE, height of enterocytes; WLP, width of lamina propria; IEL, number of intraepithelial lymphocytes; SNV, supra nuclear vacuoles. Values are presented as means ± SEM, n = 12 per diet group. If present, significant differences ($p < 0.05$) among diet groups are indicated by different superscripts (a, b, c, or d) on each row after conducting a one-way ANOVA followed by Tukey's honestly significant difference (HSD) post-hoc test or Kruskal-Wallis (for GNE, NOM, IEL and SNV) followed by Dunn's post hoc test.

3.5. Correlation between mucous cell-based histological indices and mucin and AMP gene expression data

Spearman correlation-based analysis revealed a significant correlation between histologically analysed mucous cell indices and mucin and AMP gene expression data (Fig. 6). Significant positive correlations, with correlation coefficient > 0.50) between mucous cell indices were observed for the following pairs: SME and SNE ($r = 0.60$, $p < 0.001$), GME and GNE ($r = 0.74$, $p < 0.001$). The histological index SNE tended to be positively correlated to the skin AMP gene SD1 (r 0.28, $p = 0.058$). A significant positive correlation between gene expression data was also observed for the following pair: SC1 and SD1 ($r = 0.35$, $p = 0.016$). The SM1 positively correlated with SM2 ($r = 0.49$, $p < 0.001$), SM5 ($r = 0.84$, $p < 0.001$), and ID4 ($r = 0.29$, $p = 0.049$). Likewise, SM5 positively correlated with SM2 ($r = 0.51$, $p < 0.001$), GM2 ($r = 0.31$, $p = 0.033$), and IM2 ($r = 0.30$, $p = 0.039$). In addition, ID4 and GM5 ($r = 0.22$, $p = 0.037$) were also positively correlated.

4. Discussion

In the present study, *L. plantarum* R2 Biocenol™ (CCM 8674) and *L. fermentum* R3 Biocenol™ (CCM 8675) were fed to Atlantic salmon to understand if the probiotic bacteria, when applied singly or in combination, could prevent soybean meal-induced enteritis. The LAB species were isolated from the intestinal content of rainbow trout, and they were selected based on features including tolerance to different pH values, bile acids and temperature, antagonistic activity against the two salmonid pathogens *A. salmonicida* subsp. *salmonicida* CCM 1307 and *Y. ruckeri* CCM 6093 and growth properties in vitro (Fečkaninová et al., 2019). These probiotic species have the capacity to prevent diseases in aquaculture. The results from the present study indicate that the single and combined administration of the probiotic bacteria are effective to prevent soybean meal-induced enteritis, the latter approach was found to have a better effect.

An earlier study with Atlantic salmon showed a shift in gut microbiota composition when Atlantic salmon was fed diets supplemented with *L. plantarum* and *L. fermentum* (Gupta et al., 2019), and is most likely the reason for the changes in SCFAs observed in the present experiment. The enterocytes in the villi absorb metabolites and utilize the SCFAs as energy sources. A legume-based diet (mix of soybean meal and wheat gluten – SBMWG) presented a high relative abundance of LAB (Gajardo et al., 2017) and increased the plasma osmolality and water content of the distal intestine chyme (Hu et al., 2016). A shift in microbiota composition can sometimes favour SCFA production. In the

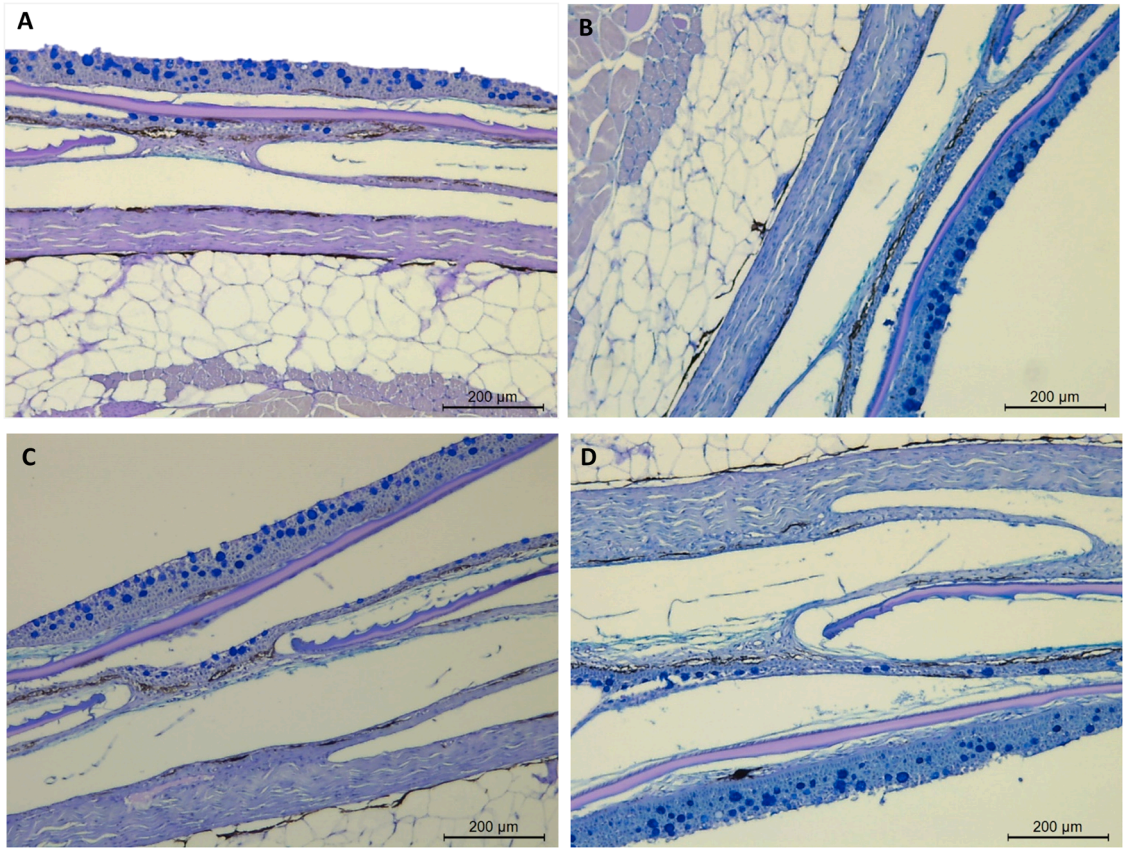

Fig. 2. Histological micro-photographs of the skin from post-smolt Atlantic salmon fed with soybean meal-based control diet (A), control diet coated with *Lactobacillus plantarum* (B), control diet coated with *Lactobacillus fermentum* (C) and control diet coated with both *L. plantarum* and *L. fermentum* (D). Images were acquired with Leica camera fitted on DM 3000 light microscope at 10X magnification.

present study, supplementation of feeds with LAB, singly or in combination, did not alter the concentration of formic, acetic or butyric acids. However, acetoacetic, lactic, succinic and propionic acids including total SCFAs were altered by probiotics. Fish fed the LP diet had more lactic acid compared to those fed LF, while those fed the LF diet had more propionic acid than the LP group as well as those fed the LP&LF diet. The end product of *L. fermentum* might have influenced the propionate-producing bacteria; either by shifting the microbiota composition to favour the pyruvate-lactate-propionate pathway or succinate-propionate pathway under anaerobic conditions (Hati et al., 2019; Kusumo et al., 2019; Meenakshi Malhotra, 2015). Propionate is absorbed through enterocytes and transported via vena porta to the liver where it plays a role in lipid synthesis in hepatocytes. An overview of the potential effects of propionate on cholesterol level and lipid synthesis in humans is given by Hosseini et al. (2011). In brief, this review cited different in vivo and in vitro studies that reported propionate intake-caused decrease in hepatic and blood cholesterol levels and liver lipogenesis. Newer literature also points to the role of propionic acid in autoimmune and neurodegenerative diseases in humans (Duscha et al., 2020). Long-term consumption of propionic acid had positive effects on the health of these patients; their relapse rate was less, and brain atrophy was reduced. The LP&LF fed fish (mixture of two LAB species) had more acetoacetic acids compared to LP or LF fed groups (one of the species of LAB), as well as a higher concentration of succinic and total SCFAs. These observations suggest that each LAB species has its metabolic pathways and metabolic fingerprint. Our findings are in line with other studies on the influence of probiotic supplementation on the SCFAs in the fish gastrointestinal tract (Allameh et al., 2017; Asaduzzaman et al., 2018; Burr et al., 2005). In fish, acetic acid is transported from the intestinal lumen into the portal blood and used either as an energy source for skeletal muscle or lipid synthesis (Asaduzzaman et al., 2018; Titus and Ahearn, 1991). The SCFA metabolites of bacterial fermentation are also known to stimulate gut epithelial cell proliferation resulting in increased villi height (Ichikawa et al., 1999). Our study also demonstrated the LP&LF-caused beneficial effects such as increased SCFA production in the digesta, increased villi height, and reappearance of supranuclear vacuoles in Atlantic salmon fed SBM-based diet. It seems that different species of bacteria depend on the carbon sources produced by the other cohabitants (Hosseini et al., 2011; Smid and Lacroix, 2013). Thus, the two approaches had differential effects on the SCFA content and the subsequent alteration of the micromorphology of the intestine.

Earlier studies on fishes have reported the influence of probiotics on immune-related gene expression (Cingeľová Maruščáková et al., 2021; Hasan et al., 2018; Van Doan et al., 2018). The present study evaluated the expression of four mucin genes in the skin, gills and intestine and four AMP genes in the skin and intestine. The expression pattern was tissue-specific, and probiotics influenced the studied mucin and AMP genes differently. Fish fed the LP diet had a significantly higher level of skin *defensin1* mRNA, a tendency towards a significantly higher expression of skin *muc5ac1* and intestine *muc2* compared to those fed the control diet (CT). These observations in the skin are in line with a study that reported an upregulation of *beta defensin* in juvenile Atlantic cod, when probiotics were added to the rearing water (Ruangsri et al., 2014). In our study, fish fed a mixture of the LAB species (diet LP&LF) also had a strong tendency towards elevating the expression of *muc5ac2* in the gills compared to the control group (CT). These observations may suggest that the fish fed LP&LF had improved gill barrier status compared to fish fed the CT. Our observations in the skin and gills of Atlantic salmon are in line with several other studies that reported positive effects of LAB on the host innate immune responses. For example, common carp

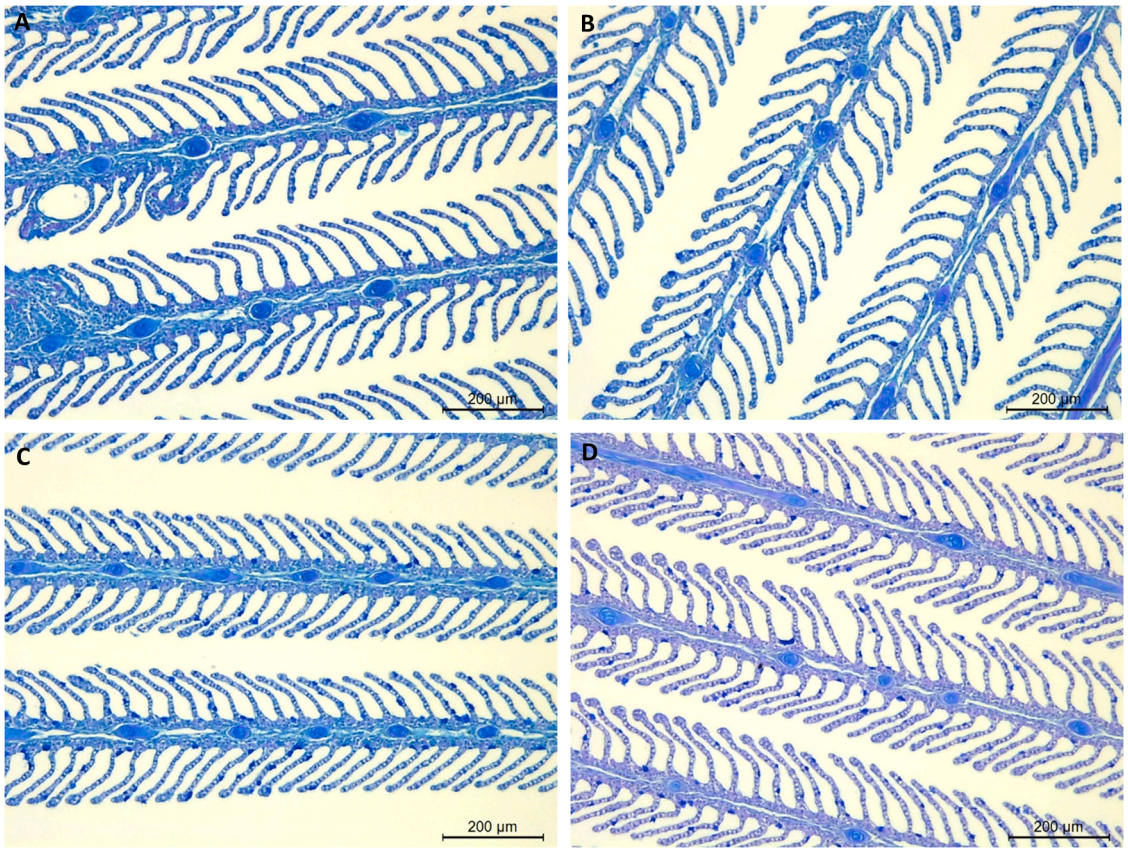

Fig. 3. Histological micro-photographs of the gills from post-smolt Atlantic salmon fed a soybean meal-based control diet (A), control diet coated with *Lactobacillus plantarum* (B), control diet coated with *Lactobacillus fermentum* (C) and control diet coated with both *L. plantarum* and *L. fermentum* (D). Images were acquired with Leica camera fitted on DM 3000 light microscope at 10X magnification.

(*Cyprinus carpio*) fed *P. acidilactici* (6×10^8 CFU per g) for 60 days had increased mucus protease activity and skin lysozyme gene expression (Hoseinifar et al., 2019), Nile tilapia (*Oreochromis niloticus*) fed *L. plantarum* (10^8 CFU per g) and *Bifidobacterium velezensis* (10^7 CFU per g) for 15 or 30 days had significantly higher innate immune markers (Van Doan et al., 2018); Olive flounder fed *L. sakei* and *L. plantarum* (10^{11} CFU /g) for 42 days had increased expression of immune genes in the gills and head kidney (Feng et al., 2018). It seems that the effects of single and combined application of the bacteria on the immune genes can vary and the target area can also be different.

Moreover, the present study found a significant positive correlation between mucus-related genes. For example, skin *muc5b* (SM5) is positively correlated with skin *muc5ac1* (SM1), skin *muc5ac2* (SM2), gills *muc5ac2* (GM2), and intestine *muc2* (IM2). These results indicate the interactions between the mucosal surfaces and the tendency to follow a pattern in gene expression in multiple mucosal sites. Moreover, the mucin and AMP gene expression positively correlated with the histologically evaluated mucous cell indices (in some cases) supporting the hypothesis that probiotics have the ability to influence gene expression and alter the histomorphometry of mucosal tissues. The gut microbiome and its metabolites are known to influence the skin microbiome and the associated immune defence (Salem et al., 2018). Gut health status in fishes like yellowtail kingfish was found to influence the skin and gill bacterial assemblages, which have a bearing on the barrier systems of these mucosal surfaces during the early onset of enteritis (Legrand et al., 2018). Hence, the possible connection, which we observed in our study, between diet, the intestine and other organs (skin and gills) could be that the metabolites absorbed by the gut epithelium are transported to the skin, gills and brain through the circulatory system where metabolites aid in modulating the mucosal tissues (Ghosh et al., 2021; Guo et al., 2022; Sharon et al., 2014; Silva et al., 2020; Thursby and Juge, 2017; Wiatrak et al., 2022). Moreover, the mechanism or the pathway could be that the dietary probiotics influence the production of mucins via stimulation of different receptors on lymphocytes resulting in a modulation of the immune system (Grondin et al., 2020).

Based on the present study, around 14% of the skin epidermis area is covered by skin mucous cells, irrespective of LAB supplementation. Mucous cell area-related indices (SME and GME) indicate the fraction of epithelium area that is covered by mucous cell area. These indices depend on mucous cell size and count, both of which reflect the overall mucus production. It should be noted that if the size or count of mucous cells increases, the SME or GME will increase, but if only one of these cell attributes increases and the other decreases, SME or GME will not change. Dietary probiotics can stimulate mucous cell formation, which is counted as one of the innate immune responses in fish (Sewaka et al., 2019). The present study revealed a non-significant, but numerical increase in skin mucous cell count when LAB was administrated via feeds compared to the feed without LAB. This is in line with our previous experiment that reported an increase in skin mucous cell count in salmon fed a combination of the two LAB *L. plantarum* and *L. fermentum* (Nimalan et al., 2022). Other studies have also reported responses in the skin when fish were fed probiotics; Porthole livebearer (*Poecilopsis gracilis*) fed *Lactobacillus* enriched *Artemia* (Hernandez et al., 2010) and catla (*Catla catla*) fed the LAB, *Bacillus* (Das et al., 2013) had a high content of protein in skin mucus. Unfavourable environmental factors may also increase the mucus cell counts of the skin (Vatsos et al., 2010). Genes encoding for secretory processes-linked proteins in mucus were altered with an increase in mucus-producing cells and hypertrophic mucous cell modelling in the gills of Atlantic salmon was a response to an oxidizing agent (Karlsen et al., 2018; Haddeland et al., 2020). The

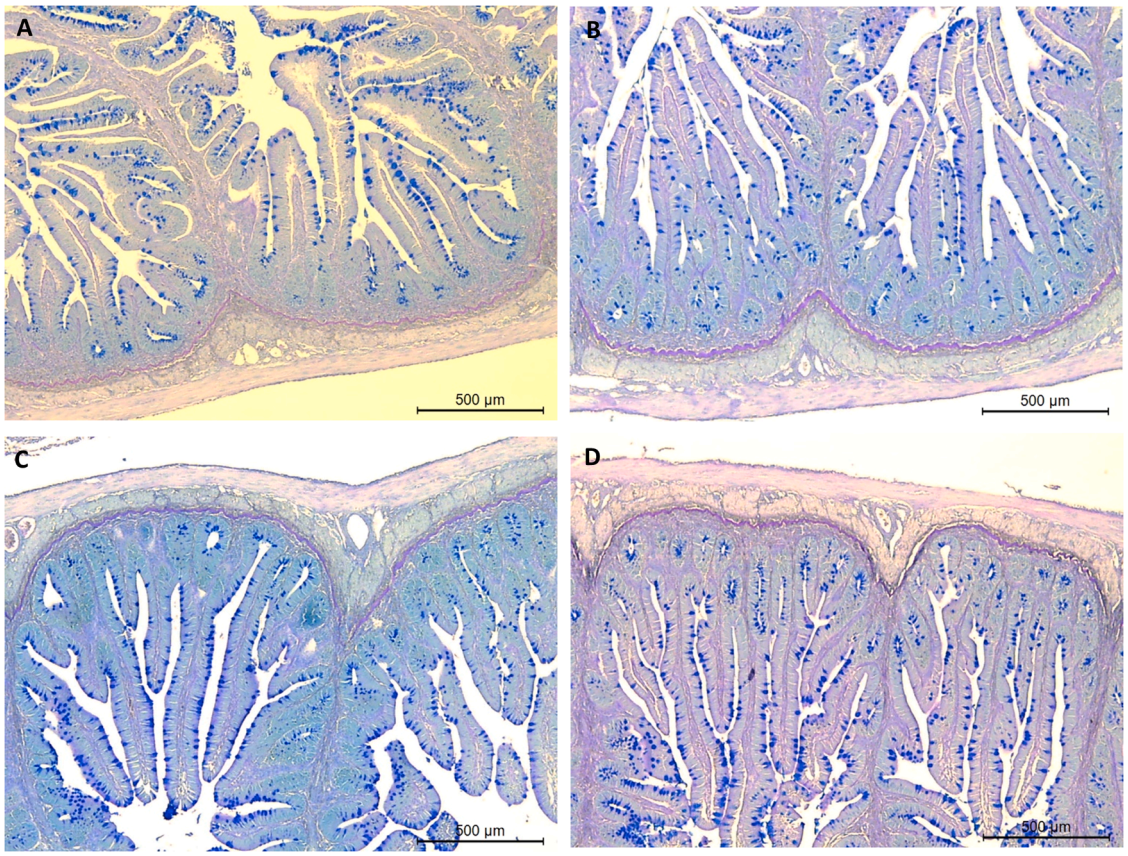

Fig. 4. Histological micro-photographs of the distal intestine from post-smolt Atlantic salmon fed a soybean meal-based control diet (A), control diet coated with *Lactobacillus plantarum* (B), control diet coated with *Lactobacillus fermentum* (C) and control diet coated with both *L. plantarum* and *L. fermentum* (D). Images were acquired with Leica camera fitted on DM 3000 light microscope at 5X magnification.

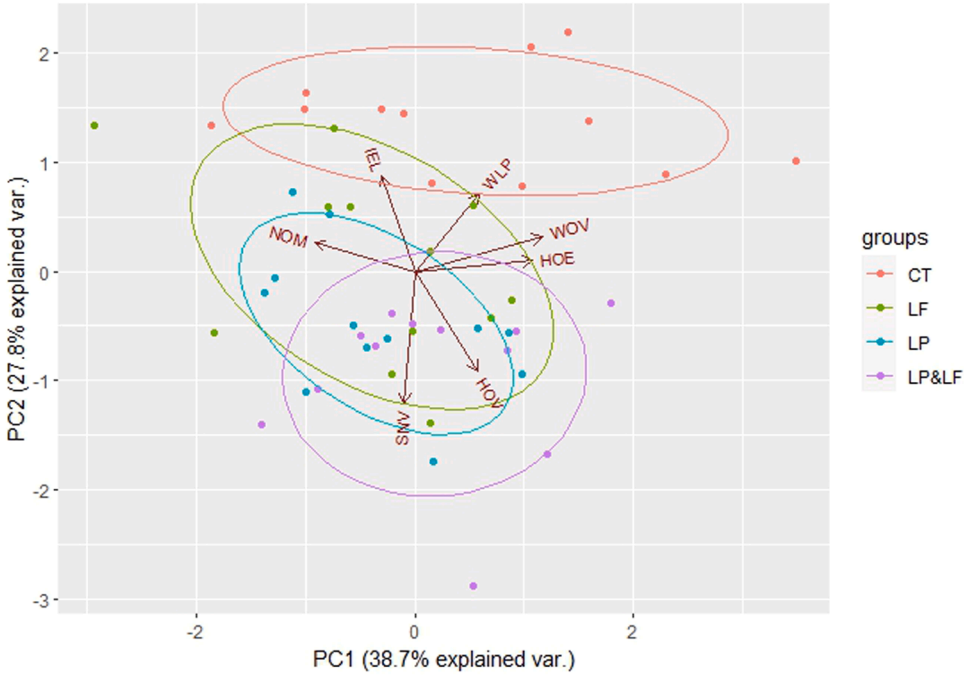

Fig. 5. Principal component analysis (PCA) biplot showing the intestine samples and loading vectors. Expression of AMP and mucin genes were used for the intestinal histology data-based dimensionality reduction. CT, control diet without probiotics; LF, control diet with probiotic species *Lactobacillus fermentum*; LP, control diet with probiotic species *Lactobacillus plantarum*; and LP&LF, control diet with a combination of *L. plantarum* and *L. fermentum*. NOM, score for number of intestinal mucous cells; HOV, height of villi; WOV, width of villi; HOE, height of enterocytes; WLP, width of lamina propria; IEL, score for number of intra-epithelial lymphocytes; SNV, score for supra nuclear vacuoles.

present study showed that the gills of fish fed LP&LF had almost two times higher GME and GNE than the CT group, and mucous cell count in the distal intestine tended to be higher than in the fish of the CT group. Our observations are in line with a study that reported an increase in mucin-secreting goblet cells in red tilapia (*Oreochromis* spp.) fed dried *L. rhamnosus* (10^8 CFU/g) for 30 days (Sewaka et al., 2019). Not only the

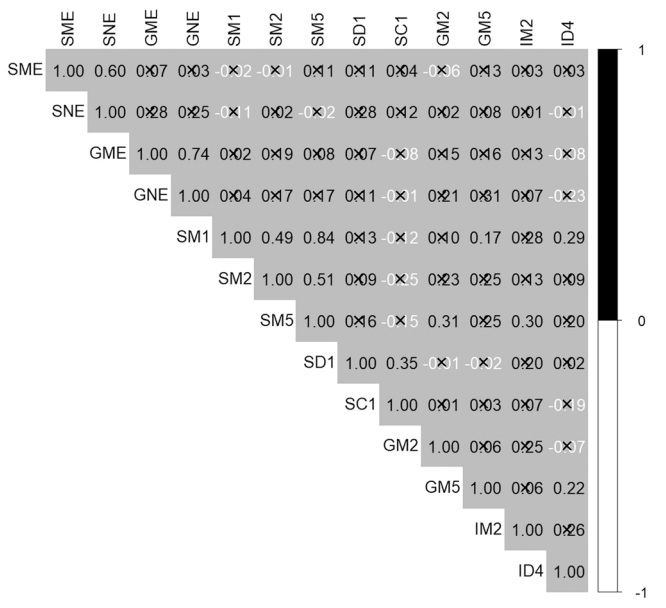

Fig. 6. Correlation plot for histologically evaluated mucous cell indices and selected mucus-related gene expression. Intestinal mucous cell number (NOM), intestine *defensin3*, intestine *cathelicidin1* are not shown because they were not significantly correlated with any of the other parameters. A cross indicates non-significant correlations ($p > 0.05$; Spearman rank correlation test). Correlation coefficients are color coded; black font indicates positive correlations and white font indicates negative correlations. We did not detect any significant negative correlations. SME, total area of mucous cells per total area of epithelium in the dorsal skin. SNE, number of mucous cells per total area of epithelium in the dorsal skin. GME, total area of mucous cells per total area of epithelium in the gills. GNE, number of mucous cells per total area of epithelium in the gills. SM1, skin *muc5ac1*. SM2, skin *muc5ac2*. SM5, skin *muc5b*. SC1, skin *cathelicidin1*. GM2, gill *muc5ac2*. GM5, gills *muc5b*. IM2, intestine *muc2*. ID4, intestine *defensin4*.

mucin secretion and the number of mucus cells, but also the mucus composition, viscosity and thickness can be changed in response to host factors and external factors such as probiotics in feeds (Paone and Cani, 2020). Interestingly, our study showed that singly or in combination, LAB can be effective to target different mucosal surfaces.

In the present study, we found that feeding a mix of *L. plantarum* and *L. fermentum* can even increase the villi height. These observations are similar to those of several other studies that reported histomorphometric effects of probiotics on the intestine of fishes (Daniels et al., 2010; Merrifield et al., 2010; Pirarat et al., 2011). A probiotic species *L. rhamnosus* significantly increased villi height in Nile tilapia fed at a rate of 10^{10} CFU/g in feed for 30 days (Pirarat et al., 2011). Yet another probiotic *L. pediococcus* enhanced the enterocyte microvilli in the anterior intestine in rainbow trout (Merrifield et al., 2010). Similarly, *Bacillus* spp. increased microvilli length and density in larvae and post-larvae of European lobster (*Homarus gammarus*) (Daniels et al., 2010). It should be noted that the aforementioned studies have assessed the effect of a single probiotic species and, most of them did not report the impact on the absorptive surface or SNVs. However, the present study results revealed the significant reappearance of SNVs in the enterocytes, likely associated with the observed increase in SCFAs and improvement in other intestine features such as villi height of fish fed the probiotics, *L. plantarum* and *L. fermentum*. These observations point to improved mucosal health of post-smolt Atlantic salmon. Therefore, both *L. plantarum* and *L. fermentum* have the potential to prevent SBMIE in Atlantic salmon. In this study, the combination of two LAB species showed a better response in mucosal tissues as they might have promoted cross feeding through positive interactions that benefit the host.

Enteritis is defined as inflammation of the intestine, and the condition is characterised by shortened intestinal villi, changes in mucus production, epithelial abnormalities, widened lamina propria as well as submucosa mainly due to the infiltration of different immune cells including neutrophils, macrophages and lymphocytes (Agboola et al., 2022; Baeverfjord and Krogdahl, 1996; Nimalan et al., 2022). In our previous studies also, we observed intestinal inflammation in Atlantic salmon fed 20% SBM in the diet (Nimalan et al., 2022; Sørensen et al., 2021). In those studies, we tested, amongst others, a marine-based diet (BG1) mainly with fish meal and fish oil, and one diet with 20% SBM diluting the marine ingredients (BG2) to study the gut health of Atlantic salmon. Though we did not have a positive control in the present study, BG1 can be considered as the positive control. The BG2 diet used in our previous studies is the CT diet in the present study. The gut barrier biomarker mucin 2, *muc2*, was significantly reduced in fish fed BG2 compared to BG1 (Nimalan et al., 2022; Sørensen et al., 2021). A comparison of the positive control in Nimalan et al. (2022) with the CT diet in the present study showed that HOV, HOE, WLP, IEL and SNV were significantly reduced and WOV tended ($P = 0.070$) to be lower in fish fed the CT (Supplementary Table 1). The number of intraepithelial lymphocytes (IELs) in CT (less score = more cells in this study, 3.67) was significantly higher compared to BG1÷ (score 4.75) in Nimalan et al. (2022). Moreover, *muc2* in the intestine was significantly reduced in CT (0.75) compared to BG1 (2.79). Therefore, reduction in supranuclear vacuoles, villi height and enterocyte height and increase in width of lamina propria and IELs indicate that fish fed the CT diet could develop enteritis in the present study.

Generally, fish fed plant ingredients tend to have more small-sized mucous cells in the intestine (Sørensen et al., 2021). This is a general response feature of inflammation. Though studies have reported probiotics-induced increase in the mucous cells, under inflammatory condition also we observed more mucous cells (Nimalan et al., 2022). In the present study, an increasing trend was observed for the distal intestinal mucous cell indices of the fish fed lactic acid bacteria, when applied singly or in combination. Since the response was not significant compared to the control group (without probiotics) NOM score may not indicate an alleviation of enteritis. Nevertheless, for more responsive organs like the gills, the mucous cell indices can be considered as a good indicator.

Assessment of various forms of enteritis like ulcerative colitis and Crohn's disease in humans is based on established histological scores (Erben et al., 2014; Ma et al., 2021). It was reported that the percentage of tissue occupied by $CD4^+$ T cells was significantly lower in inflamed tissue while that covered by macrophages were significantly higher (Naser et al., 2011). Furthermore, infiltrating intestinal T cells in active cases of inflammatory bowel disease had increased percentages of $CD4^+$ T cells, T_{reg}, and lower percentages of $CD8^+$ T cells and $CD103^+$ T cells (Smids et al., 2018). Although we were not able to immunophenotype the subpopulation of the IELs, this subset was lower in the soybean meal fed fish ($p = 0.127$). The T cell population in the IEL compartment of humans is populated mostly by induced TCRαβ CD8αβ and barely by TCRαβ CD4 (Mayassi and Jabri, 2018). Flow cytometry studies are necessary to ascertain the type of T cells that were decreased in the soybean fed fish. As for the higher number of IELs in the probiotic fed groups, another study has also reported similar results. *Lactobacillus rhamnosus* feeding increased the number of IELs in the intestine of tilapia (Pirarat et al., 2015).

Probiotic feeding can have benefits beyond immune system stimulation. *Lactobacillus* feeding can improve the growth of the probiotic-consumed fish (Abdelfatah and Mahboub, 2018; Dawood et al., 2019; Feng et al., 2019; Jami et al., 2019; Van Nguyen et al., 2019). Supplementation of a mixture of probiotic species may have stronger growth-promoting effects than a single probiotic species (Aly et al., 2008; Beck et al., 2015; Hai, 2015; Hai et al., 2009). Nonetheless, we did not observe growth-promoting effects of the two LAB in the present study, when Atlantic salmon post-smolts were fed SBM-based feed with either *L. plantarum* or *L. fermentum* or a mix of the two, at 10^8 CFU/g of

feed for 38 days. On the other hand, supplementation of a multi-strain commercial product containing probiotic strains of *Saccharomyces cerevisiae*, *Enterococcus faecium*, *L. acidophilus*, *L. casei*, *L. plantarum*, and *L. brevis*, in a diet with 20% SBM, prevented SBM-caused growth retardation in rainbow trout. Furthermore, the fish fed this product and the SBM starter diets exhibited higher digestibility and growth during the grow-out phases (Sealey et al., 2009). These findings suggest that certain microbes can promote intestinal health and prevent inflammation induced by antinutrients. Notably, these short-term effects will be erased following the cessation of supplementation, presumably because the strains do not colonize the intestinal tract to impart a sustained health-promoting effect (Sealey et al., 2009).

5. Conclusion

The present study has shown that the fish fed SBM alone had enteritis symptoms. Single or combined application of *L. plantarum* and *L. fermentum* can stimulate the formation of goblet cells at different mucosal surfaces such as the skin, gills and intestine. Though probiotics did not completely prevent the SBMIE, they had positive effects—such as increased villi height, reduced lamina propria width, and reappearance of supra nuclear vacuoles—on intestinal micro-morphometric structures. This study has demonstrated that the probiotics can prevent enteritis, possibly by altering the SCFA composition, mucous cell count, mucin and AMP genes expression, and improved endocytosis.

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CRediT authorship contribution statement

Mette Sørensen conceptualized the study, developed methodology, acquired resources, and was involved in investigation, writing, reviewing and editing the manuscript, besides supervising and coordinating the study and leading the project. Dagmar Mudroňová, Jana Koščová, Soňa Gancarčíková and Adriána Fečkaninová developed methodology, acquired resources for probiotics and short chain fatty acid study, and were involved in investigation, writing, reviewing and editing the manuscript. Solveig Lysfjord Sørensen developed the protocol and conducted the gene expression study. Nadanasabesan Nimalan carried out histological studies, analysed data and wrote the first version of the manuscript. Saraswathy Bisa, Ioannis N. Vatsos and Viswanath Kiron were involved in developing, editing and reviewing the manuscript. All co-authors have read and edited the manuscript and have approved the submission to Aquaculture Reports.

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.

Data availability

We have included all the data in the manuscript and the supplementary files.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aqrep.2022.101461.

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