Aquaculture 552 (2022) 737983

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Investigation into the potential use of dietary supplementation to reduce the impact of Amoebic Gill Disease

Anita Talbot [a,1], Michelle McCormack [a,*,1], Ankit Dwivedi [a], Ian O'Connor [a], Victoria Valdenegro [b], Eugene MacCarthy [a]

[a] Marine and Freshwater Research Centre, Galway Mayo Institute of Technology, Galway, Ireland
[b] BioMar AS, Havnegata 9, Pirsenteret, Trondheim 7043, Norway

ARTICLE INFO

Keywords:
Amoebic Gill Disease (AGD)
Paramoeba perurans
Atlantic salmon
Gene expression

ABSTRACT

Amoebic Gill Disease (AGD) is a disease affecting cultured Atlantic salmon (*Salmo salar*) which is caused by *Paramoeba perurans*. The immunomodulatory impact of functional foods has previously been demonstrated in salmon. The aim of this study was to investigate the role of a novel fish feed in mitigating the severity and progression of AGD in Atlantic salmon.

Negative and positive control fish were maintained on a base fish feed, with experimental fish maintained on a customized feed blend pre- and post-amoeba inoculation. Disease progression was evaluated using gill scoring and diagnostic quantitative real-time polymerase chain reaction (qPCR) for the detection of *P. perurans* at 14 and 21 dpi. The expression profiles of 11 immune related genes were analyzed by qPCR at 14 and 21 dpi. The customized feed blend was shown to delay the onset of clinical symptoms associated with AGD. Furthermore, the expression of genes with roles in maintenance of the mucosal layer and mucosal defense were impacted in fish fed on this novel feed formulation.

1. Introduction

The escalating demand for fish and fish products in recent years has driven the growth of the aquaculture industry. Indeed, global aquaculture production has doubled in three decades, reaching about 171 million tons in 2016. Salmonids make up about 80% of total fish trade and more than 65% of the total value of fish and fish products (FAO, 2020).

Amoebic Gill Disease (AGD), caused by the marine ectoparasite *Paramoeba perurans* (*P. perurans*), also known as *Neoparamoeba perurans,* was first described in Tasmania in 1986 (Munday, 1986) and has been acknowledged as one of the most significant health threats in salmon farming (Rodger, 2014). Gill hyperplasia and lamellar fusion, characteristic of AGD, can lead to mortality in up to 80% of cases if left untreated (Adams and Nowak, 2003; Oldham et al., 2016). Treatment primarily involves bathing affected fish in fresh water or hydrogen peroxide baths, to remove amoeba from the gill surfaces and reduce gill mucus viscosity (Roberts and Powell, 2008). However, the benefits of these treatment regimens are limited, due to incomplete removal of the parasite (Parsons et al., 2001) and acute physiological stress to fish (Martinsen et al., 2018), coupled with the high financial and labor burdens placed upon farmers (Munday et al., 2001).

More recently, studies have begun to focus on selective breeding as a means of fostering resistance to AGD in Atlantic salmon (Boison et al., 2019; Robledo et al., 2020). However, the existing highre-infection rates, whichoften require multiple treatments within a single life cycle (Martinsen et al., 2018), underline the urgent need for prophylactic strategies. Although fish have been successfully immunized against several common bacterial and viral infections in aquaculture (Ma et al., 2019), and attempts to immunize fish against *P. perurans* have been conducted (Cook et al., 2012; Valdenegro-Vega et al., 2014; Villavedra et al., 2010) no viable vaccines against *P. perurans* have been developed to date.

Nutraceutical products supplement standard nutritional regimes to provide additional health benefits (Télessy, 2019) and have been used as immune modulating agents to mitigate infection. Previous studies have demonstrated the immunomodulatory effect of supplemented fish feeds in sea lice infection (Jodaa Holm et al., 2016; Núñez-Acuña et al., 2015;

* Corresponding author at: Marine and Freshwater Research Centre, Galway Mayo Institute of Technology, Dublin Road, Galway H91 TRNW, Ireland.
E-mail address: michelle.mccormack@gmit.ie (M. McCormack).
[1] Contributed equally to this paper and present the work as joint first authors.

https://doi.org/10.1016/j.aquaculture.2022.737983
Received 11 August 2021; Received in revised form 12 January 2022; Accepted 26 January 2022
Available online 31 January 2022

Skugor et al., 2016). Other such dietary impact studies have shown an increase in the expression of genes related to immunity and a decrease in viral loads of the *piscine myocarditis* virus and the Atlantic salmon reovirus (Martinez-Rubio et al., 2014; Martinez-Rubio et al., 2012). However, the underlying immunomodulatory effects of these feeds are poorly understood. In the case of AGD, oral supplementation with the immunostimulants levamisole and β -glucan was found to be ineffective in delaying disease onset (Bridle et al., 2005; Zilberg et al., 2000). However, functional diets with a combination of micro additives, vitamins and amino acids, have shown to be a promising preventive approach to the disease (Mullins et al., 2020).

The present study aimed to investigate the nutraceutical benefits of a novel fish feed in mitigating the severity and progress of AGD in salmon. Standard base recipe and a novel functional fish feed, provided by our industrial partner BioMar, were compared to study the impact of feed upon variables such as disease onset, severity and duration. Candidate genes, many of which were previously shown to have roles in AGD (Marcos-López et al., 2018; Marcos-López et al., 2017), were identified in ongoing research investigating differential gene expression in AGD using ribonucleic acid (RNA) sequencing (RNA-seq) (data submitted for publication). To investigate the potential immunomodulatory effects of the novel feed at various timepoints post infection, high throughput qPCR was utilized to analyze the genomic expressions of mucin 5 ac (*muc5ac*), mucin 2 (*muc2*) and mucin 7 (*muc7*) the secreted gel-forming mucins (Voynow and Fischer, 2006), the membrane bound mucin 13 (*muc13*) (Maher et al., 2011) and the alpha-*N*-acetylgalactosaminide alpha-2,6-sialyltransferase 1 (*st6galnac1*) and polypeptide N-Acetylgalactosaminyltransferase 14 (*galnt14*), enzymes which contribute to mucus rigidity and diversity (Linden et al., 2008; Padra et al., 2014; Fahy and Dickey, 2010). The expressions of the innate immune molecules lysozyme g (*lys-g*) and carboxylesterase 3 (*ces3*) (Saurabh and Sahoo, 2008; Gomez et al., 2013), angiotensinogen (*agt*) which influences osmoregulation and vasoconstriction (Navar et al., 2011; Rudemiller and Crowley, 2016) and carboxypeptidase N Subunit 1 (*cpn1*) and mannan-binding lectin serine protease 1 (*masp1*) which modulate the classic and lectin complement pathways respectively (Mueller-Ortiz et al., 2009; Thiel et al., 2012) were also investigated.

2. Methods

2.1. Fish husbandry

Atlantic salmon smolts reared on a land-based freshwater hatchery in the west of Ireland, were transferred to a land-based indoor, marine recirculating facility at the Marine and Freshwater Research Centre (MFRC) at the Galway-Mayo Institute of Technology (GMIT) in Galway, Ireland. Salmon smolts (average weight 85 g ± 10 g) were distributed into six circular black 1m^3 tanks (n = 40 fish/tank) with the following conditions: water temperature 12 °C, artificial salinity of 30 parts per thousand (ppt) (Coral Pro salt, Red Sea), light cycle of 12 h light, 12 h dark. Diet A was used as the control feed, and as a base for Feed B which contained 2 additional micro additive packages. Both control Feed A and experimental Feed B were produced by an extrusion process in the BioMar Tech-Centre (Brande, Denmark); the formulations were isoenergetic and isonitrogenous and covered all known nutritional requirements for Atlantic salmon. Diet ingredients and proximate composition are detailed in Table 1. Fish were fed with 1% body weight per day. The *in-vivo* fish trial was carried out according to the ARRIVE guidelines for animal research (Kilkenny et al., 2010). This project was authorized by the Health Products Regulatory Authority (HPRA) in Ireland under project authorization number AE19137/P003 in compliance with Directive 2010/ 63/EU transposed into Irish law by S.I. No 543 of 2012.

Table 1
Formulation and estimated and proximate composition of the experimental diets.

	Diets	
Ingredients	A	B
Marine origin protein	30.0	30.0
Vegetable origin protein	37.8	39.6
Wheat	9.5	9.1
Marine origin Oil	13.9	12.1
Vegetable origin Oil	5.9	5.1
Vitamin and mineral Premix	2.28	2.71
Others [a]	0.69	0.65
Micro additives premix 1 [b]		0.43
Micro additives premix 2 [b]		0.4
Estimated composition		
Dry matter (DM) (%)	94.0	94.0
Moisture (%)	6.0	6.0
Protein - crude (% DM)	45.4	45.1
Fat - crude (% DM)	24.3	24.1
Ash (% DM)	8.6	8.7
Proximate composition		
Moisture (%)	6.02	5.4
Protein - crude (% DM)	46.3	46.9
Fat - crude (% DM)	23.6	22.4
Ash (% DM)	8.2	8.1

[a] Others include amino acids, inert markers, antioxidants and pigments.
[b] Micro additive mixes are a proprietary composition of BioMar AS and include plant extracts, nucleotides, prebiotics and MOS.

2.2. Paramoeba perurans isolation and culture

Paramoeba perurans trophozoites were isolated from AGD-affected Atlantic salmon, located on a commercial farm in the west of Ireland, by swabbing the gills and placing swabs in 0.2 μm filtered sterile seawater (SSW) (30 ppt, 4 h). Amoebae were grown on marine yeast agar plates (MYA; 0.01% malt, 0.01% yeast, 2% Bacto Agar), at 16 °C, overlaid with 7 ml SSW (Crosbie et al., 2012). The amoebae were subcultured every two weeks by transferring free-floating cells to fresh MYA plates. Confirmation of *P. perurans* identity was performed using qPCR as previously described (Downes et al., 2015). The amoebae were in culture for 33 weeks prior to challenge.

2.3. Feed trial and P. perurans challenge

All smolts were fed with BioMar Feed A (A) during the pre-trial acclimation period of 7 days. Following acclimatization both negative and positive control fish (duplicate tanks (40 fish/tank/treatment)) were maintained on Feed A throughout the duration of the trial. Experimental fish (duplicate tanks 40 fish/ tank) were changed to Feed B (Fig. 1) and maintained on this feed for 21 days prior to infection challenge with *P. perurans*. Fish in 4 of the 6 tanks were exposed to 500 amoeba/l for 4 h. Experimental conditions were (1) Feed A naive, negative control (A-), (2) Feed A AGD-affected, positive control (A +) and (3) Feed B AGD-affected (B +) (Fig. 1).

2.4. Sample collection

Gill samples were collected from six control (A-) and six experimental fish (A+ and B+) at each of the following time points: T0; 0 dpi (pre-AGD challenge), T1 (14 dpi) and T2 (21 dpi). Fish were euthanized by overdose of anesthetic (400 mg L-1 tricaine methane sulfonate (MS-222). For diagnostic PCR analyses the left fourth gill arch was retained. In each case the filaments were excised from the cartilaginous gill arch and confirmation of *P. perurans* identity was performed using real-time PCR as previously described (Downes et al., 2015). There was no preselection of the gills for amoeboid mucous patches during gill sampling.

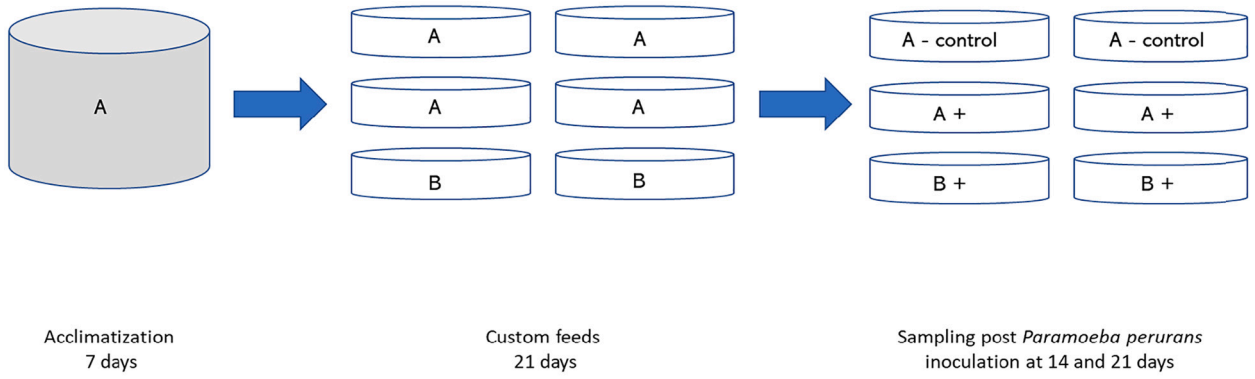

Fig. 1. Experimental design of feed trial with *P. perurans* infection. Fish were acclimatized for 7 days on Feed A (A). Fish were divided among 6 tanks and 4 tanks were maintained on Feed A (A) and 2 tanks were fed Feed B (B) for 21 days prior to *P. perurans* inoculation. Two tanks were retained as negative controls (A – control) and the remaining 4 tanks were maintained on their respective feeds and exposed to *P. perurans* (A+ and B+). Sampling was undertaken at 14 (T1) and 21 (T2) dpi.

2.5. Gill histology

Fixed gill samples (left gill arch 1) were routinely processed and embedded in paraffin wax blocks. Sections (5 μm) were stained with haematoxylin and eosin (H&E) and examined using an Olympus BX41 Microscope and CellSens software (Olympus). Gill filaments exhibiting features of AGD including lamellar fusion, hyperplasia and vesicle formation were imaged. Additional sections were also stained with Alcian blue/ Periodic-acid Schiff (AB/PAS) for mucin detection. Mucous cell quantification was performed by counting the number of stained mucous cells visible in 10 consecutive inter-lamellar units (ILU), across four randomly chosen areas, for each gill, as previously described (Minich et al., 2020).

2.6. Disease progression

Clinical symptoms of AGD were determined by macroscopic examination of the intact gills in euthanised fish. Prior to sampling, 3 fish were taken from each duplicate control and AGD-affected tank, and all 6 fish placed in an anesthetic bath (400 mg L^{-1} tricaine methane sulfonate). Naïve control fish (6) and experimental fish (6) were sampled at each time point (14 and 21 dpi) and all fish were gill scored.

Clinical symptoms of AGD were scored ranging from Gills score (GS) 0 (absence of clinical symptoms) to GS5 (extensive lesions covering most of the gills surface), in accordance with the standard protocol (adapted from Taylor et al., 2009). In compliance with the project authorization by HPRA, when 50% of AGD-affected fish displayed a gill score of 2 in any treatment group, it was determined that the humane end-point for this group was reached and the fish were removed from the trial.

2.7. PCR primers

Primers were designed using PrimerQuest (Integrated DNA Technologies, https://eu.idtdna.com/) with amplicon size in the region of 150 bp, Tm 62 °C, 50% GC content for the following genes: angiotensinogen (*agt*), carboxylesterase (*ces3*), lysozyme g (*lys-g*), carboxypeptidase N subunit 1 (*cpn1*), mannan-binding lectin-associated serine protease-1 (*masp1)*, mucin 2 (*muc2*), mucin 5 AC (*muc5AC*), mucin 7 (*muc7*), mucin 13 (*muc13*), *N*-acetylgalactosaminyltransferase 14 (*galnt14*), alpha-*N*-acetylgalactosaminide alpha-2,6-sialyltransferase 1 (*st6galnac1*), and elongation factor 1A *ef1a* (Table 2).

2.8. RNA extraction and quantification

In each case the left gill arch 3 was collected, the filaments were excised from the arch and were stored in RNAlater™ (Ambion, USA), stored at 4 °C overnight before being stored at −80 °C until required. Total RNA was extracted from 30 mg gill samples using RNeasy® Mini Plus Kit (Qiagen, Germany) according to the manufacturer's instructions. Briefly, a 30 mg sample from each individual fish gill, irrespective of the presence of absence of mucoid lesions, was homogenized using a bead mill (Fisher Scientific) and 2.8 mm ceramic beads in 350 μl RLT lysis buffer and 1 μl DX antifoam reagent (Qiagen, Germany), 5 pulses/s, 10 s, repeated 3 times. The optional DNase I (Qiagen, Germany) step was included to ensure complete elimination of gill genomic DNA. RNA was eluted in 70 μL of nuclease -free water and stored at −80 °C until required. RNA quality was visualized on a 1% agarose gel and quantified using the Qubit 2.0 fluorometer (Thermo Fisher Scientific, USA).

Table 2
List of genes with forward and reverse primers for qPCR.

Gene description	Gene ID	Forward	Reverse	Amplicon size
alpha-*N*-acetylgalactosaminide alpha-2,6-sialyltransferase 1-like	106,589,847	TGGCTACAACAATGCACCTCA	GTAGTACTTCCACGGCTGTCTG	148
mucin-13-like	106,579,100	AACACCGGAGGCCACTACAA	ACCTTGGCTTCCTCACATAGCC	165
mucin-5 AC-like	106,612,029	AGGACCTGCAGGATGCAAAACT	TGGCTTCGTGGTGTCACTCT	156
mucin-7-like	106,560,392	TGTTCTCATGCAGCTGAGGC	TGCCACAGATCTTGTTGCTGG	135
angiotensinogen	100,195,417	AGTGGATTTCTCCCAACCCCAG	CCTTCCTCCAGTTGCCTTTGAA	158
carboxylesterase 3	100,380,756	GGATACCACAGAGATGCAGGT	AGTATCAGCTCCTCCCCGTT	263
carboxypeptidase N, polypeptide 1	100,196,707	TGCTGAGGCTATACAGTTGCAC	GGACATGCGGGTGTGTTCTTT	168
lysozyme g-like	106,587,409	TGCTAGAGGTTATAGCGGGGT	ACGTATTGCTCTCCCACCTCTG	172
mannan-binding lectin serine peptidase 1	106,612,353	ACAGGACTTGTCGAGTGGAGTG	TGTCGAAGGTGTCGTCGAACT	168
mucin-2-like	106,561,012	GCTCAACATGAGCCTTCCGC	GAGTCCCCTGTGTGACTGCT	91
UDP-*N*-acetyl-alpha-D-galactosamine:polypeptide *N*-acetylgalactosaminyltransferase 14	LOC106571428	AGCACTCAACCAGGAGTGGAT	ATCGTTGTTTCCCGTCGCTG	137
Elongation factor 1 alpha	LOC100136525	GCTGCTGAGATGGGTAA	CAAACTTCCACAGGGAAATG	133

2.9. Reverse transcription

Complementary DNA (cDNA) was generated from individual gill samples by reverse transcription using GoScript (Promega) according to the manufacturer's instructions. Briefly, 0.5 μg total RNA, 0.5 μl Oligo $(dT)_{15}$ (500 μg/ml) and 0.5 μl random hexamers (500 μg/ml) and nuclease-free water were combined together in a final volume of 11.5 μl, heated at 70 °C for 5 min and then placed immediately on ice. The contents were collected by a brief centrifugation before adding 4 μl GoScript™ 5× buffer, 2 μl 25 mM $MgCL_2$ 1 μl 10 mM PCR nucleotide mix, 0.5 μl RNasin (40 U/μl) and 1 μl of GoScript™ reverse transcriptase. Reverse transcription, annealing was carried out at 25 °C for 5 min, extension at 42 °C for 1 h followed by enzyme inactivation at 70 °C for 15 min and storage at −20 °C until needed.

2.10. Gene expression analysis

Gene expression analysis was performed using qPCR as per the manufacturer's instructions using 48.48 Dynamic Array Integrated Fluidic Circuit (IFC) chips on the Biomark HD system (Fluidigm, USA). The IFC chip was primed on the Juno IFC controller (Fluidigm, USA) as per the manufacturer's instructions. Briefly, diluted cDNA samples were mixed with a primer pool consisting of 12 primer pairs (*agt, ces3, lysG, cpn1, masp1, muc2, muc5ac, muc7, muc13, galnt14, st6galnac1 and ef1a*). Each assay and sample inlet contained 5 μL of respective mix.

The assay mix consisted of 0.7 μL of 50 mM primer mix (IDT, Belgium), 3.5 μL of 2× assay loading reagent (Fluidigm), and 2.8 μL of 1× DNA elution buffer (Qiagen, Germany). The sample premix was prepared with 200 μL of 2× SsoFast EvaGreen supermix with low ROX (Bio-Rad, München, Germany) and 20 μL of 20× Binding Dye Sample Loading Reagent (Fluidigm). Further, 3.85 μL of sample pre-mix was mixed with 3.15 μL of the diluted pre-amplified PCR product for each sample inlet. The qPCR program was 95 °C for 60s, 30 cycles of 95 °C for 5 s, 58 °C for 20s followed by a melt curve protocol of 55 °C to 95 °C with a ramp rate of 1 °C / 3 s. Each sample was run in triplicate/chip and 3 chips were used for the qPCR.

Validation of *ef1a* as the housekeeping gene was performed using geNorm in qBase+ (Vandesompele et al., 2002). PCR amplification efficiency (E) was calculated for each gene of interest and the housekeeping gene by the generation of standard curves using 10-fold serial dilutions of the cDNA template (standard curve: $R^2 > 0.980$, amplification efficiency range 90–105%). Melt curve analysis was preformed to confirm the specificity of the amplified PCR product.

Relative gene expression (ddCT) of each gene at T1 (14 dpi) and T2 (21 dpi) was calculated using the delta delta CT (ddCT) method (Livak and Schmittgen, 2001) where A+ and B+ were compared to A- at that same timepoint using the Real-Time PCR Analysis software on the Biomark HD (Fluidigm, USA).

2.11. Statistical analysis

A Student's *t*-test was performed to compare mucous cell counts. The distribution of the scores for the 2 experimental diets was compared for each sampling time using an independent samples Kruskal-Wallis non-parametric test. For each gene, an unpaired Welch's t-test was performed in Excel 2016 (Microsoft, USA) to assess any significant differences in gene expressions between feed types at each time point.

3. Results

3.1. Gill score analysis

All fish scored GS0 at T0. At T1 5 of the 6 fish from the A + group were at GS2, which was deemed the humane endpoint, and the trial was terminated for this cohort. For the B + group, at T1 all 6 fish showed mild symptoms consistent with a gill score of less than 1, and by T2 2 of the 6 fish of this cohort had GS1, 3 had GS 2 and 1 fish was GS 0 (Fig. 2). Statistical analyses between fish fed the 2 diets revealed that diet B had a significantly different distribution of scores to fish fed diet A at T1 (Independent sample Kruskal-Wallis 7,2, df 2, $P = 0.028$). Individual gill score data for each fish are detailed in supplementary data Table 1.

3.2. Diagnostic qPCR

At T0 the qPCR results of A-, A+ and B+ were all negative. The negative control (A-) remained negative at T1 (14 dpi) and T2 (21 dpi). Five of the 6 fish in the A+ group at T1 were positive for the presence of amoeba. At that same time-point, 2 of 6 fish from the B+ group were positive for *P. perurans*. At T2, 4 of 6 fish from the B+ group were positive for the presence of *P. perurans* (Fig. 3). Individual qPCR data for each fish are detailed in supplementary data Table 1.

3.3. Histology

Histopathology analysis revealed no signs of AGD in control samples (Fig. 4 A). Hyperplastic lesions, interlamellar vesicles and hyperplastic lamellar fusion were evident in AGD-affected samples at T1 and T2 (Fig. 4 B – D). Staining with AB/PAS allowed the visualization and quantification of mucous cells across randomly selected areas of gill section (Fig. 5). Mucous cell counts remained consistent between A-control and A+ feed groups at T1. However, mucous cell numbers were significantly lower in the B+ groups when compared to A- at both T1 and T2 (Fig. 6).

3.4. Gene expression analysis

The immunomodulatory effects of Feed A and Feed B were investigated through the quantification of the differential gene expression of eleven innate immune genes in the gill. The results obtained are displayed in Fig. 7. Values given are the fold changes for each gene in each condition, compared to the negative control (A-) at each timepoint. Only those genes expressing fold changes of at least +/− 1.5 were considered to be biologically relevant (shaded bars in all graphs Fig. 7) (Zhao et al., 2018).

Fig. 7. ctd. Gene expression analysis of target genes: Using qPCR the expression of target genes was assessed in each AGD-affected feed group (A+ and B+) at both T1 (14 dpi) and T2 (21 dpi). Graphed values are fold change values in comparison to the negative control (A-) at each timepoint. Only those genes expressing fold changes of at least +/− 1.5 were considered to be biologically relevant (shaded bars in all graphs). Statistical analyses compared the effect of feed on gene expression *$p < 0.05$; **$p < 0.01$.

4. Discussion

The host response to AGD is a multifaceted dynamic phenomenon, the investigation of which is a complex challenge. Dietary supplementation as a means of preventative strategy is a promising proposition, which could provide a cost-effective mitigation strategy to tackle the ever-present threat of AGD in salmon aquaculture. In this study, AGD progression in fish fed a novel proprietary feed (Feed B), provided by BioMar was assessed and compared with AGD development in fish maintained on Feed A (BioMar). Significant differences in the progression of disease between the standard and novel feed were observed from the first sampling time point following exposure to *P. perurans* (14 dpi). AGD diagnosis was based on gross examination. More fish were found to have macroscopic signs of AGD pathologies at 14 dpi (T1) and 21 dpi (T2) than demonstrated by the presence of amoeba in qPCR analysis. This discrepancy may be explained by the fact that, for consistency, the fourth left gill arch was used for PCR analysis, regardless of whether there were lesions on this particular gill or not. Indeed, throughout this study, gill sampling was carried out in a manner which respected

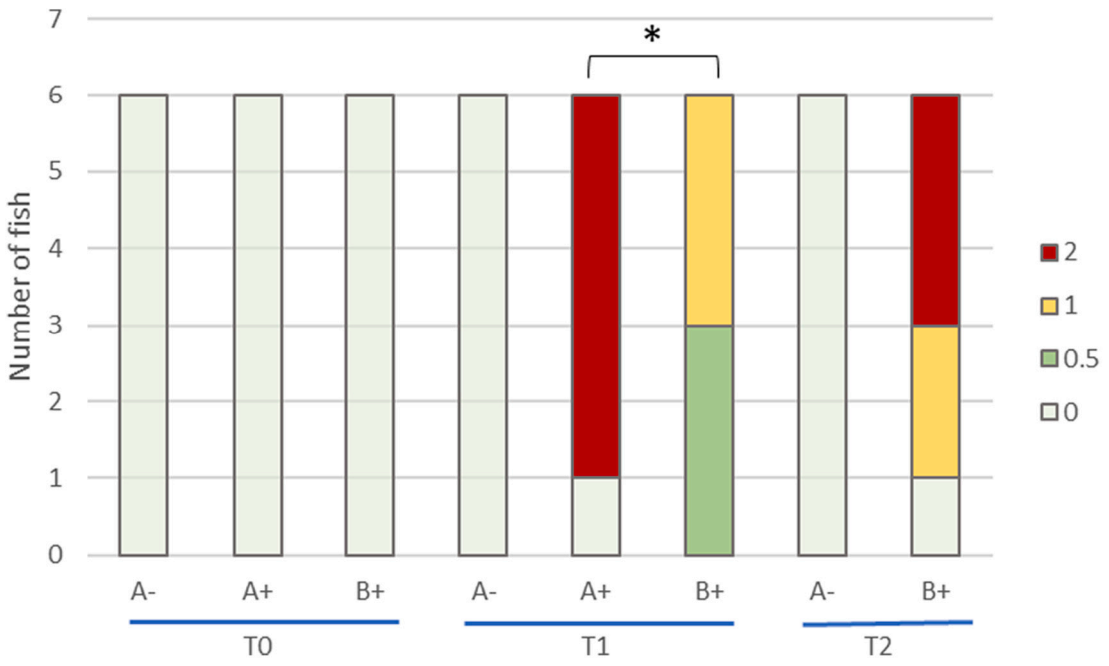

Fig. 2. Distribution of gill scores among fish fed different diets at different sampling points T0 (0dpi), T1 (14dpi) and T2 (21 dpi). * denotes significance differences between diets within each time point (Kruskal-Wallis ANOVA, $P < 0.05$).

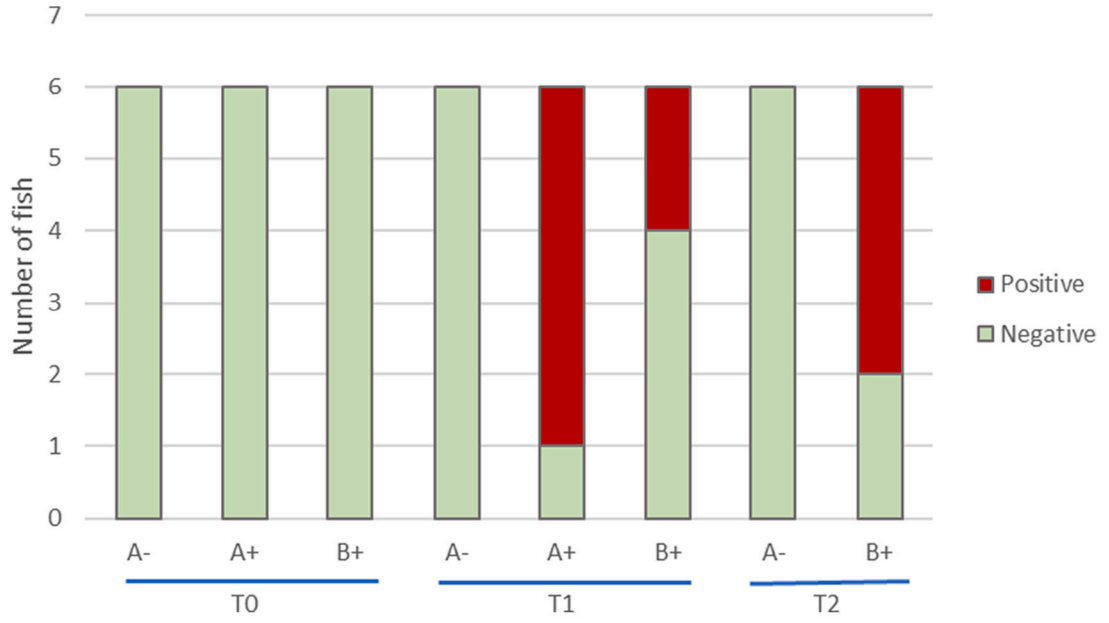

Fig. 3. Distribution of 18S N. perurans qPCR test results among fish fed different diets at different sampling points T0 (0dpi), T1 (14dpi) and T2 (21 dpi).

experimental consistency, and pre- selection of samples to specifically include or exclude areas of AGD pathology was avoided. In addition, it has been shown that diagnostic PCR carried out on a gill biopsy sample, as in this study, is more likely to yield a false negative result than analysis of a gill swab (Downes et al., 2017). In any case, given that in this study, many of the fish at T1, particularly those maintained on Feed B, exhibited little or no clinical signs of AGD, it would have been impossible to consistently sample lesioned areas. However, for future investigations, analysis of gill swabs in conjunction with gill biopsies would be preferable to ensure consistency between PCR and gill score diagnoses.

At T1 5 of the 6 fish in the Feed A group had reached GS2, the ethically approved humane end-point for this study and this cohort was removed from the study. At this same timepoint 3 of the 6 fish fed Feed B were at GS1, while the remaining 3 displayed no pathology. These fish were maintained on the same feed and were reassessed one week later (21 dpi), at which time 4 of the 6 fish maintained on Feed B had reached GS2. As fewer fish on Feed B were affected by 14 dpi, and to a much less severe degree, these observations suggest that the novel formulation feed used in this study had positive effects on both disease onset and severity.

In order to explore some of the mechanisms underlying these apparent protective effects, the expressions of 11 target genes (*agt*, *ces3*, *lys-g*, *cpn1*, *masp1*, *muc2*, *muc5AC*, m*uc7*, *muc13*, *galnt14* and *st6galnac1*),

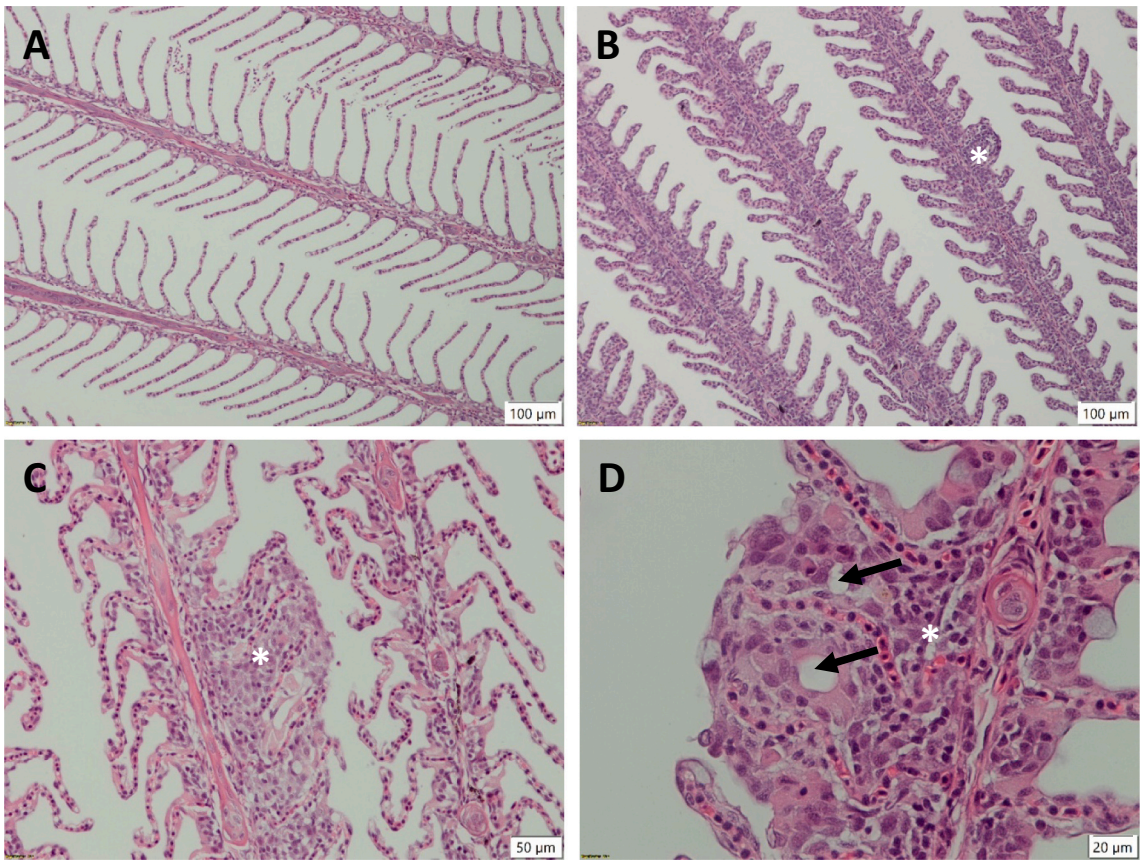

Fig. 4. Histology (H&E staining) of salmon gill samples: (A) Gill sample from pre-inoculation control (T0) (B) Gill sample from 14 dpi (T1) and (C - D) gills from salmon sampled at 21 dpi (T2). Features commonly associated with AGD including hyperplastic lesions, hyperplastic lamellar fusion (*) and interlamellar vesicles (black arrow) were evident in gill samples (B - D). Scale bars are indicated on images.

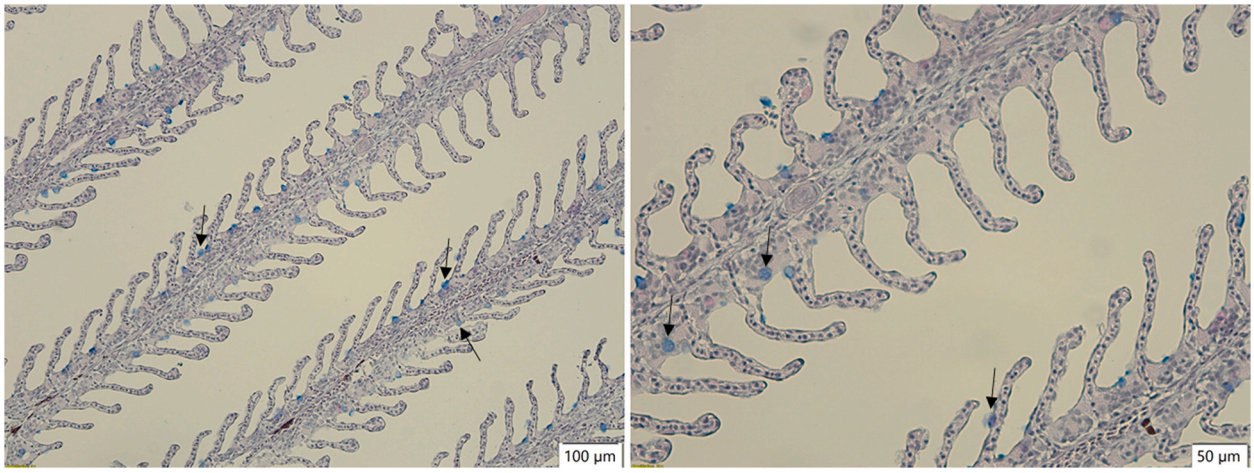

Fig. 5. Histology (AB/PAS staining) of salmon gill samples: Black arrows indicate areas with positive mucin staining. Scale bars are indicated on images.

chosen from previous studies, with a diverse range of functions, were assessed in each feed group at each timepoint. An arbitrary cut-off of +/− 1.5 fold change was applied to the qPCR data to focus on those genes which exhibited the most significant changes when compared to negative controls (Feed A naïve fish) (Zhao et al., 2018).

Of all 11 genes assessed, *galnt14* was the only gene which did not reach a sufficiently substantial fold change in any of the feed groups. However, many of the remaining genes demonstrated expression patterns which were interesting in the context of nutraceutical immune modulation in response to AGD.

Angiotensinogen (AGT) is a hormone that is part of the renin-angiotensin-aldosterone system (RAAS) which maintains the osmoregulatory and vasoconstriction mechanism in vertebrates (Navar et al., 2011; Rudemiller and Crowley, 2016). This mechanism has also been investigated in Rainbow trout (*Oncorhynchus mykiss)* (Olson et al., 1994). The *agt* gene possesses a serpin domain that is known to have protease activity and is associated with the anti-coagulation system (Wang and Ragg, 2011). The RAAS system is activated by innate and adaptive immune responses in mice (Rodriguez-Iturbe et al., 2017). An *in-vitro* study in Atlantic salmon demonstrated the role of AGT in

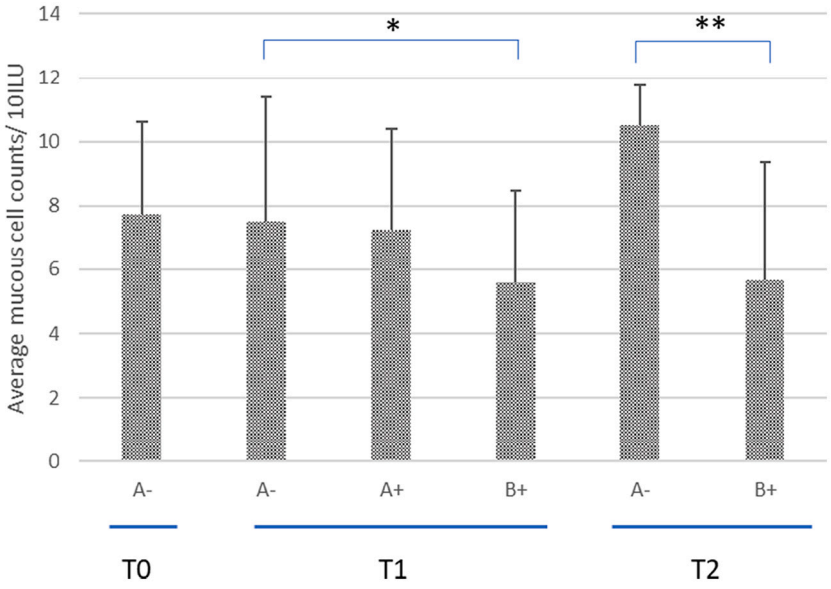

Fig. 6. Mucous cell counts of salmon gill samples: Gill sections from the naive control (T0) and control (A-) and AGD-affected fish (A+; B+) at T1 (14 dpi) and T2 (21 dpi) timepoints, were stained with AB/PAS to visualize mucous cells. The number of stained cells in 10 consecutive interlamellar units (ILU) were counted across four randomly selected areas of each section in 6 fish per condition. Average mucous cell counts per 10 ILU are displayed above. A Student's *t*-test was performed to determine statistical significance $*p < 0.05$; $**p < 0.01$.

muscular degradation in inflammation, *via* an IGF-1 mediated protein catabolism pathway (Pooley et al., 2013). An increase in AGT production has been demonstrated in stressful conditions such as crowding in Atlantic salmon (Veiseth-Kent et al., 2010). Vasoconstriction and increased systemic arterial resistance have been reported as the pathogenic outcomes of AGD that cause cardiovascular problems and mortality in affected fish (Leef et al., 2007).

In our study, our data demonstrated a marked upregulation in *agt* expression in response to the novel feed formulation (Feed B) at T1. Given that the Feed B fish at this time point were significantly healthier than the Feed A fish, and by extension were experiencing less physiological stress, it is unlikely that this elevation of *agt* represents gill degradation, or stress. By T2 (GS2), expression was downregulated, to a level which resembles that observed for Feed A at T1. *P. perurans* causes an osmotic imbalance in fish resulting in elevated plasma osmolality and raised concentrations of plasma Cl^- and Na^+ (Hvas et al., 2017). In cases of raised plasma osmolality the RAAS pathway is inhibited (Armanini et al., 2018). The data obtained in this study support the concept that the delay in disease onset conferred by the novel feed formulation reduced or postponed the characteristic imbalance in osmolality traditionally associated with AGD, thus maintaining elevated *agt* expression at T1 in Feed B. Expression is then eventually reduced, at T2, which coincides with later stage disease and more pronounced disturbances in osmolality.

Lysozyme is a vital protease of innate immunity found in mucus, lymphoid tissue, kidney, serum and plasma of fish. Lysozyme is an indicator of infection and, its level of activity may change depending upon stress and various physical, environmental and biochemical factors (Saurabh and Sahoo, 2008). Lysozyme was reported to be elevated and to contribute to resistance to furunculosis in a soybean-based salmon feed study (Krogdahl and Roed, 2000). Carboxypeptidase N Subunit 1 (*cpn1*) inactivates the complement derived anaphylactic molecules C3a and C5a which can reduce inflammation, and Bradykinin which results in vasoconstriction (Bokisch and Müller-Eberhard, 1970; Mueller-Ortiz et al., 2009; Plummer and Hurwitz, 1978) and has been shown to reduce the biological activity of the chemokine stromal-derived factor-1α (SDF-1α), which is an essential regulator of lymphocyte homing and B-cell growth (Davis et al., 2005). *Masp1* activates the Lectin pathway of the complement system (Thiel et al., 2012). *Masp1* mediated complement activity has been studied in teleosts and was found to be downregulated upon parasitic infection in common carp (Gonzalez et al., 2007; Li et al., 2016).

In the current study, these three genes demonstrated identical expression patterns. At T1 no *lys-g, cpn1 or masp1* upregulation was observed for fish exhibiting GS2 (Feed A). However, a substantial increase in all three expressions was noted at T2 in the novel feed cohort, which coincided with GS2 in this group. The data suggest that the increase in *lys-g, cpn1* and *masp1* expression observed at 21 dpi may not therefore be linked to disease severity but may be associated with the novel feed impacting on the immune response and the reduction of inflammation.

Carboxylesterase 3 (*ces3*) belongs to a family of carboxylesterase enzymes which are associated with pro-inflammatory and leucocyte chemotaxis *via* 2-arachidonoylglycerol (2-AG) hydrolysis in humans (Kishimoto et al., 2006; Oka et al., 2004) and are indicators in environmental monitoring in aquatic species (Barron et al., 1999; Wheelock et al., 2008). The presence of esterases, along with many other innate immune molecules, in fish skin mucus offers a first line defense against pathogen invasion (Gomez et al., 2013). Previously our group reported higher esterase activity at GS1 and in freshwater treated fish in comparison to GS0 and GS2, in AGD-affected salmon (Marcos-López et al., 2017). However here the only substantial upregulation was noted at T2 in the B Feed cohort. This disparity may be explained as previously, lesioned gill was selected for analysis while in this current study, no preselection for lesions was carried out.

This study investigated whether the dietary supplements influenced the expression of previously investigated mucins (Marcos-López et al., 2018). Mucins are high molecular weight O-glycosylated glycoproteins that are the chief components of mucus and are classified structurally into two major categories; large secreted gel-forming mucins (SGFM) and membrane-bound mucins (MBM) and are vital for defense against pathogens (Moniaux et al., 2001). *N*-acetylglucosamine (GlcNAc), *N*-acetylgalactosamine (GalNAc), and sialic acids are bonded with O-glycans in mucins rendering their complex structure (Brockhausen and Schachter, 2008). The structural complexity diversifies with the binding of a range of saccharides that may change according to disease state, organ, and other factors within a species (Linden et al., 2008; Padra et al., 2014). Skin mucins in Atlantic salmon are post-translationally modified with O-glycosylated N-Acetylneuraminic acid (Neu5Ac), N-Glycolylneuraminic acid (Neu5Gc), single deaminoneuraminic acid (Kdn) and GalNAc molecules (Jin et al., 2015; Padra et al., 2014). The diversity of mucus constituents enriches the defense against the invasion of pathogens which can release enzymes such as proteases, glycosidases and sialidase to digest the mucosa (Corfield et al., 1992; Hoskins and

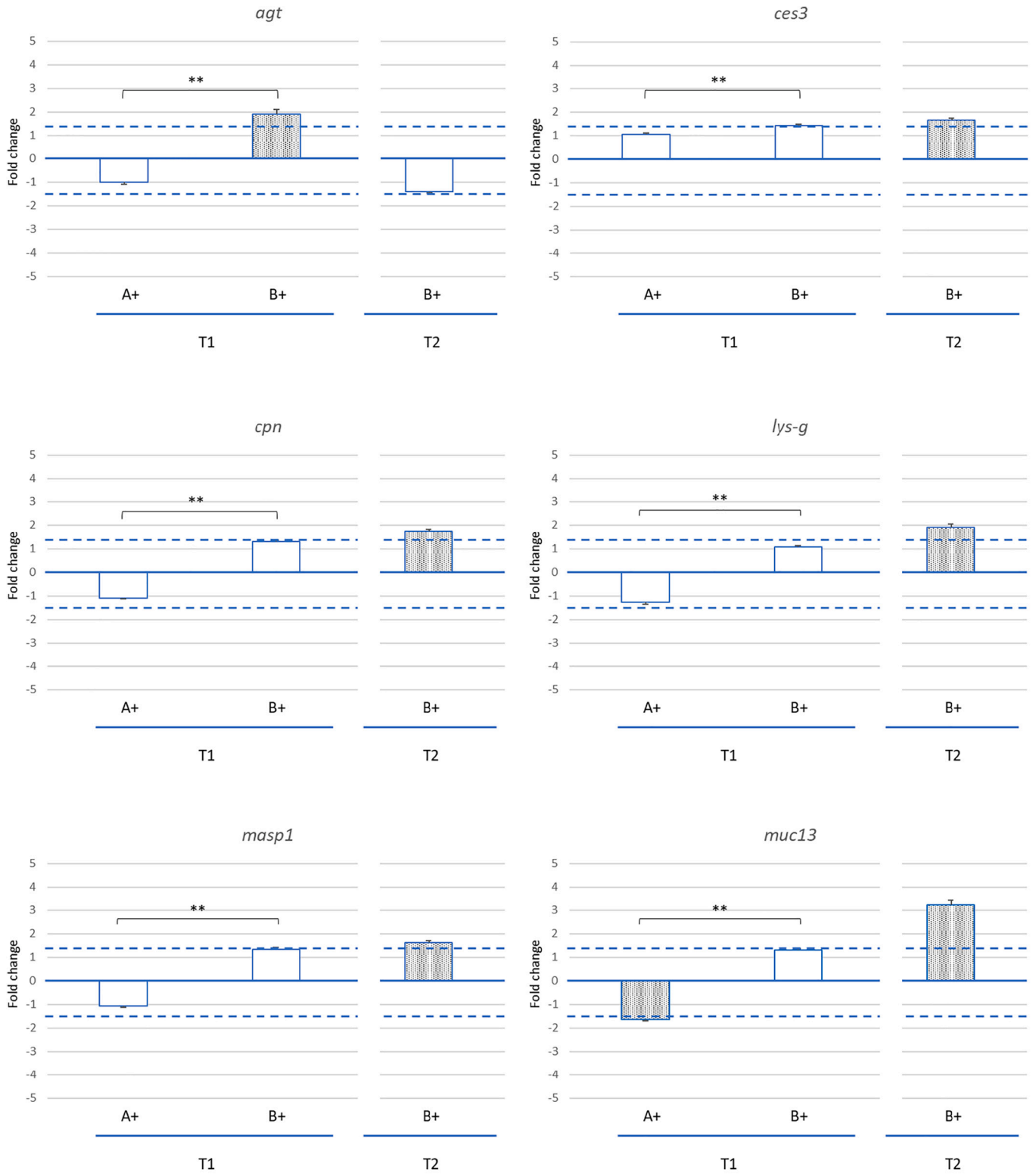

Fig. 7. Gene expression analysis of target genes: Using qPCR the expression of target genes was assessed in each AGD-affected feed group (A+ and B+) at both T1 (14 dpi) and T2 (21 dpi). Graphed values are fold change values in comparison to the negative control (A-) at each timepoint. Only those genes expressing fold changes of at least +/− 1.5 were considered to be biologically relevant (shaded bars in all graphs). Statistical analyses compared the effect of feed on gene expression *$p < 0.05$; **$p < 0.01$.

Boulding, 1981).

Mucin-13 is a membrane-bound mucin (MBM), which does not form oligomers, possesses three epidermal growth factor-like domains and is thought to play a role in cell signaling, potentially through ErbB2-related pathways. These pathways are involved in cellular proliferation, migration and apoptosis (Chauhan and Moore, 2006; Olayioye,

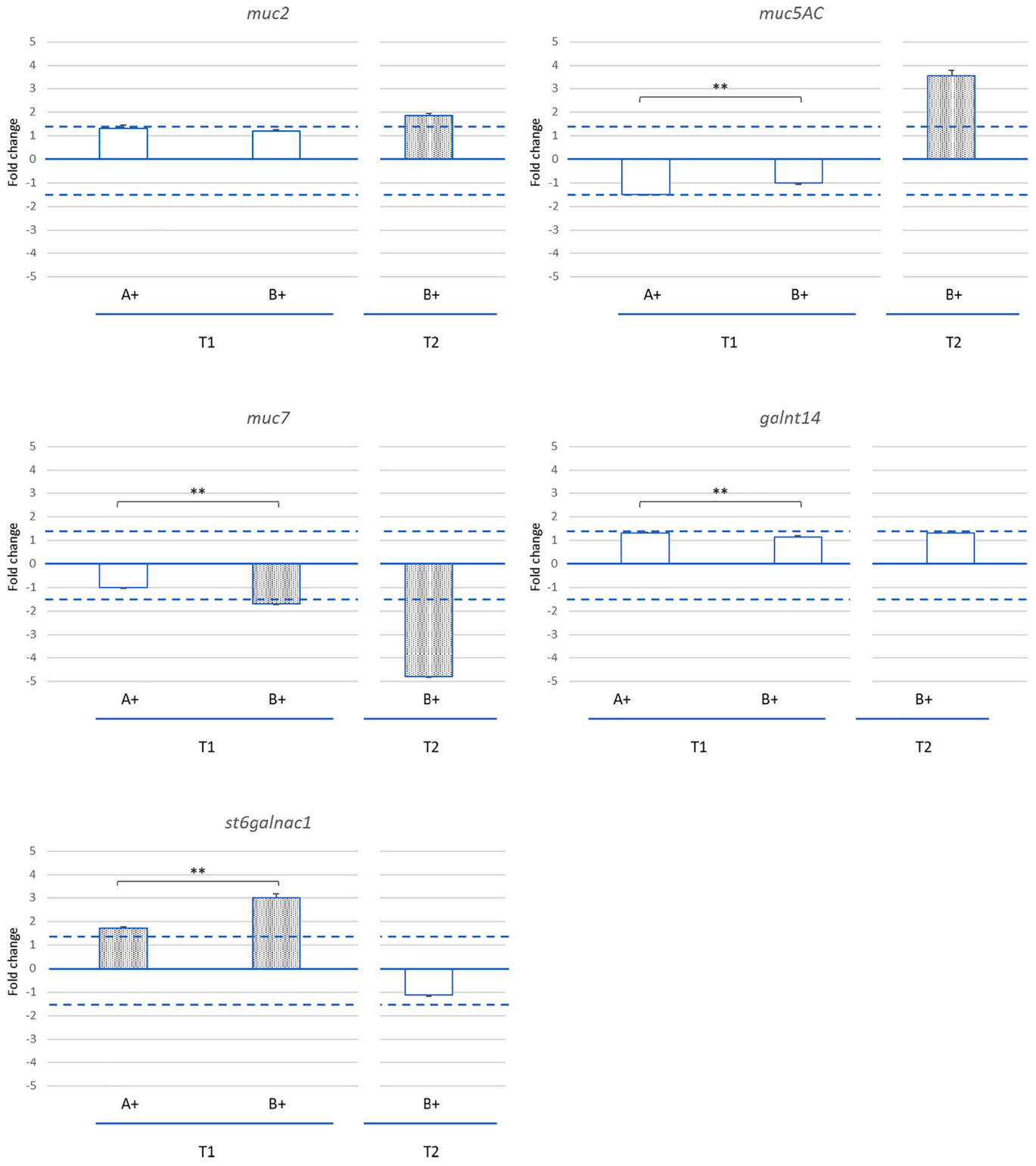

Fig. 7. (*continued*).

2001). Membrane-bound mucins contain the extracellular proteolytic cleavage site that is present in several mucins found in *Salmo trutta* (Malachowicz et al., 2017). In this study, the most significant changes in *muc13* expression, relative to the negative control, were in those fish which exhibited the most advanced disease (Feed A at T1 and Feed B at T2). However, the pattern of *muc13* expression in Feed A at T1 diverges from that obtained for Feed B at T2. Given that many of the fish in these conditions reached GS2, these data suggest that the observed differences in *muc13* expression are not attributable to differences in the health or disease severity in the fish but may instead be ascribed to the feeds used in each of the groups. *Muc13* expression is commonly associated to the gut in bream (Pérez-Sánchez et al., 2013), and in murine gut models *muc13* has been shown to protect against mechanically induced inflammation (Sheng et al., 2011). An elevation in IL-1b and TNF-alpha has been demonstrated at the gene level in AGD (Pennacchi et al., 2014). It therefore may be suggested that the elevation of *muc13* observed in

fish maintained on the novel feed formulation may be indicative of a diet stimulated protective response to inflammation.

Muc5ac like muc2 and muc7 is a secreted gel-forming mucin (Voynow and Fischer, 2006), which is reported to provide a 3-D scaffold for host secreted immuno-protective enzymes including lysozymes, proteases and antiproteases (Ganesan et al., 2013). *Muc5* was previously reported to increase with AGD progression in salmon naturally challenged in sea cages (Marcos-López et al., 2018). In this study, an increase in *muc5ac* in the Feed A cohort at T1, was not observed. However, an increase in *muc5ac* expression, coinciding with GS2, was evident at T2 in the B group. Given that Feed A at T1 and Feed B at T2 are the cohorts which had the most advanced disease stages in this study, the inconsistencies in *muc5ac* expression are unlikely to be attributable to differences in disease state, but are more likely to be ascribed to the feeds. The data suggest that Feed B enhanced *muc5ac* expression as a protective response to disease progression. In two recent studies, multiple muc-2 like isoforms were found to be upregulated in AGD (Talbot et al., 2021; Botwright et al., 2021) Here, as with *muc5ac*, the most significant expression of muc2 was detected at the latest timepoint. However the overall lower expression levels of *muc2* may be due to the fact that expression of this gene in salmon has been chiefly confined to the intestine (Sveen et al., 2017).

The expression of muc7 is reported to have direct candidacidal activity (Linden et al., 2008). A recent study investigating transcriptomic response in early stage AGD demonstrated a consistent down regulation of muc7-like gene expression across various timepoints (Talbot et al., 2021). In this study while the two cohorts which experienced the most advanced stages of disease (Feed A at T1 and Feed B at T2) demonstrated minor and significant muc7 downregulation respectively, the relatively healthy Feed B group at T1 showed moderately reduced muc7 expression. It is therefore difficult to elucidate a relationship between feed, disease stage and muc7 expression.

It is interesting to note that the mucous cell counts were consistently and significantly lower in fish maintained on the experimental diet, when compared to all cohorts of diet A, regardless of infection status. Given that mucus secretion has been linked to host response in AGD (Benktander et al., 2020), this finding may seem to deviate from expectations. However, the AB/PAS stain does not distinguish between mucin subtypes, and therefore this analysis does not allow specific identification of the mucins in each case. The reduced mucous cell count combined with the delayed disease onset in the experimental group in this case indicates that these findings warrant further investigation into the nature of mucin secretion in AGD fish and those maintained on supplemented feeds.

Sialylation in skin mucus has been shown to have a protective role against *A. salmonicida* infection in Atlantic salmon, by protecting internal GlcNAc residues on mucosa from consumption by pathogens (Padra et al., 2017). In the current study, at T1 both feed cohorts exhibited elevated expressions of *st6galnac1*. This gene codes for the enzyme Alpha-*N*-acetylgalactosaminide alpha-2,6-sialyltransferase 1, which is involved in the biosynthesis of the carbohydrate moiety of mucin-type O-linked glycan chains by transfer of the sialic acid acetylneuraminic acid (Neu5Ac), in an alpha-2,6 linkage to O-linked GalNAc residues on substrate molecules (Delannoy et al., 2009). Given that the production of sialic acid has been used as a parameter to gauge mucus production in fish for many years (Arillo et al., 1979; Eddy and Fraser, 1982), this increased expression may be perceived as an indicator of mucin production in response to pathogen exposure. However, it is interesting to note that, although at T1 the fish maintained on Feed A are in the most advanced stage of disease, expression of *st6galnac1* in Feed B at this same time point is significantly higher. As the protective role of *st6galnac1* has been previously established, the elevated expression with Feed B may demonstrate an immuno-protective effect of this feed, which may in part be responsible for the delay in disease onset at this time point. However, at T2 the reduced expression levels for this feed group, suggests that this perceived immuno-protective effect does not persist during more advanced disease stages.

The results of this study support the potential use of dietary based immunomodulatory agents as practical and effective preventative interventions in the mitigation of AGD. The effect of the novel feed formulation on the rate of AGD progression, as assessed by gill score, was significant, with Feed B slowing disease progression and reducing the number of positive fish when compared to standard feed. The impact of these feeds on the expression of *muc13, muc5ac, lys-g* and *st6galnac*, all of which have roles in the maintenance of the mucosal layer and mucosal defense, is particularly interesting in the context of AGD. The data obtained in this preliminary study are representative of AGD in a controlled setting. More extensive studies in an industry setting are required to validate the findings of this study and to enhance our understanding of the mechanisms underlying the protective action observed in this study.

CRediT authorship contribution statement

Anita Talbot: Methodology, Formal analysis, Investigation, Writing – review & editing. **Michelle McCormack:** Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Ankit Dwivedi:** Methodology, Investigation, Writing – review & editing. **Ian O'Connor:** Conceptualization, Methodology, Investigation, Writing – review & editing, Supervision. **Victoria Valdenegro:** Methodology, Investigation, Writing – review & editing. **Eugene MacCarthy:** Conceptualization, Methodology, Investigation, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

Dr. Victoria Valdenegro currently holds a post at BioMar AS. The base and supplemented feeds used in this study were supplied by BioMar.

Acknowledgement

This material is based upon research supported by the Department of Agriculture, Food and the Marine under the Grant award No. 15 S 745.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2022.737983.

References

Adams, M.B., Nowak, B.F., 2003. Amoebic gill disease: sequential pathology in cultured Atlantic salmon, *Salmo salar* L. J. Fish Dis. 26, 601–614. https://doi.org/10.1046/j.1365-2761.2003.00496.x.

Arillo, A., Margiocco, C., Melodia, F., 1979. The gill sialic acid content as an index of environmental stress in rainbow trout, Salmo gairdneri, Richardson. J. Fish Biol. 15, 405–410. https://doi.org/10.1111/j.1095-8649.1979.tb03623.x.

Armanini, D., Bordin, L., Dona, G., Andrisani, A., Ambrosini, G., Sabbadin, C., 2018. Relationship between water and salt intake, osmolality, vasopressin, and aldosterone in the regulation of blood pressure. J. Clin. Hypertens. 20, 1455–1457. https://doi.org/10.1111/jch.13379.

Barron, M.G., Charron, K.A., Stott, W.T., Duvall, S.E., 1999. Tissue carboxylesterase activity of rainbow trout. Environ. Toxicol. Chem. 18, 2506–2511. https://doi.org/10.1002/etc.5620181117.

Benktander, J., Padra, J.T., Maynard, B., Birchenough, G., Botwright, N.A., McCulloch, R., Lindén, S.K., 2020. Gill mucus and gill Mucin O-glycosylation in healthy and amebic gill disease-affected Atlantic Salmon. Microorg. https://doi.org/10.3390/microorganisms8121871.

Boison, S.A., Gjerde, B., Hillestad, B., Makvandi-Nejad, S., Moghadam, H.K., 2019. Genomic and Transcriptomic analysis of amoebic gill disease resistance in Atlantic Salmon (Salmo salar L.). Front. Genet. 10, 68. https://doi.org/10.3389/fgene.2019.00068.

Bokisch, V.A., Müller-Eberhard, H.J., 1970. Anaphylatoxin inactivator of human plasma: its isolation and characterization as a carboxypeptidase. J. Clin. Invest. 49, 2427–2436. https://doi.org/10.1172/JCI106462.

Botwright, N.A., Mohamed, A.R., Slinger, J., Lima, P.C., Wynne, J.W., 2021. Host-parasite interaction of Atlantic salmon (Salmo salar) and the Ectoparasite

Neoparamoeba perurans in amoebic gill disease. Front. Immunol. 12, 672700 https://doi.org/10.3389/fimmu.2021.672700.
Bridle, A.R., Carter, C.G., Morrison, R.N., Nowak, B.F., 2005. The effect of β-glucan administration on macrophage respiratory burst activity and Atlantic salmon, Salmo salar L., challenged with amoebic gill disease – evidence of inherent resistance. J. Fish Dis. 28, 347–356. https://doi.org/10.1111/j.1365-2761.2005.00636.x.
Brockhausen, I., Schachter, H., 2008. Glycosyltransferases Involved inN–andO–Glycan Biosynthesis. In: Glycosciences. Wiley-VCH Verlag GmbH, pp. 79–113. https://doi.org/10.1002/9783527614738.ch5.
Chauhan, A.K., Moore, T.L., 2006. Presence of plasma complement regulatory proteins clusterin (Apo J) and vitronectin (S40) on circulating immune complexes (CIC). Clin. Exp. Immunol. 145, 398–406. https://doi.org/10.1111/j.1365-2249.2006.03135.x.
Cook, M., Maynard, B., Crosbie, P., Prideaux, C., Elliott, N., 2012. AGD Vaccine Phase III: Sea-based Trials, Vaccine Refinement and Commercialisation.
Corfield, A.P., Wagner, S.A., Clamp, J.R., Kriaris, M.S., Hoskins, L.C., 1992. Mucin degradation in the human colon: production of sialidase, sialate O- acetylesterase, N-acetylneuraminate lyase, arylesterase, and glycosulfatase activities by strains of fecal bacteria. Infect. Immun. 60, 3971–3978. https://doi.org/10.1128/iai.60.10.3971-3978.1992.
Crosbie, P.B.B., Bridle, A.R., Cadoret, K., Nowak, B.F., 2012. In vitro cultured Neoparamoeba perurans causes amoebic gill disease in Atlantic salmon and fulfils Koch's postulates. Int. J. Parasitol. 42, 511–515. https://doi.org/10.1016/j.ijpara.2012.04.002.
Davis, D.A., Singer, K.E., De La Luz Sierra, M., Narazaki, M., Yang, F., Fales, H.M., Tosato, G., 2005. Identification of carboxypeptidase N as an enzyme responsible for C-terminal cleavage of stromal cell-derived factor-1α in the circulation. Blood 105, 4561–4568. https://doi.org/10.1182/blood-2004-12-4618.
Delannoy, P., Harduin-Lepers, A., Krzewinski-Recchi, M.-A., 2009. ST6GALNAC1 (ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 1). Atlas Genet. Cytogenet. Oncol. Haematol. 13, 70–74. https://doi.org/10.4267/2042/44389.
Downes, J.K., Henshilwood, K., Collins, E.M., Ryan, A., O'Connor, I., Rodger, H.D., Ruane, N.M., 2015. A Longitudinal Study of Amoebic Gill Disease on a Marine Atlantic Salmon Farm Utilising a Real-Time PCR Assay for the Detection of Neoparamoeba Perurans.
Downes, J.K., Rigby, M.L., Taylor, R.S., Maynard, B.T., MacCarthy, E., O'Connor, I., Cook, M.T., 2017. Evaluation of non-destructive molecular diagnostics for the detection of *Neoparamoeba perurans*. Front. Mar. Sci. https://doi.org/10.3389/fmars.2017.00061.
Eddy, F.B., Fraser, J.E., 1982. Sialic acid and mucus production in rainbow trout (Salmo gairdneri Richardson) in response to zinc and seawater. Comp. Biochem. Physiol. Part C, Comp. 73, 357–359. https://doi.org/10.1016/0306-4492(82)90135-6.
Fahy, J.V., Dickey, B.F., 2010. Medical progress: Airway mucus function and dysfunction. N. Engl. J. Med. 363, 2233–2247. https://doi.org/10.1056/NEJMra0910061.
FAO, 2020. The State of World Fisheries and Aquaculture (SOFIA) [WWW Document]. URL. http://www.fao.org/documents/card/en/c/ca9229en (accessed 11.18.20).
Ganesan, S., Comstock, A.T., Sajjan, U.S., 2013. Barrier function of airway tract epithelium. Tissue Barriers 1, e24997. https://doi.org/10.4161/tisb.24997.
Gomez, D., Sunyer, J.O., Salinas, I., 2013. The mucosal immune system of fish: the evolution of tolerating commensals while fighting pathogens. Fish Shellfish Immunol. 35, 1729–1739. https://doi.org/10.1016/j.fsi.2013.09.032.
Gonzalez, S.F., Buchmann, K., Nielsen, M.E., 2007. Complement expression in common carp (Cyprinus carpio L.) during infection with Ichthyophthirius multifiliis. Dev. Comp. Immunol. 31, 576–586. https://doi.org/10.1016/j.dci.2006.08.010.
Hoskins, L.C., Boulding, E.T., 1981. Mucin degradation in human colon ecosystems. Evidence for the existence and role of bacterial subpopulations producing glycosidases as extracellular enzymes. J. Clin. Invest. 67, 163–172. https://doi.org/10.1172/JCI110009.
Hvas, M., Karlsbakk, E., Maehle, S., Wright, D.W., Oppedal, F., Cooke, S., 2017. The Gill Parasite Paramoeba Perurans Compromises Aerobic Scope, Swimming Capacity and Ion Balance in Atlantic Salmon. https://doi.org/10.1093/conphys/cox066.
Jin, C., Padra, J.T., Sundell, K., Sundh, H., Karlsson, N.G., Lindén, S.K., 2015. Atlantic Salmon carries a range of novel O-glycan structures differentially localized on skin and intestinal Mucins. J. Proteome Res. 14, 3239–3251. https://doi.org/10.1021/acs.jproteome.5b00232.
Jodaa Holm, H., Wadsworth, S., Bjelland, A.K., Krasnov, A., Evensen, Ø., Skugor, S., 2016. Dietary phytochemicals modulate skin gene expression profiles and result in reduced lice counts after experimental infection in Atlantic salmon. Parasites and Vectors 9. https://doi.org/10.1186/s13071-016-1537-y.
Kilkenny, C., Browne, W., Cuthill, I.C., Emerson, M., Altman, D.G., 2010. Animal research: reporting in vivo experiments: the ARRIVE guidelines. Br. J. Pharmacol. 160, 1577–1579. https://doi.org/10.1111/j.1476-5381.2010.00872.x.
Kishimoto, S., Oka, S., Gokoh, M., Sugiura, T., 2006. Chemotaxis of human peripheral blood eosinophils to 2-arachidonoylglycerol: comparison with other eosinophil chemoattractants, in: international archives of allergy and immunology. Int Arch Allergy Immunol 3–7. https://doi.org/10.1159/000092704.
Krogdahl, Bakke-Mckellep, Roed, Baeverfjord, 2000. Feeding Atlantic salmon Salmo salar L. soybean products: effects on disease resistance (furunculosis), and lysozyme and IgM levels in the intestinal mucosa. Aquac. Nutr. 6, 77–84. https://doi.org/10.1046/j.1365-2095.2000.00129.x.
Leef, M.J., Hill, J.V., Harris, J.O., Powell, M.D., 2007. Increased systemic vascular resistance in Atlantic salmon, Salmo salar L., affected with amoebic gill disease. J. Fish Dis. 30, 601–613. https://doi.org/10.1111/j.1365-2761.2007.00840.x.
Li, M.F., Li, J., Sun, L., 2016. CsMAP34, a teleost MAP with dual role: a promoter of MASP-assisted complement activation and a regulator of immune cell activity. Sci. Rep. 6, 1–14. https://doi.org/10.1038/srep39287.
Linden, S.K., Sutton, P., Karlsson, N.G., Korolik, V., McGuckin, M.A., 2008. Mucins in the mucosal barrier to infection. Mucosal Immunol. https://doi.org/10.1038/mi.2008.5.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25, 402–408. https://doi.org/10.1006/meth.2001.1262.
Ma, J., Bruce, T.J., Jones, E.M., Cain, K.D., 2019. A review of fish vaccine development strategies: conventional methods and modern biotechnological approaches. Microorganisms 7, 569. https://doi.org/10.3390/microorganisms7110569.
Maher, D.M., Gupta, B.K., Nagata, S., Jaggi, M., Chauhan, S.C., 2011. Mucin 13: structure, function, and potential roles in cancer pathogenesis. Mol. Cancer Res. 9, 531–537. https://doi.org/10.1158/1541-7786.MCR-10-0443.
Malachowicz, M., Wenne, R., Burzynski, A., 2017. De novo assembly of the sea trout (Salmo trutta m. trutta) skin transcriptome to identify putative genes involved in the immune response and epidermal mucus secretion. PLoS One 12, e0172282.
Marcos-López, M., Espinosa Ruiz, C., Rodger, H.D., O'Connor, I., MacCarthy, E., Esteban, M.Á., 2017. Local and systemic humoral immune response in farmed Atlantic salmon (Salmo salar L.) under a natural amoebic gill disease outbreak. Fish Shellfish Immunol. 66, 207–216. https://doi.org/10.1016/j.fsi.2017.05.029.
Marcos-López, M., Calduch-Giner, J.A., Mirimin, L., MacCarthy, E., Rodger, H.D., O'Connor, I., Piazzon, M.C., 2018. Gene expression analysis of Atlantic salmon gills reveals mucin 5 and interleukin 4/13 as key molecules during amoebic gill disease. Sci. Rep. 8, 13689. https://doi.org/10.1038/s41598-018-32019-8.
Martinez-Rubio, L., Morais, S., Evensen, Ø., Wadsworth, S., Ruohonen, K., Vecino, J.L.G., Tocher, D.R., 2012. Functional feeds reduce heart inflammation and pathology in Atlantic Salmon (Salmo salar L.) following experimental challenge with Atlantic salmon reovirus (ASRV). PLoS One 7, e40266. https://doi.org/10.1371/journal.pone.0040266.
Martinez-Rubio, L., Evensen, Ø., Krasnov, A., Jørgensen, S., Wadsworth, S., Ruohonen, K., Tocher, D.R., 2014. Effects of functional feeds on the lipid composition, transcriptomic responses and pathology in heart of Atlantic salmon (Salmo salar L.) before and after experimental challenge with piscine myocarditis virus (PMCV). BMC Genomics 15, 462. https://doi.org/10.1186/1471-2164-15-462.
Martinsen, K.H., Thorisdottir, A., Lillehammer, M., 2018. Effect of hydrogen peroxide as treatment for amoebic gill disease in Atlantic salmon (Salmo salar L.) in different temperatures. Aquac. Res. 49, 1733–1739. https://doi.org/10.1111/are.13627.
Minich, J.J., Poore, G.D., Jantawongsri, K., Johnston, C., Bowie, K., Bowman, J., Allen, E. E., 2020. Microbial ecology of Atlantic Salmon (Salmo salar) hatcheries: impacts of the built environment on fish mucosal microbiota. Appl. Environ. Microbiol. 86 https://doi.org/10.1128/AEM.00411-20.
Moniaux, N., Escande, F., Porchet, N., Aubert, J.P., Batra, S.K., 2001. Structural organization and classification of the human mucin genes. Front. Biosci. https://doi.org/10.2741/moniaux.
Mueller-Ortiz, S.L., Wang, D., Morales, J.E., Li, L., Chang, J.-Y., Wetsel, R.A., 2009. Targeted disruption of the gene encoding the murine small subunit of carboxypeptidase N (CPN1) causes susceptibility to C5a anaphylatoxin-mediated shock. J. Immunol. 182, 6533–6539. https://doi.org/10.4049/jimmunol.0804207.
Mullins, J., Nowak, B., Leef, M., Røn, Ø., Eriksen, T.B., McGurk, C., 2020. Functional diets improve survival and physiological response of Atlantic salmon (Salmo salar) to amoebic gill disease. J. World Aquac. Soc. 51, 634–648. https://doi.org/10.1111/jwas.12692.
Munday, B.L., 1986. Diseases of salmonids. In: Proceedings of the Workshop on Diseases of Australian Fish and Shellfish, pp. 127–141.
Munday, B.L., Zilberg, D., Findlay, V., 2001. Gill disease of marine fish caused by infection with Neoparamoeba pemaquidensis. J. Fish Dis. 24, 497–507. https://doi.org/10.1046/j.1365-2761.2001.00329.x.
Navar, L.G., Prieto, M.C., Satou, R., Kobori, H., 2011. Intrarenal angiotensin II and its contribution to the genesis of chronic hypertension. Curr. Opin. Pharmacol. https://doi.org/10.1016/j.coph.2011.01.009.
Núñez-Acuña, G., Gonçalves, A.T., Valenzuela-Muñoz, V., Pino-Marambio, J., Wadsworth, S., Gallardo-Escárate, C., 2015. Transcriptome immunomodulation of in-feed additives in Atlantic salmon Salmo salar infested with sea lice Caligus rogercresseyi. Fish Shellfish Immunol. 47, 450–460. https://doi.org/10.1016/j.fsi.2015.09.009.
Oka, S., Ikeda, S., Kishimoto, S., Gokoh, M., Yanagimoto, S., Waku, K., Sugiura, T., 2004. 2-Arachidonoylglycerol, an endogenous cannabinoid receptor ligand, induces the migration of EoL-1 human eosinophilic leukemia cells and human peripheral blood eosinophils. J. Leukoc. Biol. 76, 1002–1009. https://doi.org/10.1189/jlb.0404252.
Olayioye, M.A., 2001. Intracellular signaling pathways of ErbB2/HER-2 and family members. Breast Cancer Res. 3, 385. https://doi.org/10.1186/bcr327.
Oldham, T., Rodger, H., Nowak, B.F., 2016. Incidence and distribution of amoebic gill disease (AGD) — an epidemiological review. Aquaculture 457, 35–42. https://doi.org/10.1016/j.aquaculture.2016.02.013.
Olson, K., Chavez, A., Conklin, D., Cousins, K., Farrell, A., Ferlic, R., Veldman, T., 1994. Localization of angiotensin ii responses in the trout cardiovascular system. J. Exp. Biol. 194.
Padra, J.T., Sundh, H., Jin, C., Karlsson, N.G., Sundell, K., Lindén, S.K., 2014. Aeromonas salmonicida binds differentially to mucins isolated from skin and intestinal regions of Atlantic salmon in an N-acetylneuraminic acid-dependent manner. Infect. Immun. 82, 5235–5245. https://doi.org/10.1128/IAI.01931-14.
Padra, J.T., Sundh, H., Sundell, K., Venkatakrishnan, V., Jin, C., Samuelsson, T., Lindén, S.K., 2017. *Aeromonas salmonicida* Growth in Response to Atlantic Salmon Mucins Differs between Epithelial Sites, Is Governed by Sialylated and N-

Acetylhexosamine-Containing O-Glycans, and Is Affected by Ca 2. https://doi.org/10.1128/IAI.00189-17.
Parsons, H., Nowak, B., Fisk, D., Powell, M., 2001. Effectiveness of commercial freshwater bathing as a treatment against amoebic gill disease in Atlantic salmon. Aquaculture 195, 205–210. https://doi.org/10.1016/S0044-8486(00)00567-6.
Pennacchi, Y., Leef, M.J., Crosbie, P.B.B., Nowak, B.F., Bridle, A.R., 2014. Evidence of immune and inflammatory processes in the gills of AGD-affected Atlantic salmon, *Salmo salar* L. Fish Shellfish Immunol. 36, 563–570. https://doi.org/10.1016/j.fsi.2013.12.013.
Pérez-Sánchez, J., Estensoro, I., Redondo, M.J., Calduch-Giner, J.A., Kaushik, S., Sitjà-Bobadilla, A., 2013. Mucins as diagnostic and prognostic biomarkers in a fish-parasite model: transcriptional and functional analysis. PLoS One 8, e65457.
Plummer, T.H., Hurwitz, M.Y., 1978. Human plasma carboxypeptidase N. Isolation and characterization. J. Biol. Chem. 253, 3907–3912.
Pooley, N.J., Tacchi, L., Secombes, C.J., Martin, S.A.M., 2013. Inflammatory responses in primary muscle cell cultures in Atlantic salmon (Salmo salar). BMC Genomics 14, 1–20. https://doi.org/10.1186/1471-2164-14-747.
Roberts, S.D., Powell, M.D., 2008. Freshwater bathing alters the mucous layer of marine Atlantic salmon Salmo salar L. J. Fish Biol. 72, 1864–1870. https://doi.org/10.1111/j.1095-8649.2008.01853.x.
Robledo, D., Hamilton, A., Gutiérrez, A.P., Bron, J.E., Houston, R.D., 2020. Characterising the mechanisms underlying genetic resistance to amoebic gill disease in Atlantic salmon using RNA sequencing. BMC Genomics 21, 271. https://doi.org/10.1186/s12864-020-6694-x.
Rodger, H.D., 2014. Amoebic gill disease (AGD) in farmed salmon (Salmo salar) in Europe. Fish Vet. J. 16–27.
Rodriguez-Iturbe, B., Pons, H., Johnson, R.J., 2017. Role of the immune system in hypertension. Physiol. Rev. 97, 1127–1164. https://doi.org/10.1152/physrev.00031.2016.
Rudemiller, N.P., Crowley, S.D., 2016. Interactions between the immune and the renin-angiotensin Systems in Hypertension. Hypertension. https://doi.org/10.1161/HYPERTENSIONAHA.116.06591.
Saurabh, S., Sahoo, P.K., 2008. Lysozyme: an important defence molecule of fish innate immune system. Aquac. Res. 39, 223–239. https://doi.org/10.1111/j.1365-2109.2007.01883.x.
Sheng, Y.H., Lourie, R., Lindén, S.K., Jeffery, P.L., Roche, D., Tran, T.V., McGuckin, M.A., 2011. The MUC13 cell-surface mucin protects against intestinal inflammation by inhibiting epithelial cell apoptosis. Gut 60, 1661–1670. https://doi.org/10.1136/gut.2011.239194.
Skugor, S., Jodaa Holm, H., Bjelland, A.K., Pino, J., Evensen, Ø., Krasnov, A., Wadsworth, S., 2016. Nutrigenomic effects of glucosinolates on liver, muscle and distal kidney in parasite-free and salmon louse infected Atlantic salmon. Parasites Vectors 9, 1–17. https://doi.org/10.1186/s13071-016-1921-7.
Sveen, L.R., Grammes, F.T., Ytteborg, E., Takle, H., Jørgensen, S.M., 2017. Genome-wide analysis of Atlantic salmon (Salmo salar) mucin genes and their role as biomarkers. PLoS One 12. https://doi.org/10.1371/journal.pone.0189103.
Talbot, A., Gargan, L., Moran, G., Prudent, L., O'Connor, I., Mirimin, L., MacCarthy, E., 2021. Investigation of the transcriptomic response in Atlantic salmon (Salmo salar) gill exposed to Paramoeba perurans during early onset of disease. Sci. Rep. 11, 20682. https://doi.org/10.1038/s41598-021-99996-1.
Taylor, R., Muller, W., Cook, M., Kube, P., Elliott, N., 2009. Gill observations in Atlantic salmon (*Salmo salar*, L.) during repeated amoebic gill disease (AGD) field exposure and survival challenge. Aquaculture 290, 1–8. https://doi.org/10.1016/j.aquaculture.2009.01.030.
Télessy, I.G., 2019. In: Singh, R.B., Watson, R.R., Takahashi, T.B.T. (Eds.), Chapter 24 - Nutraceuticals. Academic Press, pp. 409–421. https://doi.org/10.1016/B978-0-12-813148-0.00024-4.
Thiel, S., Jensen, L., Degn, S.E., Nielsen, H.J., Gál, P., Dobó, J., Jensenius, J.C., 2012. Mannan-binding lectin (MBL)-associated serine protease-1 (MASP-1), a serine protease associated with humoral pattern-recognition molecules: normal and acute-phase levels in serum and stoichiometry of lectin pathway components. Clin. Exp. Immunol. 169, 38–48. https://doi.org/10.1111/j.1365-2249.2012.04584.x.
Valdenegro-Vega, V.A., Crosbie, P.B.B., Cook, M.T., Vincent, B.N., Nowak, B.F., 2014. Administration of recombinant attachment protein (r22C03) of Neoparamoeba perurans induces humoral immune response against the parasite in Atlantic salmon (Salmo salar). Fish Shellfish Immunol. 38, 294–302. https://doi.org/10.1016/j.fsi.2014.03.034.
Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3 https://doi.org/10.1186/gb-2002-3-7-research0034 research0034.1.
Veiseth-Kent, E., Grove, H., Færgestad, E.M., Fjæra, S.O., 2010. Changes in muscle and blood plasma proteomes of Atlantic salmon (Salmo salar) induced by crowding. Aquaculture 309, 272–279. https://doi.org/10.1016/j.aquaculture.2010.09.028.
Villavedra, M., To, J., Lemke, S., Birch, D., Crosbie, P., Adams, M., Wallach, M., 2010. Characterisation of an immunodominant, high molecular weight glycoprotein on the surface of infectious Neoparamoeba spp., causative agent of amoebic gill disease (AGD) in Atlantic salmon. Fish Shellfish Immunol. 29, 946–955. https://doi.org/10.1016/j.fsi.2010.07.036.
Voynow, J.A., Fischer, B.M., 2006. In: Laurent, G.J., Shapiro, S.D.B.T. (Eds.), MUCINS. Academic Press, Oxford, pp. 56–62. https://doi.org/10.1016/B0-12-370879-6/00248–9.
Wang, Y., Ragg, H., 2011. An unexpected link between angiotensinogen and thrombin. FEBS Lett. 585, 2395–2399. https://doi.org/10.1016/j.febslet.2011.06.021.
Wheelock, C.E., Phillips, B.M., Anderson, B.S., Miller, J.L., Miller, M.J., Hammock, B.D., 2008. Applications of carboxylesterase activity in environmental monitoring and toxicity identification evaluations (TIEs). Rev. Environ. Contam. Toxicol. https://doi.org/10.1007/978-0-387-77030-7_5.
Zhao, B., Erwin, A., Xue, B., 2018. How many differentially expressed genes: a perspective from the comparison of genotypic and phenotypic distances. Genomics 110, 67–73. https://doi.org/10.1016/j.ygeno.2017.08.007.
Zilberg, D., Findlay, V., Girling, P., Munday, B., 2000. Effects of treatment with Levamisole and Glucans on mortality rates in Atlantic Salmon (Salmo salar L.) suffering from amoebic gill disease. Bull. Eur. Assoc. Fish Pathol. 20.