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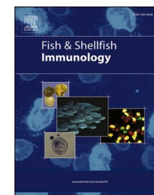

Full length article

# *In-vivo* analysis of Protec™ and β-glucan supplementation on innate immune performance and intestinal health of rainbow trout

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ABSTRACT

Functional diets are often given to fish during key stages to improve health through the interaction of the feed components with the host intestine. The additional factors added in these diets are known to modulate the immune response and as such may also offer protection against pathogenic challenges. The present study was undertaken to evaluate whether β-glucan supplementation for 6 weeks can alter the magnitude of immune response to immunological challenges and subsequently offer an improved innate immune response to bacterial challenge in rainbow trout. Two experimental diets were used to study these effects: a basic commercial diet supplemented with β-glucan and a commercially available functional diet (Protec™) that has β-glucan as a functional component in addition to other components were compared to a basic commercial control diet. No significant differences were observed in biometric data. Histological analysis revealed a significantly greater number of goblet cells in the fish fed Protec™ and β-glucan diets compared to those fed a control diet. Cell marker gene expression of distal intestine leucocytes indicated higher expression of T- and B-cells marker genes to both the β-glucan containing diets in comparison to control. The Protec™ diet demonstrated modulation of innate immune markers after 6 weeks of feeding with key antimicrobial genes (*SAA, HAMP, IL-1β* and *TNFα*) showing significant increases compared to the other diets. After stimulation with both PAMPs and an immune challenge with *A. salmonicida* fish fed the β-glucan diet and the Protec™ exhibited modulation of the innate immune response. An immune challenge with *A. salmonicida* was carried out to identify if dietary composition led to differences in the innate immune response of rainbow trout. Modulation of the magnitude of response in some immune genes (*SAA, IL-1β* and *HAMP*) was observed in both the distal intestine and head kidney in the Protec™ and β-glucan fed fish compared to those fed the control diet.

## 1. Introduction

Functional feeds are now used as part of health management and aquaculture in response to increased intensification of rearing, for prevention of disease outbreaks and improving general health in farmed fish. These feeds aim to not only to sustain normal physiological function but also to improve responses to pathogenic threats which subsequently aids/improves survival. These functional feed ingredients can be split into several main groups: prebiotics, probiotics, immunostimulants and micronutrients. Prebiotics are often added to support the growth of a favourable microbial communities in the intestine by acting as a nutrient source for bacteria, these nutrients often being indigestible by the animal itself and are commonly oligosaccharides made up of repeating monomers of sugars (Mannose, Galactose, Fructose) with a variety of different lengths and structures. The molecules can also act directly upon the immune cells present in the gut with effects on macrophage activation, lysozyme activity, respiratory burst and leucocyte activity [1]. The addition of these oligosaccharides can alter gene expression in the intestinal tissue with indication there is enhancement of the innate immune response [2].

β-glucans are commonly used prebiotics, they can have a variety of different structures that can induce different immune responses [3,4], they are comprised of a D-glucose backbone with side chains connected by β-glycosidic bonds. These naturally occurring compounds are found

*Abbreviations:* VHSV, Viral haemorrhagic septicaemia virus; PAMP, pathogen associated molecular pattern; GALT, Gut associated lymphoid tissue; rIL-1β, recombinant Interleukin-1β.

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in the cell wall of yeast, algae, and many other species of plants and fungi. The innate immune system acts on a broad range of molecules and is thought to bind to β-glucans through C-type lectins [4], these are receptors that bind carbohydrate molecules and trigger immune pathways through the C-type lectin signalling pathways which induces both innate and adaptive immune responses [5]. C-type lectin 4 has been previously characterised in rainbow trout (*Oncorhynchus mykiss*), where the gene is induced following infection with the bacterial pathogen *Yersinia ruckeri* [6]. Upregulation of the C-type lectin pathway can lead to amplification of a panel of genes known to be drivers of inflammation including *COX-2, IL-1β, TNFα, IL-6,* and IFNγ as demonstrated in a number of studies [3,4,7–10]. The increase in expression of these key proinflammatory genes suggests β-glucans can prime the innate immune system and hence protect the fish against disease, leading to higher survival against pathogens as previously demonstrated for viral haemorrhagic septicaemia virus (VHSV) [8], *Aeromonas salmonicida* [11,12] and *A. hydrophilia* challenges [7].

Immunostimulants are of great research interest in health management for both production animals and human health, and as a result has led to the discovery of many different immunostimulants; polysaccharides, vitamins, hormones, and bacterial components (PAMPs) [13]. Protec™ diet is a commercial aquafeed (Skretting) which contains several functional compounds including β-glucans, vitamin E, vitamin C and zinc. The Protec™ diet enhances the innate and adaptive immune response of rainbow trout through the enhancement of respiratory burst, leucocyte proliferation and increased production of specific IgM against *Lactococcus garvieae* [14] and can be considered to have both immunostimulatory and prebiotic effects in the host. A feeding trial using Protec™ in rainbow trout following an infection with *L. garvieae* showed increased leucocyte respiratory burst activity and increased levels of *L. garvieae* antibodies occurred at both 4- and 8-weeks post-infection compared to a control diet before and after vaccination against *L. garvieae*. Protec™ has also been demonstrated to protect against viral pathogen infections such as viral haemorrhagic septicaemia virus (VHSV) [8], where fish fed the Protec™ diet mounted a greater immune gene response (*MX, IgM, IgT* and *IgD)* to VHSV and showed increased levels of natural IgM in sera after being fed for 30 days on the functional diet. Further research using *in-vitro* approaches in rainbow trout demonstrated improvements in head kidney leucocyte proliferation and respiratory burst activity after stimulation with sonicated Protec™ immune support pack which is a combination of glucans, vitamin C, vitamin E and zinc [14].

To measure the effectiveness of functional ingredients, both *in-vitro* and *in-vivo* methods can be used. Traditionally, feeding trials are commonly used, followed by assessment of growth and immune parameters. To complement such studies, recent developments of cell culture techniques such as the use of cell lines, RTS11 [9] and primary cell cultures such as head kidney macrophages [4], have been used to further elucidate the role of functional ingredients on specific cell types. This use of cell lines gives added information to mechanisms of response, but also reduces the number of fish being used in feeding trials. Gut associated lymphoid tissue (GALT) leucocytes have been used in assays to identify responses to PAMPs [15,16] and have also been used in previous research to assess nutritional ingredients [10]. GALT leucocytes extracted from the gut contain multiple different cell types and enable greater interpretation of responses to whole tissue or specific cell lines used in cell culture, where the cell lines are restricted to a single phenotype. These immune cells can interact directly with the functional ingredients and may demonstrate how they are likely to respond in the gut at the interface between the host and the feed sources.

In this study, the responses in rainbow trout following a 6-week feeding trial of two diets containing β-glucans compared to fish fed control diet was examined. The three diets were a commercial functional feed diet containing β-glucan and other components (Protec™, Skretting), a diet with β-glucan alone as prebiotic, and a commercial diet. To assess the *in-vivo* effects on the fish; growth parameters, histology of the distal intestine, gene expression of a panel of immune cell markers and expression of innate immune genes in primary cultures of GALT leucocytes were used. To identify how the functional diets modulated the innate immune response, GALT leucocytes were extracted and stimulated with Poly I:C and recombinant IL-1β followed by gene expression analysis. To further understand how diets, modulate the innate immune response to pathogenic challenges an immune challenge was carried out using *A. salmonicida* and was followed by targeted gene expression analysis of innate immune related genes. The data collected indicates the effectiveness of β-glucans and the Protec™ diet in modulating the gut innate immune response potentially allowing the fish to be better equipped to fight disease during stressful life events.

## 2. Materials and methods

### 2.1. Fish

All procedures were carried out under the UK Animals (Scientific Procedures) Act 1986 and Home Office code of practice guidance, under Home Office project licence PFF8CC5BE. Rainbow trout (~75 g) were obtained from a commercial rainbow trout farm and kept in 1 m diameter fiberglass tanks with recirculating freshwater at 14 ± 1 °C, within the aquarium facilities in the School of Biological Sciences, University of Aberdeen, UK.

### 2.2. Feeding trial

For the feeding trial 270 fish were distributed randomly between nine tanks; with tanks being run in triplicate for each diet giving 90 fish per diet group, a diagram of the experimental set up can be seen in Fig. 1. After a two-week acclimatization period (during this acclimation fish were feed on standard commercial diet, Skretting), a six-week feeding trial was carried out. Three diets were used; acclimatization as a control diet and two experimental diets one with β-glucan supplementation (0.1%) and another the Protec™ diet produced by Skretting ARC (commercially available). The composition and formulation of experimental diets are shown in Table 1. All diets were prepared by Skretting Aquaculture Innovation. Fish were fed continuously throughout daylight hours (using automated Arvotec feeders) with fish being fed an amount equal to 1.5% of their average body weight per day. All fish were weighed at the beginning of the feeding trial and ten fish per tank were weighed every two weeks until the end of the trial with the amount of feed adjusted accordingly.

### 2.3. Growth parameters

Specific Growth Rate (SGR), Fulton's Condition Factor (K), Feed Conversion Ratio (FCR), Weight Gain (WG), weights and fish lengths were calculated as biometric data.

*SGR* was calculated using the following formula to calculate the percentage gain per day:

$$SGR = \left[\frac{(LN_{W2} - LN_{W1})}{Number\ of\ Days}\right] \times 100$$

where W1 is starting weight and W2 is final weight.

*FCR* was calculated using the following formula:

$$FCR = \frac{Feed\ Fed\ (g)}{Gain\ in\ weight\ of\ fish\ (g)} \times 100$$

*Fulton's Condition Factor* was calculated using the following formula:

$$K = \frac{100 \times weight\ (g)}{Length^3\ (cm)}$$

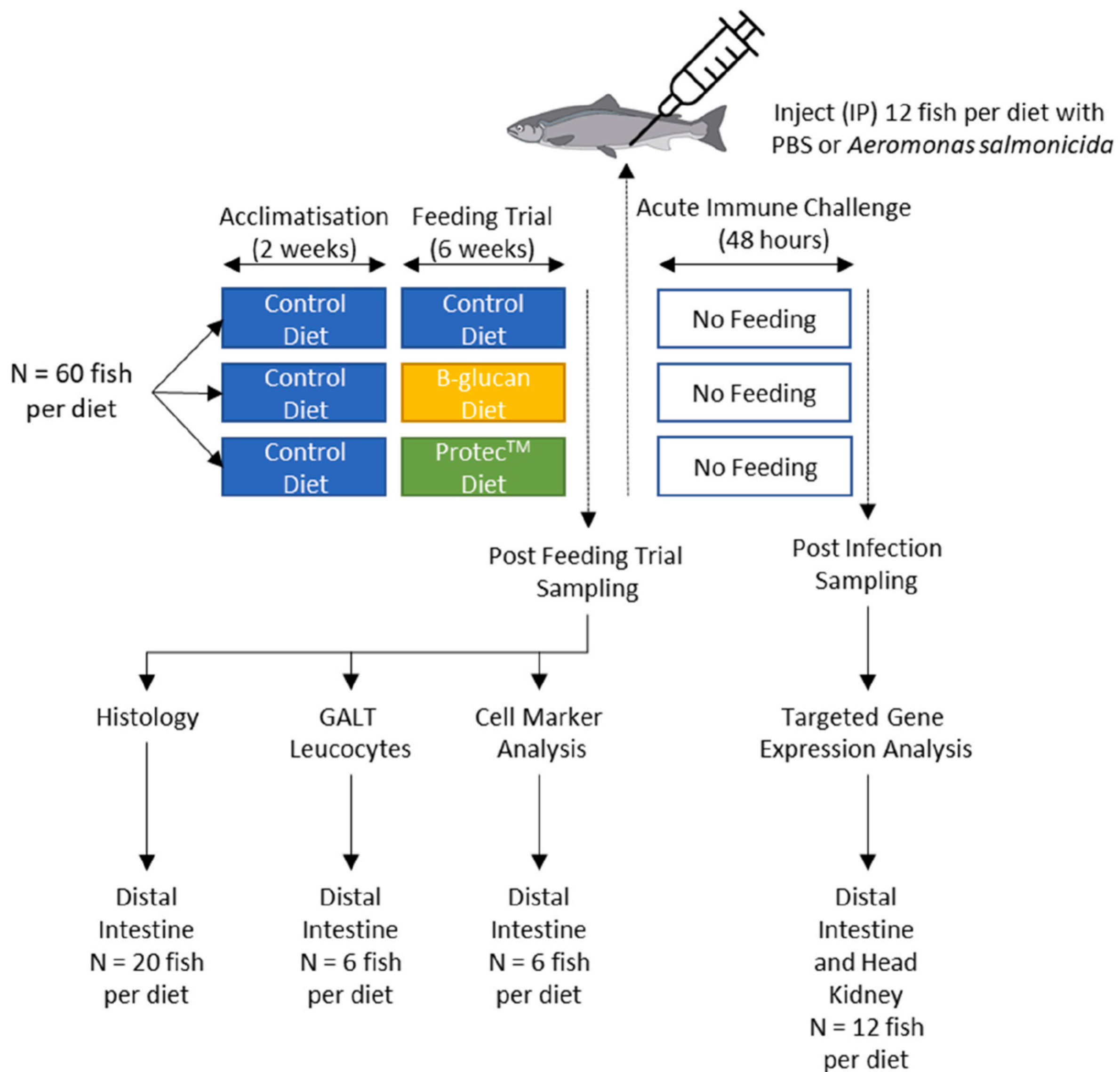


**Fig. 1.** Diagram of the experimental set-up. A 6-week feeding trial was carried out with a range of immunological sampling taken at the end of the 6 weeks. After which 12 fish per diet were injected with Aeromonas salmonicida for 48hrs before whole tissue samples of head kidney and distal intestine were collected to measure the acute response to bacterial infection.

### *2.4. Histological examination of distal intestine*

Following week 6 of the feeding trial, fish were randomly selected from each diet (20 fish per diet, 60 total) and killed by overdose on anaesthetic before confirmation of death by the destruction of the brain (schedule 1 killing) before distal intestine tissue samples were taken (approximately 1 cm in length). The tissue samples were rinsed with PBS (Sigma) to remove excess digesta before being stored in 10% neutral buffered formaldehyde (CellPath). Sample preparation, and image acquisition were carried out at the University of Aberdeen Microscopy and Histology Core Facility. Distal intestine samples were routinely dehydrated in ethanol, equilibrated in xylene, and embedded in paraffin wax according to standard protocols [17]. Cross sections (3 μm) of the distal intestine were cut with a microtome and mounted onto microscope slides. These sections where subjected to haematoxylin and eosin (H&E) staining with alcian blue. Sections were imaged using the Zeiss AxioScan Z1 slide scanner. The resultant images were randomised to ensure blinded assessment and then scored using a semi-quantitative criterion using the Zen Blue software (V3.4). The criteria measured 6 variables (goblet cells, eosinophilic granulocytes, lamina propria, mucosal folds, supranuclear vacuoles and sub-epithelial mucosa) and each were given a score between 1 and 5 as previously described by Uran et al [18] and can be seen in Table 2. An increasing score represents increasing inflammation. The morphology of the mucosal folds (MF) is an indicator of gut health, as MFs are important for increasing surface area for the absorption of nutrients. The presence and size of supranuclear vacuoles (SNV) is measured as SNVs contains lysosomal proteins. The abundance of goblet cells was measured due to their roles in mucous production and are a characterised inflammatory marker. The widening of the lamina propria (LP) and thickness of the sub-epithelial mucosa (SEM) were measured to monitor the health of mucosal membranes with a larger LP or thicker SEM suggesting higher levels of inflammation. The infiltration of eosinophilic granulocytes (EGs) into the SEM or LP was measured as an indicator of the immune capacity/migration of immune cells to the gut (site of inflammation). These scores were analysed using a two-way Anova in R (V4.1.0).

### *2.5. Isolation of primary GALT leucocytes*

GALT cells were isolated as previously outlined by Porter et al [10].

**Table 1**
Dietary formulation and proximate composition of the experimental diets (% dry matter).

| | Control | β-glucan | Protec |
|---|---|---|---|
| Wheat | 12.93 | 12.83 | 11.69 |
| Corn gluten meal | 8.55 | 8.57 | 8.87 |
| Faba bean meal | 10.00 | 10.00 | 10.00 |
| Soy protein concentrate | 30.00 | 30.00 | 30.00 |
| Guar meal | 12.00 | 12.00 | 12.00 |
| Fish oil | 7.46 | 7.46 | 7.52 |
| Fish meal | 10.00 | 10.00 | 10.00 |
| Rapeseed oil | 7.58 | 7.56 | 7.36 |
| Phosphate | 0.72 | 0.72 | 0.71 |
| dl-Methionine | 0.10 | 0.10 | 0.10 |
| Astaxanthin | 0.01 | 0.01 | 0.01 |
| Vitamin and mineral premix[a] | 0.649 | 0.649 | 0.842 |
| Beta-glucans | | 0.10 | |
| Micro ingredients[b] | | | 0.91 |
| Proximate composition | | | |
| Ash | 4.3 | 4.3 | 4.4 |
| Crude fat | 19.7 | 19.5 | 19.7 |
| Moisture | 7.4 | 7.2 | 7 |
| Crude Protein | 42.6 | 43.1 | 43.3 |

[a] Vitamin and mineral premix (Trouw Nutrition, Boxmeer, The Netherlands. Proprietary composition Skretting AI).
[b] These include plant extract, nucleotides, and beta glucans (Proprietary composition Skretting AI).

The distal intestine was removed from fish and stored in cold PBS (Sigma) until washing. Samples were rinsed in PBS three times before being cut longitudinally and then into 1 $cm^2$ sections to aid with rinsing. Samples were then rinsed for 30 min in an orbital shaker at 40 rpm in 30 ml of PBS. Following one final rinse in PBS samples were added to falcon tubes containing 25 ml of predigestion solution (HBSS (Gibco, 14025092) + 0.145 mg/ml DTT (Sigma, D9779) + 0.37 mg/ml EDTA (Fisher, D/0700/53)) and put in an orbital shaker at 40 rpm for 20 min. The supernatant (S1) was filtered into 50 ml falcon tubes using 100 μm cell strainers (Greiner). before being rinsed with PBS twice, the pelleted cells were stored in growth media (Leibovitz L-15 media (Gibco) + 10% FBS + 1% P/S) in a 20 °C incubator. The gut segments were rinsed using washing solution (HBSS + 0.05 mg DNase1/ml (Sigma, DN25) + 5% FBS + 1% Penicillin/Streptomycin) to remove EDTA and DTT. After which the gut segments were added to 25 ml of digestion solution (washing solution + 0.37 mg collagenase IV/ml (Gibco, 17104-019) in 50 ml falcon tubes before being placed in an orbital shaker for 2 h at 40 rpm. The supernatant was filtered into the tube containing S1 using 100 μm cell strainers (Greiner). The mixture was washed three times with PBS +1% P/S to remove any collagenase before being resuspended in 5 ml of growth media. The cell suspension was slowly layered on top of a discontinuous percoll gradient (25% and 75%) (GE healthcare, 17-0891-01) and centrifuged for 30 min at 400 g, at 4 °C. Cells were then collected from the interface between the 25 and 75% percoll gradients and washed twice in PBS +1% P/S before being resuspended in stimulation media (Leibovitz-15 media +1% FBS + 1% P/S). Collected cells were adjusted to $1 \times 10^6$ cells/ml before 1 ml was added to 6 well plates to which stimulants were added.

### 2.6. Immune stimulation of GALT leucocytes

Two different stimuli were used to stimulate the GALT leucocytes; poly I:C (100 μg/ml, Sigma, P1530) to induce an antiviral response and, recombinant IL-1β (20μgml$^{-1}$, Provided by Dr. Tiehui Wang (Scottish Fish Immunology Research Centre)) [19]. A stock solution for poly I:C was diluted in L-15 media with aliquots stored in −20 °C until use. rIL-1β was stored in −80 °C in aliquots and diluted in L-15 media. The stimulants were then added to wells containing $1 \times 10^6$ cells diluted in 1 ml of stimulation media to give the working solutions. All experiments were performed in a randomized design with sextuplicate wells that were treated independently through the entire protocol (n = 6).

### 2.7. Immune challenge with *Aeromonas salmonicida*

After week 6 of the feeding trial, 24 fish were randomly selected for immune challenge from each diet. These fish were then split into two challenge tanks each containing 12 fish for each diet. Fish were injected interperitoneally with 1 ml of live *Aeromonas salmonicida* ($2 \times 10^6$ CFU/ml) or PBS for control fish. Head kidney and distal intestine tissues (~1 cm) were harvested 48-hrs after infection. Samples were stored in RNA later and stored in the −80 °C freezer until RNA extraction.

### 2.8. Transcriptional analysis

RNA was extracted from cells in individual wells or tissue samples using 750 μL or 1 ml of TRI Reagent (Sigma) respectively following the manufacturer's instructions. The RNA pellet was washed using 80% ethanol, dissolved in RNase-free water, and stored at −80 °C until use. Quality control of the RNA was determined by nanodrop spectrophotometer for quantity and Agilent bioanalyzer 2100 to determine RNA integrity. RNA (500 ng for cells and 1 μg for tissues) was used as template for reverse transcription using the Qiagen QuantiTect Reverse Transcription Kit following manufacturer's instructions. The generated cDNA was diluted 10x with RNase-free water and stored at −20 °C until use.

Gene expression was determined for proinflammatory (*Interleukin-1 beta,* (*IL-1β*)*, Interleukin-8* (*IL-8*)*, Interleukin-10* (*IL-10*) and Tumour Necrosis Factor-alpha (*TNFα*)), antimicrobial genes (Hepcidin antimicrobial peptide (*HAMP*) *and Serum Amyloid A* (*SAA*)), anti-viral markers (*MX, Viperin* (*RSAD2*), *Proteasome subunit gene 9* (*PSMB9*), *gamma inducible protein* (*γIP*) *and Interferon-1 alpha* (*IFN-1α*)), mucins (*Mucin* 5-AC (*MUC5AC*) and *Mucin 2A* (*MUC2A*)), *C-type lectin 4* (*CLEC4T*), cellular markers for T cells/antigen presenting cells (*Interleukin-4/13* (*IL-4/13*) and *Interferon-gamma* (*IFN-γ*)) and B cells (*CD4, CD8α, Major Histocompatibility complex II-beta* (*MHCIIβ*)*, CD208, Secretory immunoglobulin M* (*sIgM*) and *secretory immunoglobulin T* (*sIgT*)) responses by qPCR. Amplification was performed using Agilent Brilliant III Ultra-Fast SYBR and specific primers that had been tested for their efficiency previously (Table 3) [6,16,20–22] using a Roche Lightcycler 480 platform using PCR cycles of initial denaturation of 3mins at 95 °C followed by 40 cycles (5s at 95 °C, 10s at 60 °C and 1s at 72 °C). Following amplification all products were subjected to by melting curve analysis to ensure single product was amplified. The expression of target genes was normalised to the relative expression of three housekeeping genes *Elongation Factor-1 alpha* (*ELF-1α*), *Ribosomal Protein* L4 (*RPL4*) and *Ribosomal Protein S29* (*RPS29*) (Table 3). Three housekeeping genes were used to minimize random errors involved with qPCR. The gene expression was calculated using the Genex 5 software (Multid) to generate relative gene expression which was then used to calculate fold change in comparison to the control samples. No tank effect was present in the data sets in relation to growth so gene expression methods were not altered. Samples were collected randomly from different tanks in equal amounts to remove any tank bias.

### 2.9. Statistical analysis

Data handling, calculation of fold change and statistical analysis was performed in Microsoft Excel 2016. Statistical analysis carried out in Excel was the two-tailed students T-test. Graphical representation and one-way and two-way Anova's of the data were completed using GraphPad Prizm 5 and R (V4.1.0).

**Table 2**
Distal intestine histological scoring chart taken from Uran et al. (17).

| Mucosal Folds (MF) | |
|---|---|
| Scoring | Definition |
| 1 | Basal length |
| 2 | Some shrinkage and bloating |
| 3 | Diffused shrinkage and onset of tissue disruption |
| 4 | Diffused tissue disruption |
| 5 | Total tissue disruption |
| **Lamina Propria (LP)** | |
| **Scoring** | **Definition** |
| 1 | Normal size |
| 2 | Increased size |
| 3 | Medium size |
| 4 | Large size |
| 5 | Largest Size |
| **Eosinophilic Granulocytes (EC)** | |
| **Scoring** | **Definition** |
| 1 | Few in sub-epithelial mucosa |
| 2 | Increased number in sub-epitelial mucosa and some migration into lamina propria |
| 3 | Increased migration into LP |
| 4 | Diffused number in sub-epithelial mucosa and LP |
| 5 | Dense EG in sub-epithrlil mucosa and LP |
| **Goblet Cells (GC)** | |
| **Scoring** | **Definition** |
| 1 | Scattered Cells |
| 2 | Increased number and sparsely distributed |
| 3 | Diffused number widely spread |
| 4 | Densely grouped cells |
| 5 | Highly abundant and tightly packed cells |
| **Supranuclear Vacuoles (SNV)** | |
| **Scoring** | **Definition** |
| 1 | Basal size |
| 2 | Some size reduction |
| 3 | Diffused size reduction |
| 4 | Onset of extinction |
| 5 | No vacuoles |
| **Sub-epithelial Mucosa (SEM)** | |
| **Scoring** | **Definition** |
| 1 | Normal size |
| 2 | Increased size |
| 3 | Medium size |
| 4 | Large size |
| 5 | Largest Size |

## 3. Results

### 3.1. Growth parameters

A feeding trial was carried out using a β-glucan supplemented diet, Protec™ diet and a commercial control diet, all fish survived and remained healthy throughout the trial. No significant differences were found in the biometric parameters measured (Table 4) or between tanks for each diet. Overall, the fish had an FCR of 0.95 ± 0.17, 0.96 ± 0.11, 1.23 ± 0.12 for control, β-glucan and Protec™ diets respectively .

### 3.2. Histological analysis of dietary modulation of the distal intestine

Histological analysis revealed all three diets had healthy levels of all parameters measured (examples of intestinal sections for each diet see Fig. 2). The number of goblet cells was significantly different ($p < 0.05$) across all three diets with fish fed the Protec™ showing the highest number of goblet cells followed by the fish fed the β-glucan diet with the lowest numbers were observed in the fish fed the control diet. Scoring of the mucosal folds indicated a significant difference ($p < 0.05$) between all three diets with the β-glucan diet showing the highest scoring followed by the Protec™ diet with the lowest scoring found in the control diet. These results suggest that both the β-glucan and Protec™ had increased bloating and shrinkage of the mucosal folds compared to the control diet. The supranuclear vacuoles demonstrated significant decreases in size ($p < 0.001$) in both the Protec™ and the β-glucan diet compared to the control diet, 2.35 (Table 5). The lamina propria showed some significant increases ($p < 0.001$) in size and thickness in both the β-glucan diet, 1.87, and Protec™ diets, 1.79 compared to the control diet, 1.56 (Table 5). There were no significant differences observed for sub-epithelial mucosa (Table 5) or the eosinophilic granulocytes () Table 5 between the different diet treatments.

### 3.3. Identification of GALT leucocyte cell markers

Gene expression for specific cellular markers were used to identify T-cell (*CD4-1, CD8α*), dendritic cell (*MHCIIβ, CD108)* and B-cell (*sIgM* and *sIgT*) populations to determine if there were any differences between dietary groups which may signify changes in cellular abundance/activity of these cell types within the distal intestine (Fig. 3). T-cell marker

**Table 3**
qPCR primer sequences, product size and GenBank accession numbers.

| Gene | Forward Primer (5′-3′) | Reverse Primer (5′-3′) | Product Size (bp) | Accession Number | Source |
|---|---|---|---|---|---|
| **EF1-α** | CAAGGATATCCGTCGTGGCA | ACAGCGAAACGACCAAGAGG | 327 | AF498320 | [16] |
| **RPL4** | CCTTCAGAAACATCCCTGGTATCAC | GGGCAGATTGTAGTCTACCTTGAGAG | 182 | BT057966 | [20] |
| **RPS29** | GGGTCATCAGCAGCTCTATTGG | AGTCCAGCTTAACAAAGCCGATG | 167 | BT043522 | [20] |
| **MX-1** | CGTCCCAGACCTCACACTCATC | TGCCATCTTCAAAGCCTCTGTG | 187 | OMU30253 | [21] |
| **IFN-1α** | GTGTGTCATTGCTGTGACTGGA | TTTGTGATATCTCCTCCCATCTG | 95 | AJ580911 | [21] |
| **γIP** | GACATCAACGGTCCTCATCAGC | CCGTTCCTCAGAGTGACAATGAT | 201 | AJ417078 | [21] |
| **PSMB9 (LMP2)** | GTGCTGGGCTCTGACTCTCG | CTCCAACCTCAATGCTGTGC | 179 | AF112117 | [21] |
| **RSAD2 (Viperin)** | TCCCAAGTGTCAGTATTGTCAGC | AGGTTGTCCAGGTGGCTCTT | 166 | AF076620 | [21] |
| **SAA** | AGTCATCAGTAATGGCCGGGA | AAAAGCTTGTTTGGAATTTGGTCCT | 205 | NM_001124436 | |
| **HAMP (Hepcidin)** | GCTGTTCCTTTCTCCGAGGTGC | GTGACAGCAGTTGCAGCACCA | 93 | HQ711993.1 | [22] |
| **TNFα** | CTGTGTGGCGTTCTCTTAATAGCAGCTT | CATTCCGTCCTGCATCGTTGC | 99 | AJ401377 | [16] |
| **IL-8** | GAAACTCGCCACAGACAGAGAA | AGTGTGTTGTTATCTCGCTGGTAA | 114 | HG917307.1 | |
| **IL-10** | ACATCCCTGCTGGACGAAGG | GGCAGCACCGTGTCGAGATA | 101 | NM_001245099.1 | |
| **IL-1β** | CTGCACCTAGAGGAGGTTGCG | GAAACGCACCATGTCGCTCT | 72 | NM_001124347.2 | |
| **CD4** | GTGTGGAGGTGCTACAGGTTTTTTC | ATCGTCACCCGCTGTCTGTG | 394 | AY973028 | [16] |
| **CD8α** | CAAGTCGTGCAAAGTGGGAAA | TCTGTTGTTGGCTATAGGATGT | 214 | AF178053 | [16] |
| **MHCIIβ** | TGTCAGAGTCAGGTGGACCAGGA | GGCTCACCTCAGGTTCCCAGAT | 203 | XM_036950450.1 | [16] |
| **CD208** | ACATGAAAAGCTGTTCCCAACTGC | AGCCCAGCACTCAACCTCCTC | 163 | NM_001281412 | [16] |
| **sIgM** | TACAAGAGGGAGACCGGAGGAGT | CTTCCTGATTGAATCTGGCTAGTGGT | 221 | X65261 | [16] |
| **sIgT** | CATCAGCTTCACCAAAGGAAGTGA | TCACTTGTCTTCACATGAGTTACCCGT | 361 | AY870268 | [16] |
| **MUC2A** | GAGTGGGCTCTCAGATCCAG | ACGATGCGGACGGTAGTTTT | 112 | XM_036968563.1 | |
| **MUC5AC** | ACCTGTATTACTGCCACCTGC | AAAACAAATACTGTTGTGGTCGG | 140 | XM_036934544.1 | |
| **CLEC4T** | CACCACTGACCACAGCGAATTGG | GAACATTTCTCATCATTCCACCAC | 124 | FN667662 | [6] |

**Table 4**
Growth parameters from the feeding trial for each diet with means and standard deviations given.[a] and[b] denote a significant difference where $p < 0.05$.

| Parameter | Diet | | |
|---|---|---|---|
| | Control (A) | β-glucan (B) | Protec™ (C) |
| **FCR** | 0.95 ± 0.17[a] | 0.96 ± 0.11[a] | 1.23 ± 0.12[a] |
| **SGR** | 1.46 ± 0.21[a] | 1.45 ± 0.19[a] | 1.15 ± 0.08[a] |
| **Weight Gain (%)** | 84.9 ± 15.7[a] | 84.0 ± 14.6[a] | 62.2 ± 5.3[a] |
| **Fulton's Condition Factor** | 1.44 ± 0.05[a] | 1.42 ± 0.07[a] | 1.37 ± 0.04[a] |
| **Final Weight (g)** | 270.5 ± 22.2[a] | 274.6 ± 26[a] | 246.9 ± 12.3[a] |

CD4 was expressed at a significantly more abundant level ($p < 0.05$) in the fish fed the β-glucan diets compared to the control diet, however no significant differences were observed for CD8α. *sIgM* as marker for B-cells was expressed at significantly higher abundance in the β-glucan diet, additionally the fish fed Protec™ diet showed an increase but did not reach significance. Expression of *sIgT* was not altered as result of diet. No differences were detected for the dendritic cell markers between diet treatments.

### 3.4. Dietary modulation of key immune markers in GALT

Expression of key anti-viral and proinflammatory response genes were examined in the GALT leucocytes (Fig. 4). Targeted gene expression analysis highlighted differences in the expression of proinflammatory genes studied and clustering of the β-glucan and control diets (Fig. 5). GALT cells from fish fed the Protec diet had significantly higher expression of the two proinflammatory cytokines, *IL-1β* and *TNFα*, these cells also had significantly higher expression of the anti-microbial peptide *HAMP* and the acute phase protein *SAA* in comparison to both cells from the control and β-glucan fed fish. Analysis of the genes associated with anti-viral activity indicated an increased expression of both *MX* and *γIP* in the cells from both β-glucan and Protec™ fed fish but was not significantly increased. However, *IFN-1α*, *RSAD2* and *PSMB9* were not modulated by diet (Fig. 4).

### 3.5. Dietary mediated responses of GALT leucocytes to poly I:C

Poly I:C was used to generate anti-viral activity in GALT cells fed the different diets and were compared against non-stimulated cells (Fig. 6a, b, c). The GALT cells isolated from fish fed all three diets showed significant increases to the poly I:C with *IFN-1α, RSAD2* and *γIP* all being significantly increased compared to the unstimulated cells. The GALT cells from the fish fed the β-glucan and Protec™ diets both had significantly increased the magnitude of *MX* gene expression compared to unstimulated cells. Finally, the GALT cells from the Protec™ fed fish also had a significant increase expression of *PSMB9* compared to unstimulated cells, which was not observed in the other diet groups. Although the GALT cells all had capacity to respond to the diets, further analysis of the magnitude of these changes suggested the diet did not affect the response between diets other than for the *PSMB9* gene that only responded to the poly I:C in the Protec™ fed fish (Fig. 6d).

### 3.6. Dietary mediated responses of GALT leucocytes to rIL-1β

The rIL-1β induced expression of *IL-1β, IL-8*, and *TNFα* in GALT cells derived from fish fed all diets (Fig. 7a, b, c). *SAA*, an acute phase response gene was significantly upregulated in both the β-glucan and Protec™ fed fish but not in the fish fed the control diet compared to their respective unstimulated samples. No significant difference in fold change was observed in *HAMP* in the fish fed the β-glucan and control diets, however there was a significant decrease in expression of the fish fed the Protec™ diet. When the magnitude of the response to the rIL-1β was examined in the GALT cells, the cells from the fish fed the Protec™ had a significant larger increase (fold change) in *TNFα* compared to the other diets (Fig. 7d).

### 3.7. Dietary mediated responses to *Aeromonas salmonicida*

To further assess the impact of the diets on the immune response of rainbow trout, fish were challenged with *A. salmonicida*, a bacterial pathogen of salmonid fish. Proinflammatory genes were measured in both the head kidney and the distal intestine alongside genes relating to mucins and secretory antibodies in the distal intestine (Fig. 8).

***Immune Responses Head Kidney following bacterial infection:*** *IL-1β* was significantly upregulated in the head kidney of fish fed all three diets in compared to PBS treated controls. Fish the β-glucan diet had a significantly lower induction, (2-fold change), compared to the control diet and Protec™ diet (6.7- and 5.6-fold respectively). *IL-8* was upregulated compared to unstimulated samples in fish fed both the control diet and the Protec™ diets (1.8- and 2.2-fold change respectively) but was not changed in the fish fed β-glucan diet. *TNFα* was significantly

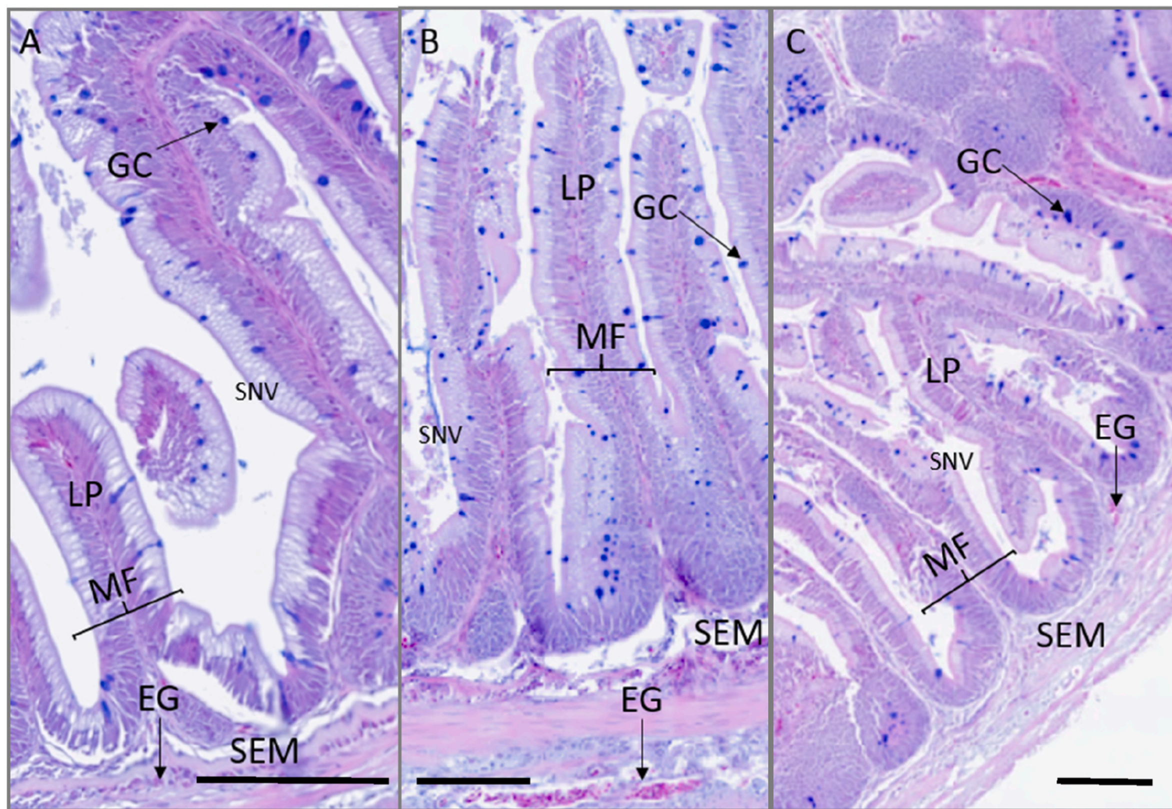


**Fig. 2.** Distal intestine of rainbow trout after feeding for 6 weeks on three diets. SNV supranuclear vacuoles, GC goblet cells, LP lamina propria, EG eosinophilic granulocytes, MF mucosal folds, SEM sub-epithelial mucosa. **(A) Control Diet. (B) β-glucan diet. (C) Protec™ Diet.** H&E Staining with Alcian blue. Bar represents 200 µm.

**Table 5**
Means and standard deviations of scoring parameters for histology,[a,b] and[c] denote significant difference.

| Descriptive Parameter | Diet | | | | | |
|---|---|---|---|---|---|---|
| | Diet A (Control) | | Diet B (B-glucans) | | Diet C (Protec) | |
| | Mean | SD | Mean | SD | Mean | SD |
| **Mucosal Folds** | 1.62[a] | 0.63 | 2.08[c] | 0.43 | 1.77[b] | 0.42 |
| **Subepithelial mucosa** | 1.77[a] | 0.78 | 1.67[a] | 0.47 | 1.72[a] | 0.45 |
| **Supranuclear Vacuoles** | 2.35[a] | 0.64 | 2.10[b] | 0.30 | 2.06[b] | 0.23 |
| **Lamina Propria** | 1.56[a] | 0.73 | 1.87[b] | 0.54 | 1.79[b] | 0.41 |
| **Eosinophilic Granulocytes** | 1.69[a] | 0.69 | 1.66[a] | 0.58 | 1.65[a] | 0.50 |
| **Goblet Cells** | 2.03[a] | 0.67 | 2.28[b] | 0.50 | 2.58[c] | 0.52 |

upregulated in all three diets with no significant differences between the control and Protec™ fed fish. However, the fish fed β-glucan diet showed significantly less magnitude of response ($p < 0.05$) compared to both control and Protec™ fed fish with only a 2.8-fold increase. *SAA* and *HAMP* showed a similar trend in both antimicrobial genes with all diets showing a significant upregulation in response to *A. salmonicida* infection. However, for both genes, both *SAA* and *HAMP* showed significantly lower magnitude of response in both the β-glucan and Protec™ diets compared to the control diets.

***Distal Intestine Immune Responses following bacterial infection:*** *IL-1β* was significantly upregulated in all three diets compared to PBS treated samples. The fish fed β-glucan diet showed a significantly higher magnitude of response (16.3-fold change) compared to fish fed both the control and Protec™ diets (4.5- and 2.8-fold change respectively). The *HAMP* gene followed the same trend as observed in the head kidney with both the fish fed β-glucan and Protec™ diets, 6.2- and 3.8-fold change, showing a much lower magnitude of response compared to the control

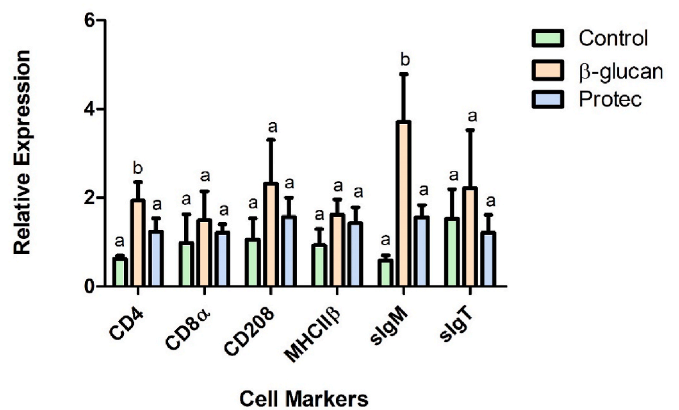


**Fig. 3.** Cellular markers for B-Lymphocytes (sIgM & sIgT), T-Lymphocytes (CD4 & CD8α) and Dendritic Cells (CD108 & MHCIIβ) in isolated GALT leucocytes from rainbow trout fed two different functional diets. The marker gene expression was determined by qPCR and is presented as relative expression after normalisation against three reference genes. Bars are means plus SEMs of 6 fish.

diet, 34.1-fold change. Conversely, *SAA* had a higher magnitude of response in both the fish fed β-glucan and Protec™ diets (17.5- and 21.4-fold change) compared to the control diet (6.4-fold change). The genes relating to secretory immunoglobulins T and M both exhibited a similar trend with the fish being fed the β-glucan diet having a higher magnitude of response ~3.4-fold for both genes compared to both the control

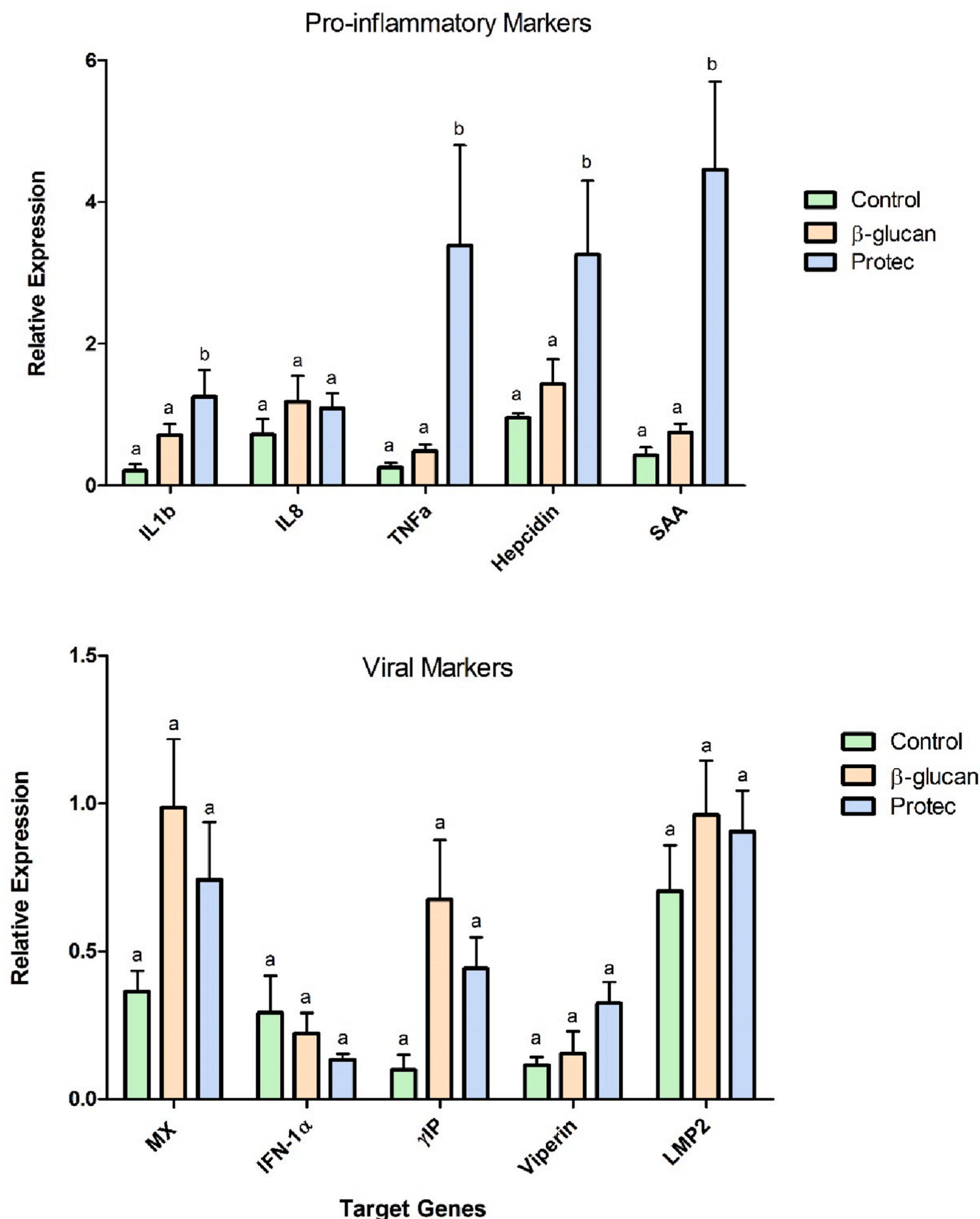


**Fig. 4.** Basal gene expression of key immune markers in isolated GALT leucocytes from Rainbow Trout fed two functional diets. Basal gene expression of proinflammatory and viral genes was determined by qPCR and is presented as relative expression after normalisation against three reference genes. Bars are means plus SEMs of 6 fish. Significance was tested via two-way Anova, followed by a post hoc Tukey test where [a] and [b] denote significance where $p < 0.05$.

and Protec™ fed groups. Fish fed the Protec™ diet also showed a significantly lower magnitude of response to both the control diet and β-glucan diet in *sIgT*. *CLEC4T* was significantly upregulated in both the control and β-glucan diet groups after the immune challenge, no change in expression was observed in the fish fed the Protec™ diet. Both the control and β-glucan diets showed a higher magnitude of response for *CLEC4T*, (3.0- and 3.1-fold change) compared to the Protec™ diet (0.8-fold change) after immune stimulation. A significantly higher relative expression of *CLEC4T* was observed in the Protec™ diet in PBS treated samples, compared to both the control and β-glucan diets. Two mucin genes *MUC5AC* and *MUC2A* were examined, *MUC5AC* had a higher magnitude of response in both the control and β-glucan diet groups (~2.4-fold change) compared to those fed Protec™ diet (0.1-fold change). However, fish fed the Protec™ diet showed a significant increase in relative expression in the PBS samples compared to fish fed both the control and β-glucan groups. The *MUC2A* gene showed no

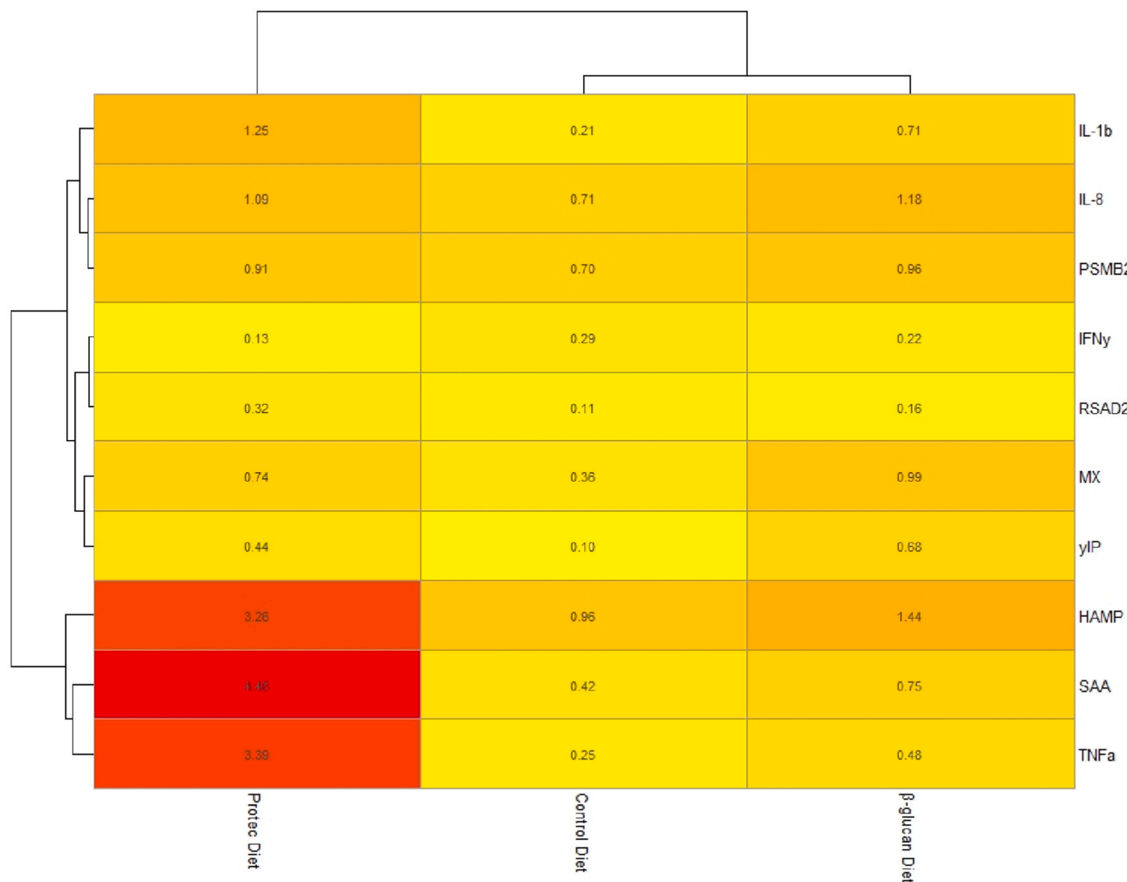


**Fig. 5.** Heatmap of basal expression between diets. qPCR was used to quantify gene expression where samples were normalised against three housekeeping genes. Genex Multi-iD 5 was then used to calculate relative expression values. Values are mean expression levels relative to each other.

significant difference to PBS treated samples across all three diets.

The proinflammatory response and antimicrobial genes *IL-1β, SAA and HAMP* were measured across both the head kidney and the distal intestine. Interestingly *IL-1β* showed a higher magnitude of response in the fish fed the β-glucan diet in the distal intestine compared to the head kidney, 16.3- compared to 2-fold change respectively. For fish fed both the control and Protec™ diets a lower magnitude of response was observed in the distal intestine compared to the head kidney. For both anti-microbial genes *SAA* and *HAMP,* a higher magnitude of response was observed in the head kidney compared to the distal intestine.

## 4. Discussion

In this current study, we have demonstrated that the administration of immunostimulant in the form of either purified β-glucan or β-glucan with additional factors (Protec™) can modulate the innate immune activity in the intestinal cells of rainbow trout. The data indicates that the diets are modulating the immune response through both the localised response within the distal intestine and the systemic response within the head kidney. The results of this trial elucidate that diets containing β-glucans are able to alter the magnitude of the response against bacterial challenges.

Supplementation with immunostimulants had no significant effect on growth performance as assessed by growth rate, food conversion and condition factor, indicating that all three diets were performing well regarding biometric performance. Detailed examination of the distal intestine by histological analysis revealed that the fish fed the β-glucan supplemented and Protec™ diets had a higher number of goblet cells distal intestine compared to the control fed fish. This result may reflect the immunostimulatory molecules found in both experimental diets that can cause an increase in the proinflammatory response which can lead to upregulation of mucins which are secreted by this cell type [23]. Goblet cells are specialised epithelial cells that maintain barrier function through secreting mucous, and secrete important antimicrobial peptides, mucins, and cytokines [24]. Goblet cells aid the innate immune system by creating goblet cell-associated antigen passages which allow the delivery of microbial products and other antigens to be presented to antigen presenting cells in the lamina propria and trigger the innate immune response. Increased goblet cell numbers have been implicated in a wide number of different detrimental conditions such soybean induced enteritis caused by the supplementation of high concentrations of soybean meal to fish diets although such high concentrations are not commonly used in commercial diets [18,25–27]. However, with increased levels of goblet cells, if associated enteritis, often, higher scores for lamina propria and other variables measured [27]. As all other variables measured demonstrated either no change or an improvement in gut health in the supplemented diets the increased goblet cell number may be due to the immunomodulation caused by β-glucans. The higher levels of goblet cells seen by the supplemented diets may offer increased protection against pathogens but not necessarily representing an inflamed status on the distal intestine.

The analysis of transcriptional cell markers that can be used as a proxy to represent the abundance of immune cell types and they indicate the two function ingredients can alter the leucocyte composition in the distal intestine. The fish fed the β-glucan diet had higher expression of the B-cell marker, *sIgM* and the T-cell marker, *CD4,* compared to both the control and Protec™ fed fish. The Protec™ diet also showed non-significant increased relative expression levels in genes relating to B- and T-cell markers. Higher levels of *CD4* transcripts would suggest there are higher numbers of $CD4^+$ T-cells, a key cell in mediating the adaptive

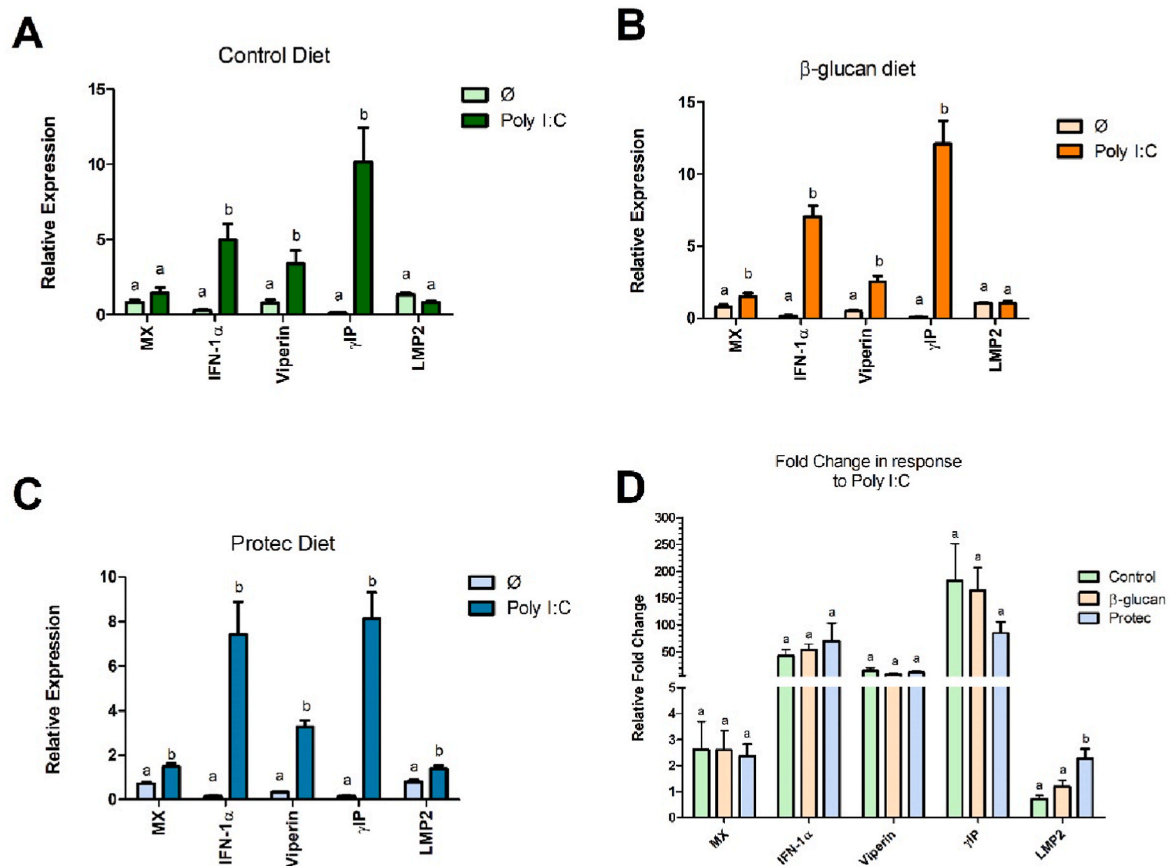


**Fig. 6.** Response to poly I:C. All results were quantified by qPCR. qPCR results were normalised against three housekeeping genes. A, B, C are relative expression of stimulated samples verses their unstimulated samples for each diet. D represents the fold change of each diet calculated from the relative expression. [a] and [b] denote significant difference where $p < 0.05$. Significance was identified using an Anova followed by a Tukey post hoc test.

response [28]. Multiple subsets of $CD4^+$ T-cells have been characterised in teleost fish and are the dominant subtype of T-cells in the lamina propria [29]. Levels of *sIgM* transcripts were also observed to be higher in the fish fed the β-glucan supplemented diet. IgM secreting B-cells are the prominent B-cell subtype in the intestine and as such play a key role in barrier maintenance and mucosal immunity [29]. IgM is responsible for helping maintain homeostasis in the microbiome and is secreted during the first stages of the immune response to infection. IgM is released by B-cells where it can bind to antigens found within the gut microbiome and acts as a marker for phagocyte recruitment, activation of the complement pathways and destroy antigens through opsonisation [30,31]. Supplementation with β-glucans has previously shown to cause increased expression of IgM in serum in rainbow trout [8] before infection and also increased expression in head kidney RNA in gilthead sea bream (*Sparus aurata*) [32] after one week of being fed β-glucan supplemented diets. Further studies may include the use of immunoglobulin D to identify specific B-cell responses to functional diets.

GALT leucocytes are a useful tool in identifying specific localised responses of the distal intestine immune cells between the host and external factors [33]. Isolated GALT leucocytes gene expression was examined after 4hrs with no stimulation to ensure cells were healthy and the responses observed were not just due to the extraction method. The GALT leucocytes extracted from fish fed the Protec™ diet had higher expression levels of the proinflammatory cytokines *IL-1β*, *SAA*, *HAMP*, and *TNFα* after 6 weeks of feeding compared to fish fed both the control and β-glucan diets potentially confirming the immunostimulatory nature of β-glucans, which is in agreement in previous research [8,9,12,34]. However, further research is needed to identify if these higher expression levels were directly due to β-glucans or other components within the Protec™ diet. Regarding the modulation of anti-viral genes in the GALT following the feeding trial we find there is minimal impact on these genes with only a non-significant increase in relative expression of *MX* transcripts observed in both the β-glucan and Protec™, suggesting neither the β-glucans nor the Protec™ diets are not triggering an anti-viral response. Viral responses also showed similar responses to previous research [8] with *MX* showing no significant difference between control and Protec™ diets with all other genes tested showing similar responses across all diets.

In order to examine the if the different diets impacted the capacity of the GALT cells to responds to immunological stimulation a panel of stimulants (rIl-1β or Poly I:C) known to drive proinflammatory or anti-viral responses were as previously described to work well on GALT cells from rainbow trout [16]. All cells no matter which diet the fish had been fed responded in the predicted way to the stimulants after 4hrs. For the anti-viral activity driven by poly I:C the only significant difference in response between diets was for *PSMB9* [35] which encodes a proteasome subunit involved in antigen presentation and known to be increased under type 1 interferon stimulation [36]. The increased magnitude of response of this gene may suggest an accelerated response to the poly I:C in the GALT cells isolated from the fish fed the Protec™ diet, which could improve the response to viral pathogens as shown by Leal et al [8]. Following the exposure to rIL-1β, there was a strong increase in the three cytokines examine at 4hrs. TNFα was the only gene that was found to respond differently depending on the diet the fish had been fed prior to GALT extraction and stimulation, where we found that the fish fed the Protec™ diet showed significantly increased expression of this gene. TNFα is a key proinflammatory cytokine and like *Il-1β* drives the proinflammatory response [37]. The protein is secreted by

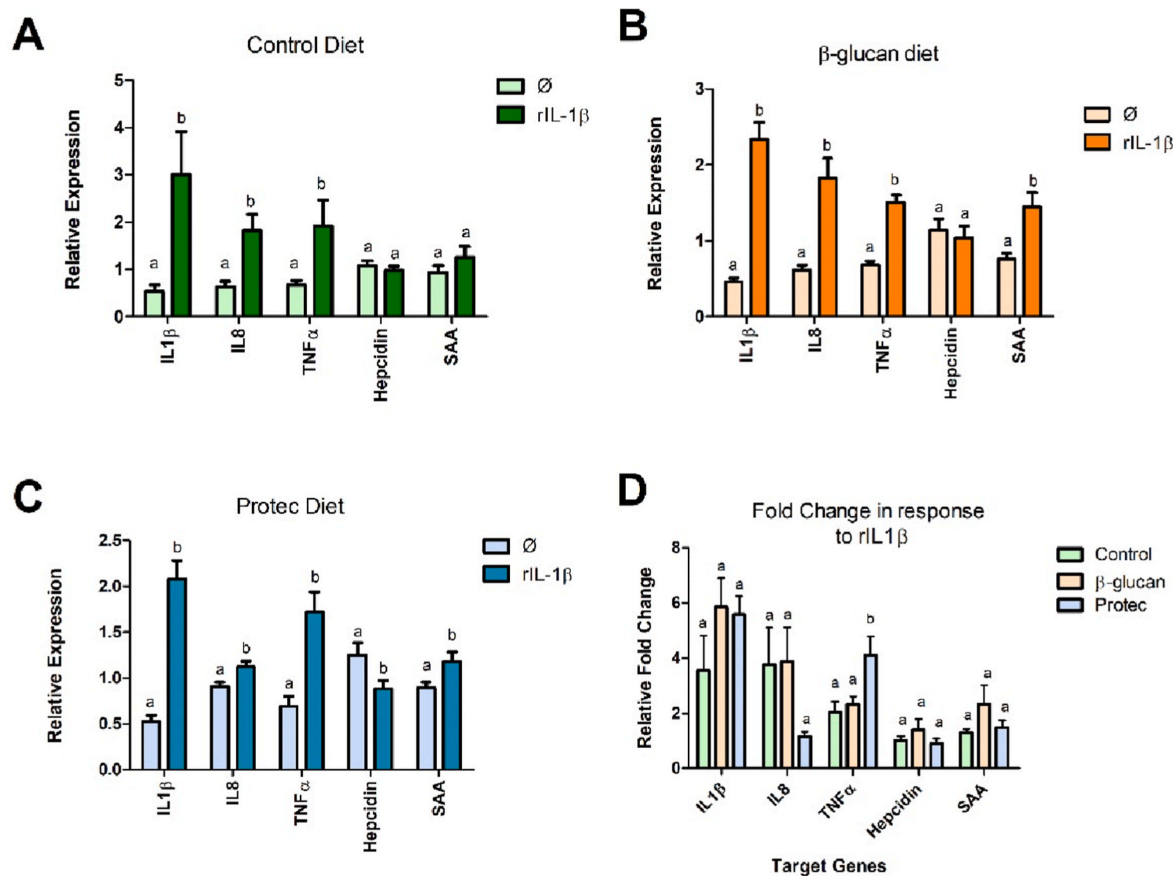


**Fig. 7.** Response to rIL-1β. All results were quantified by qPCR. qPCR results were normalised against three housekeeping genes. A, B, C are relative expression of stimulated samples verses their unstimulated samples for each diet. D represents the fold change of each diet calculated from the relative expression. [a] and [b] denote significant difference where $p < 0.05$. Significance was identified using an Anova followed by a Tukey post hoc test.

macrophages and monocytes [38,39]. This increased expression in response to the proinflammatory stimulus would suggest the fish being fed the Protec™ diet have an accelerated response and may increase their capacity to deal with immunological insults in the intestine quicker than fish fed the β-glucan or control diet. TNFα was also found to be expressed at significantly higher levels in the distal intestine.

To understand how the changes in magnitude of these immune genes (seen to be upregulated in the GALT leucocytes) may impact the immune response, fish were challenged with *A. salmonicida* for 48hrs. *A. salmonicida* was chosen due to the known effects of β-glucan on the proinflammatory response [40] and has previously shown to improve survival *A. salmonicida* with higher levels of inclusion into the diet. However, in future studies a titrated dose may provide a greater resolution in teasing out key changes in datasets when exploring the immune modulatory capacity of feed additives. Our results show that in RNA extracted from head kidney the key antimicrobial genes *SAA* and HAMP show a lower magnitude of response to *A. salmonicida* infection in the fish fed the β-glucan and Protec™ diets compared to those fed control diet. A similar result was also observed in the distal intestine for *HAMP* whilst *SAA* was conversely affected. These results suggest that fish are offered some protection due to these immunostimulatory molecules found in the diets, due to their innate immune response already being modulated in PBS-injected fish. *IL-1β* was observed to be upregulated in all diets and in both the head kidney and distal intestine showing similar levels of relative expression in both the head kidney and distal intestine after infection with *A. salmonicida*. *IL-1β* has been previously demonstrated to be modulated by supplementation with β-glucans [3,7,9,40]. Genes relating to mucins, which are likely to be involved in mucus production [41] were observed to show a much lower magnitude of response in the Protec™ fed fish, however this is likely due to these genes being expressed at much higher levels in PBS treated samples. Significantly higher numbers of goblet cells as observed in the histology which may offer increased mucosal immunological activity. *CLEC4T* has a lower magnitude of response in the Protec™ diets after infection, however, this gene was expressed at much higher levels in PBS-treated samples compared to the control and β-glucan diets. C-type lectins have previously been identified in carp as potential receptors for β-glucans in carp [4] and have been linked to upregulation of the C-type lectin signalling pathways. Relative expression of CLEC4T in *A. salmonicida* infection suggested there was significant upregulation of this gene in both the control and β-glucan diets, generating similar relative expression as observed in the Protec™ diets, indicating the C-type lectin pathways is also triggered by *A. salmonicida* infection. Secretory immunoglobulin M had significantly higher relative expression compared to fish fed both the control and β-glucan samples in PBS treated samples which has previously been shown to be increased in the Protec™ diet [8]. To identify potential benefits for in terms of disease resistance and immunity a mortality trial would need to be carried out.

Research into gut immune function is limited in fish, with responses unique to individual species [42] more research is needed to identify exactly how functional feeds modulate the gut and subsequently cause a systemic immune response in each species. Traditionally, key immune organs such as the spleen and the head kidney are the focus of immune challenges due to their immunological importance in fish. However, the use of GALT leucocytes in this experiment has elucidated that GALT is able to mount a sizeable immune response to stimulation and this response can be modulated by diet and as such may be a useful tool to measure immune response against pathogens. One method to reduce

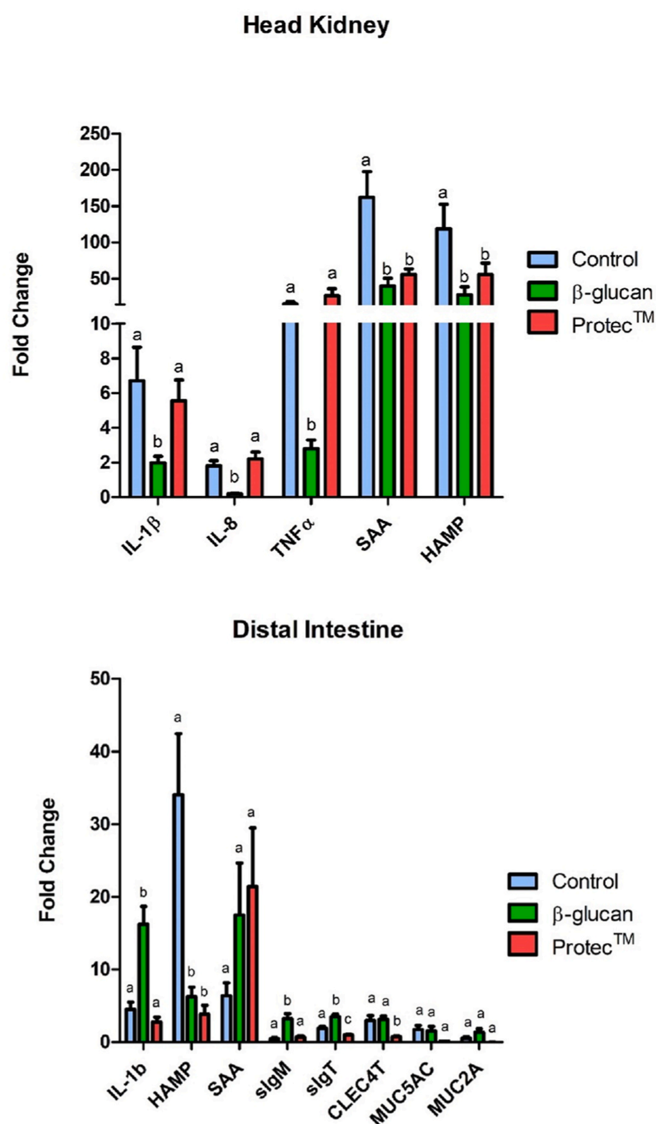


**Fig. 8.** Immune challenge with A. salmonicida for the head kidney and distal intestine. All results were quantified by qPCR. qPCR results were normalised against three housekeeping genes. Graphs show fold change relative to samples stimulated with PBS for each diet. [a], [b], and [c] denote significant difference where $p < 0.05$. Significance was identified using an Anova followed by a Tukey post hoc test.

economic loss due to disease is to train immunity through the modulation of GALT tissue and may provide a viable alternative to allow for the reduction in the use of antibiotics in aquaculture. Previous research by Petit et al [43] has already demonstrated that macrophages can be trained to protect against bacterial stimuli *in-vitro* through the use of immunostimulants (β-glucans and peptidoglycans) however further research would be needed to confirm that the same would be true *in-vivo*. Research into zebrafish injected with β-glucans, revealed short lived protection against spring viremia of carp virus (*SVHV*) after 7 days but not after 35 days post infection [44]. The data presented demonstrates the potential of functional diets and more studies are required to understand how gut immunity can be modulated to better protect against infection.

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## CRediT authorship contribution statement

**D. Porter:** Methodology, Validation, Formal analysis, Investigation, Writing – original draft. **D. Peggs:** Conceptualization, Writing – review & editing, Supervision. **C. McGurk:** Conceptualization, Writing – review & editing, Supervision. **S.A.M. Martin:** Conceptualization, Methodology, Investigation, Writing – review & editing, Supervision.

## Declaration of competing interest

**C. McGurk** and **D. Peggs** are employees of Skretting AI.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The authors declare that this study received funding from Skretting AI. The funder had the following involvement in the study: review of manuscript and provision of experimental and control diets.

## Data availability

No data was used for the research described in the article.

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