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# Immune responses to prebiotics in farmed salmonid fish: How transcriptomic approaches help interpret responses

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ABSTRACT

Within aquaculture, prebiotics are composed of complex carbohydrate molecules that cannot be digested by the fish directly but are metabolised by the microbial communities within the host gut, with the desire that "healthy" bacterial species are promoted with subsequently improved performance of the fish, there are likely some direct responses of intestinal cells to these dietary components. The sources and processing of prebiotics, which fall under the overarching theme of "functional feeds" are highly varied between species and types of prebiotics administered. How these feeds exert their effect, and the host responses are hard to determine, but new technologies and the development of high-throughput technologies (omics) are enabling the mechanisms and methods of action to be further understood. The recent advances in the availability of '*omics*' technologies with the transition from single gene assays to microarray and RNA-seq in fish health have enabled novel functional ingredients to be analysed. This review will focus on recent studies on targeted gene expression and '*omics*' technologies to characterize immune responses. Comparisons between the immunomodulatory effect of different prebiotics have been made and specific examples of how transcriptomics techniques have been used to identify immune responses to prebiotics are given.

## 1. Introduction

The role of food and nutrition is recognised as a key factor in health and disease prevention through the maintenance of a fully functioning intestinal system and associated local and systemic immune system. This is especially true for aquaculture where health management is a central theme of a sustainable and economic industry. In recent decades, the development of fish feeds has shown dramatic changes, with diets being designed to meet the basal nutritional requirements of the fish but also aid in the prevention of diseases [1,2]. Globally 598 species are commercially farmed in aquaculture, with 379 species of finfish, some of which are new to aquaculture for which nutritional requirements are relatively unknown with diets for these being constructed on limited knowledge [3,4]. The rapid growth of the aquaculture industry, globally 5.8% per year from 2000 to 2016 [3], has required new protein and oil sources to be identified and used, with many aquaculture diets having changed for almost 90% marine sourced protein and oils to currently 12–15% marine derived ingredients [5,6]. Many farmed teleosts such as Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*) are carnivorous and naturally have a diet high in marine proteins and oils, but availability and costs of marine ingredients have driven changes to terrestrial rich feeds which began during the late 1990s and have continued to the present day [7,8]. These diets bring a new set of challenges for fish physiology and health. Plant proteins are often deficient in essential amino acids (lysine and methionine), with this shortfall being compensated for by using mixtures of plant protein concentrates and crystalline amino acids [9,10]. The essential marine fatty acids: eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are not found in vegetable oils and lead to further unknown impacts on fish health [11]. Other important issues with plant rich diets are reduced palatability and the presence of antinutritional factors [12].

*Abbreviations:* DEG, Differentially expressed genes; DHA, Docosahexaenoic acid; EPA, Eicosapentaenoic acid; FOS, Fructose oligosaccharides; GALT, Gut-associated lymphoid tissues; GL, Glucosinolates; GOS, Galactooligosaccharides; KEGG, Kyoto Encyclopaedia of Genes and Genomes; MOS, Mannanoligosaccharides; MALT, Mucosa-associated lymphoid tissues; PAMP, Pathogen associated molecular pattern; PRR, Pathogen recognition receptor; SCFA, Short chain fatty acids; qPCR, quantitative polymerase chain reaction.

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Although these new diets match the requirements for farmed fish, the mechanisms by which these impact on health and immune capacity remains poorly understood, especially in relation to plant based ingredients, as such there is a need for further research into the nutrition health interface for aquaculture species. There are new questions regarding intestinal health, and how these diets can impact systemic responses and disease resistance.

### 1.1. Functional feeds

To improve fish feed further, additional factors can be added to the feeds that can be beneficial to fish health, both to improve physiology and growth, and to enhance the fish's immune capacity and disease resistance. Optimal gut health and function are central to overall fish welfare, as such these functional feed additives must provide additional benefits beyond direct nutritional requirements [13,14]. Functional feeds are foods that *deliver additional or enhanced benefits over and above their basic nutritional value* and normally include prebiotics, probiotics, micronutrients, and immunostimulants that are used to enrich the feed, tailored to a specific issue or life stage. Prebiotics are nondigestible carbohydrates such as mannanoligosaccharides (MOS), fructose oligosaccharides (FOS), and β-glucans extracted from microbes and yeast cell wall [15]. These prebiotics are used to promote the development of a healthy intestinal microbiome, promoting beneficial bacterial species, and preventing pathogenic and harmful bacteria from becoming established. There may also be direct effects on the intestinal epithelium and immune responses [16]. For example, MOS has a range of effects: immune modulator, blocking pathogen colonisation, and improvement of intestinal morphology [17]. Probiotic diets contain living microbes (probiotic organisms) such as *Lactobacillus buchneri* which are regarded as desirable intestinal bacterial [18]. Probiotics have also been shown to aid in the prevention of diseases and stress, improve water quality and enhance immunity although the mode of action is still scarcely known [19]. Functional feeds may also contain specific metabolites (nucleotides), or specific micronutrients (such as Zinc and Selenium). Nucleotides have an important role in normal intestinal functions including growth, metabolism, immune function, and tissue repair [20]. Dietary nucleotides have been shown to increase the efficacy of alternative protein utilization especially during stressful conditions where the *de novo* pathway of nucleotide synthesis becomes limiting [21]. The addition of minerals such as Zinc and Selenium can have beneficial effects on viral responses in fish as they act as cofactors for many important enzymes [22,23]. In aquaculture functional feeds are usually administered for a short period (weeks rather than months), which usually coincides with a stressful event such as sea water transfer in Atlantic salmon, coinciding with vaccination. Functional feed additives are believed to exert their actions locally upon the gut barrier, microbiome, immune and some metabolic functions [16].

Disease outbreaks in aquaculture often occur when fish are stressed, suggesting a link between hormonal and nutrient status in host defences, additionally recovery from infection is also highly variable with fish being able to clear pathogens at different rates. Functional feeds can be used at key life-history stages to improve health status. Essential aquaculture management procedures: such as handling, transport of fish, transfer of salmon to saltwater cages and other physical treatments can lead to higher mortality rates [24]. These events are likely to increase stress, respiratory processes, and impact immune function [25]. These changes in the physiological processes are likely to impair resistance to pathogens and any improvement that can be added by functional feeds can reduce not only mortalities but reduce recovery time and use of chemotherapeutic treatments [26]. The ability of fish's immune system to protect against disease and cope with stress is dependent on their nutritional status.

### 1.2. Teleost immune system

The teleost immune system is split into two distinct components: the innate immune system and the adaptive immune system. The innate immune system acts as the first line of defence against pathogens and is comprised of physical barriers as well as cellular and humoral responses. The adaptive immune system is dependent upon cellular and humoral responses but is characterised by specific antigen recognition that drives a secondary pathogenic-specific response and immune memory [27]. Whilst they act in different ways, the adaptive and innate immune systems interact heavily with each other through the cross talk between cells and molecules involved in the immune response. Human studies show nutrition has a major role in well-functioning innate and adaptive responses with negative outcomes during nutritional deficiencies [28]. These deficiencies lead to modulations in cell-mediated immunity, phagocyte function, the complement system, cytokine production, mucosal secretory antibody response, and affinity.

Like higher vertebrates (tetrapods), fish have a set of lymphoid organs with the main haemopoietic tissues being the head kidney. The head kidney, thymus, and spleen are the key immune organs in fish, but of great importance in fish are the mucosa-associated lymphoid tissues (MALTs) which have large populations of leucocytes present in these tissues and are highly immunologically active tissues [29]. There are four different types of MALTs in fish: skin, gills, gut, and the nasopharynx.

### 1.3. Gut-associated lymphoid tissue

The gut-associated lymphoid tissue (GALT) is central to how fish respond to the direct and indirect (via microbial communities) effects of functional feeds. The gut is constantly exposed to the external environment, through the uptake of food, and as such GALT is a fundamental interface between the host and the environment [30,31]. GALT is a key component of the fish immune defences and can interfere with pathogens by acting as a physical barrier or through the presence of antimicrobial peptides and antibodies in the mucus [32,33]. The interface consists of a rich and diverse microbial community that is maintained in homeostasis moderated by immunological surveillance. Mucosal B cells play a key role in this homeostasis through secreting immunoglobulins [34]. The gut is rich in both passive beneficial microflora and pathogens; it is coated in a layer of mucus that separates the epithelial cells and the microflora. The mucosal surface is a major surface barrier containing a large variety of secretory immunoglobulins, leucocytes, antimicrobial peptides, and other innate immune components [35]. These leucocytes and dendritic cells present in the mucosal surface can identify immunostimulatory molecules from prebiotics or their metabolites; short chain fatty acids (SCFAs), binding to them at pathogen associated molecular patterns and present these antigens to trigger further immune responses.

The epithelial layer of cells that lie underneath the mucus includes epithelial cells, goblet cells, endocrine cells, and the immune cells which form a monolayer [36]. At an immunological level GALT is composed of two main populations: (1) Lamina Propria Leucocytes (LPL), which includes resident granulocytes, lymphocytes, macrophages (M1 and M2), plasma cells, T cells ($CD4^+$) and B cells (IgT+ and IgM+), (2) Intraepithelial leucocytes (IEL), which include T cells ($CD8^+$) and B cells (IgT+ and IgM+) located among the epithelial cells [37,38]. M1 and M2 macrophages play important roles in the innate immune response with M1 detecting PAMPs whilst M2 are triggered by Th2 cytokines and have roles in anti-inflammatory pathways [39]. IgT + B cells are the dominant phenotype in the gut and are thought to play a key role in pathogen recognition within the intestine [34]. The presence of $CD4^+$ cells in the LPL are regulated by mucosal dendritic cells and microbiota signalling which can cause these cells to turn into T-regulatory cells. Treg and Th17 cells have important roles in mucosal immunity with inflammatory and anti-inflammatory pathways being regulated [40]. Alongside

**Table 1**
Fish studies using transcriptomic technologies to characterize immunological responses to β-glucans.

| Fish Species | Omics Technology | Dietary Manipulation | Source/ Conformity | Disease Challenge | Comparison and Sampling | Tissue Analysed | Main findings | Reference |
|---|---|---|---|---|---|---|---|---|
| Rainbow Trout (*Oncorhynchus mykiss*) | qPCR | Intraperitoneal injection with β-glucans and LPS | Laminaran, Pronova, Norway β 1,3 d-glucan | - | *In vivo* stimulation with 20 mg/kg β-glucan or 6 mg/kg LPS dissolved in PBS. After 48hrs fish were bled and samples collected. *In-vitro* stimulation of head kidney macrophages stimulated with 10 mg/kg of LPS or β-glucans | Head kidney, spleen, and liver | *IL1β1, IL1β2, IL6, C3-1, C3-2* ↑. *C3-2, C3-3*↓ | [99] |
| Gilthead Sea Bream (*Sparus aurata*) | qPCR | Supplementation with 1,3/1,6 β-glucans and *Shewanella putrefaciens* | Laminarina digitata, Sigma, β 1,3/1,6 glucans | - | Control vs β-glucan (0.1%) vs pdp11 ($10^9$ cfu) vs β-glucans (0.1%) + pdp11 ($10^9$ cfu) for four weeks | Head kidney | ↑ IgM for all diets after 1 week. Upregulation of *IFNγ* and *IL1β* in weeks 1 and 4 but only β-glucans week 4 was significant | [73] |
| Rainbow Trout *(Oncorhynchus mykiss)* | qPCR | Supplementation with β-glucans | Angel Yeast Co., China β 1,3 glucan | *Aeromonas salmonicida* | Control vs 0.05% β-glucans, 0.1% β-glucans, 0.2% β-glucans for 6 weeks | Head kidney | Survival rates increased significantly in those diets containing β-glucans. T-SOD, POD, CAT, LYZ activities and RNA expression were increased to higher levels and reached them quicker in the β-glucan diets compared to the control. 0.2% provided the optimal concentration. ↑*HSP70* in the infected with β-glucans. | [74] |
| Rainbow Trout (*Oncorhynchus mykiss*) | qPCR | Supplementation with Macrogard (β-glucans) | Macrogard – β 1,3/ 1,6 glucans | *Aeromonas hydrophilia* | Control vs 0.1% MG vs 0.2%MG vs 0.5% MG for 5 weeks | Gills, spleen, kidney | Spleen showed changes in gene expression in both healthy and bacterial challenged fish: MG02 diet showed ↑ *TNFα-1, IL-1β, COX-2 and TGFβ* after 15 and 30 days and ↑ *IL-10* after 15 days.↑ *IL-1β & TGF-β* in MG05 diets. Only occasional effects were noticed in gill and kidney. | [70] |
| Zebrafish (*Danio rerio)* | qPCR | Supplementation with β-glucans | Zymozan, Sigma β 1,3 glucan | *SVCV* | Control vs stimulation with SVCV infection vs SVCV + β-glucans (10 μg/ml 24hr prior to infection) | Zebrafish fibroblast (ZF4) Cells and in-vivo experiments on mortality | Enhanced response to SVCV (higher responsiveness and protection), ↑ IFN related genes: *gig2, tlr2, ifnphi1* and the inflammatory cytokines: il1b, il6, il8, il10 and tnfa transcripts which were observed after 48hrs but declined over 2 weeks. | [94] |
| Rainbow Trout *(Oncorhynchus mykiss)* | qPCR | Supplementation with β-glucans | Angel Yeast Co., China β 1,3 glucan | *Aeromonas salmonicida* | Control vs 0.05% β-glucans, 0.1% β-glucans, 0.2% β-glucans for 6 weeks | Head kidney or spleen | Survival rates were significantly higher in 0.1% and 0.2% 6 dpi. Head kidney: ↑*tnfα, il1b, ighm (after 42 days of administration), cxcl8 and ighm (Upregulated in response to the infection 0.2% significantly ↑ by 6 dpi).* ↑*oncymkdab, C3, tmek and myd88 post infection.* Spleen: ↑ *tlrm5, tlr5s, tmek, myd88* (All increased with β-glucan administration, further increased after infection) | [71] |
| Rainbow Trout (*Oncorhynchus mykiss*) | qPCR | Supplementation with Protec™ (β-glucans, Vitamin C, Vitamin E and zinc) | Skretting | *VHSV* | Control vs Protec for 5 weeks | Kidney, gut, and gills | ↑*IgM, IgT, IgD, MX and IFN-γ* in fish fed the Protec diet 6dpi with VHSV. IgM, IgT and *IgD* were all increased before infection. | [69] |
| Common Carp | RNA-seq | Stimulated with 25 μg/ ml of Curdlan or Macrogard | Curdlan, C7821, Sigma Aldrich β 1,3 glucan, Macrogard, Zilor β 1,3/1,6 glucans | - | Control vs Curdlan vs Macrogard | Head kidney macrophages | RNA sequencing showed 528 DEGs with Curdlan and 781 DEGs using Macrogard of which 85% and 80% were upregulated, respectively. CLR pathway activation/ upregulation of downstream targets. *CLEC4C* and *SCLRA* represent the best genes for future studies as potential β-glucan receptors. | [67] |

(*continued on next page*)

**Table 1** (*continued*)

| Fish Species | Omics Technology | Dietary Manipulation | Source/ Conformity | Disease Challenge | Comparison and Sampling | Tissue Analysed | Main findings | Reference |
|---|---|---|---|---|---|---|---|---|
| Caspian Trout *(Salmo trutta caspius)* | qPCR | Supplementation with 3 g/kg β-glucans, 4 g/kg MOS and *Lactobacillus plantarum* | Angel Yeast Co., China β 1,3 glucan | - | Basal diet vs basal diet +β-glucan vs basal diet + MOS vs basal diet + L. plantarum vs basal diet +β-glucan + L. plantarum vs basal diet + MOS + L. plantarum vs basal diet + MOS +β-glucan vs basal diet + MOS +β-glucan + L. plantarum over 8 weeks | Head kidney | *TNFa 1, IL1β and IL8* ↑ *IL1β* greatest increase by 8.75-fold in fish supplemented with only β-glucans. bβLp and bβ significantly enhanced the relative *IL*-8 mRNA expression in the head kidney ↑2.75 and 1.9-fold, respectively. | [72] |
| Rainbow Trout (*Oncorhynchus mykiss*) | RNA-seq | Supplementation with 0.2% β-glucans | Angel Yeast Co., China β 1,3 glucan | *Aeromonas salmonicida* | Control vs 0.2% β-glucans | Spleen | In CG vs BG, 378 and 406 DEG were identified on 4 and 6 dpi respectively. 46 DEGs were common enriching in GO terms: compliment activation, inflammatory response, and metabolic process. Kegg analysis revealed DEGs were involved in immune or metabolic signalling pathways such as complement and coagulation cascades, NFκB, TLR signalling pathways, antigen processing and presentation and platelet activation. 15 random DEGs such as *fgg, fgb, f5, c9, c3, c5, tlr5*, and *myd88*, were analysed using qPCR in CG vs BG on 4 dpi and 6 dpi with a correlation coefficient of 0.93 | [77] |
| Rainbow Trout (*Oncorhynchus mykiss*) | qPCR | Stimulated with 50 μg/ml of soluble β-glucans (SBG) or particulate β-glucans (M) | Soluble β-glucan (95% purity), particulate β-glucan (60% purity) Biotec BetaGlucans AS (Norway) | *Aeromonas salmonicida* subsp. *salmonicida CECT4237* | Control vs SBG vs M/Control vs SBG + A. salmonicida vs M + A. salmonicida/ tolerance tests using multiple exposure of M or LPS | RTS11 and RTgutGC cells | RTS11 cells were more responsive than RTgutGC to β-glucans. M glucans was able to cause ↑ of *TNFα, IL6, IL8 & IL1β* in RTS11 at all concentrations, only *IL6, IL8* and *IL1β* were upregulated in the highest concentration in RTgutGC. SBG didn't have an effect on RTS11 or RTgutGC. Supplementation of β-glucans and inactivated *A.salmonicida* caused a larger response than those treated with just *A. salmonicida.* Stimulation with M β-glucans had only minor tolerance effects against *A. Salmonicida.* | [95] |

these immune cells, epithelial cells, goblet cells, and endocrine cells work together to produce and regulate gut immune responses [41]. GALT is the first immunological component that is exposed to the functional feed components, and the microbial changes associated with the feed additives, with potential direct impacts on the lamina propria leucocytes and the intraepithelial leucocytes.

### 1.4. Nutrigenomic approaches for aquaculture

Functional feeds impact the local and systemic immune system, but the different processes that this involves are not resolved. Nutrigenomics integrates nutritional sciences and genomics; incorporating the application of high throughput technologies (*Omics*) such as transcriptomics, proteomics, and metabolomics to investigate the effects of nutrition on health [42].

Recent advances of high throughput sequencing of farmed fish species have allowed the development of transcriptomics and proteomics to be used in fish nutrition studies [40]. Microarrays were developed for many farmed fish species including Atlantic salmon [43–45] and rainbow trout [46] that used available cDNA sequences, and although new insights to the transcriptional responses were generated, they were constrained by genes that were present on the array. In recent years with many more whole genomes now completed for farmed fish whole transcriptome analysis by RNA-seq is becoming the method of choice for transcriptomic experiments in many labs. The whole genome of over 198 assembled species of fish is now present on NCBI and is assembled at chromosome level which includes most aquaculture fish species [47] with a further 800 available currently as scaffolds or contigs. This massive increase in genomic resources facilitates the study of complex interactions involved in the biological processes that regulate fish health, nutritional responses, host genetics, and symbiotic microbiota [48]. To understand the mechanisms fully, a multifactorial approach needs to be used that considers a variety of "omic" approaches including transcriptomics and proteomics, providing a much clearer picture of both the local and systemic effects of supplementation with different additives [49].

Historically feeding trials have been used as the primary method for the testing of novel aquafeeds with whole tissue histology, performance-based metrics, and transcriptomics/proteomics. To complement whole fish/tissue examination there are benefits of using either primary cultures of cells or established cell lines to examine the effects of functional feed ingredients directly on distinct cell types. Such approaches enable a deeper understanding of molecular mechanisms on cellular function [50]. There are several cell lines developed for salmonid fish, including intestinal derived (RTgutGC) [51], gill (RTgill-W1) [52], and the monocyte/macrophage cell line RTS11 [53], the use of fish cell lines is reviewed by Collet, Collins, and Lester, [54]. However, the phenotype of these cells is likely to have changed considerably from their original tissues and the choice of cell lines needs consideration for specific experiments, even the RTgutGC cells are unlikely to reflect the complex nature of the intestine with multiple cells phenotypes present. Primary cell culture models are also under development and when appropriate may be used alongside feeding trials and give a better reflection of responses due to multiple cell types and if the cell phenotype has been impacted by dietary components. The primary cells may be used as an assay to test the effect of functional feeds on cells before testing on fish [55–57]. These models would characterize the response of specific cell types whilst tissues of interest can also be taken to understand the systemic response to different stimuli.

This review will highlight the use of high-throughput technologies and targeted gene expression to investigate the mechanisms underlying the use of prebiotics and their effects on the immune response in aquaculture. The use of transcriptomics in functional feed studies enables specific pathways that are being modulated to be identified and subsequently, the effectiveness of different additives can be illustrated.

## 2. Targeted gene expression and transcriptomic responses to prebiotic feeds

The method by which prebiotics exert beneficial changes on the host immune system as a result many studies have used targeted gene expression (qPCR) or transcriptomics to identify this mechanism. Prebiotics are digested by bacteria resulting in increases in the levels of short-chain fatty acids (SCFAs) or immunostimulatory molecules. Bacteria such as *Bifidobacterium* and *Lactobacillus* are often termed beneficial bacteria and are targets for enrichment by functional feeds [58]. The increase in beneficial bacteria enables them to outcompete pathogenic bacteria [59]. Additionally, some prebiotics can act as immunostimulants through binding to host cellular receptors in the epithelium and induce an immune response directly, leading to an increase in the inflammatory response or complement pathways [60].

### 2.1. Molecular responses of the host caused by the supplementation of β-glucans

β-glucans are naturally occurring polysaccharides that are found in the cell walls of certain plants, and fungi [61]. β-glucans are polymers of repeating units of glucose linked by glycosidic bonds and are characterised as being branched or unbranched, long, or short, and insoluble or soluble [62]. β-glucans have been extensively used in aquaculture feeds as they induce both the cellular and humoral immune responses in intestinal epithelial tissues [63,64]. β-glucans modulate immune function through increased respiratory burst, lysozyme action, activation of leucocytes, and stimulate inflammatory mediators and promote both antibacterial and antiviral activity in fish [61,65,66]. Irrespective of the administration route, either through diet, injections, or immersion, β-glucan administration generates a short-lived immune stimulatory effect and can result in increased resistance to both viral and bacterial infections [67]. The direct effects of β-glucans are thought to act as pathogen-associated molecular patterns (PAMPs) and are recognised by pattern recognition receptors (PRRs) present on macrophages [66]. Examination of β-glucan interaction with immune cells suggests toll-like receptor 2 (TLR2) may be involved in sensing as well as through a C type lectin receptor homolog of the mammalian Dectin-1 [67,68]. The binding of the dectin-1 homolog to β-glucans is thought to lead to the activation of NF-κB leading to the inflammatory profile seen when stimulated by β-glucans. β-glucans are being used in commercial diets alongside other vitamins and minerals as part of a combination of different additives to stimulate fish health, these diets being now widely used especially during periods of stress or potential infection and recovery.

Numerous studies have used targeted gene expression to identify the response to β-glucans. Functional feeds such as the Protec™ diet (Skretting) contain several nutritional supplements; β-glucans, vitamin E, vitamin C and zinc, and have been shown to stimulate the immune response in rainbow trout where fish fed this diet had increased immunoglobulin levels (*IgT, IgM, IgD*) before infection with viral haemorrhagic septicaemia virus [69] (Table 1). Six days post-infection fish fed the functional feed diet showed increased survival rates and upregulation of both immunoglobulins and anti-viral immune genes: *IgM, IgT, IgD, MX, and IFN-γ* as determined by real-time PCR in comparison to those fish fed the control diet.

Rainbow trout juveniles fed a commercial Macrogard® (Biorigin) supplemented feed showed increased transcription of proinflammatory cytokines (*TNFα-1, IL-1β, COX-2,* and *TGFβ)* after 15 and 30 days and anti-inflammatory cytokine *IL-10* after 15 days in head kidney and gill [70] in the spleen of both healthy and fish challenged with *A. hydrophilia* (Table 1). With the response varying between the inclusion of the supplementation (1 and 2%). The effects of β-glucans in the spleen were also seen in all three diets, with 2% supplementation giving the best results, 1% supplementation only exhibited differential immune response after stimulation with *A. hydrophilia.* A similar response was observed

following a 42-day feeding trial using different levels β-glucan by a study by Ji et al., [71] where immune gene expression following *Aeromonas salmonicida* was increased in those fish fed the β-glucan (Angel Yeast Co., China) at up to 6 days post-infection in the spleen (*tlrm5, tlr5s, tmek, myd88)* and head kidney (Table 1). In this trial, increased survival to pathogen challenge was significantly greater at 0.2% inclusion compared to 0.1%, 0.05%, or a control diet. Similar responses in Caspian Trout (*Salmo trutta*) [72], and in gilthead sea bream [73], also showed improved bacterial survival after infection (Table 1).

The immunostimulatory effects of β-glucans have been demonstrated frequently although the detailed mechanisms of action are not fully understood [61]. Several reports indicate β-glucans enhance transcription of inflammatory cytokines in a variety of fish [70,72–74] (Table 1). The immune response observed can be considered tissue-dependent with the head kidney and spleen showing upregulation of inflammatory cytokines in trout but in carp, the intestines have shown downregulation [70,75]. These differences could reflect tissue-specific responses or other experimental interactions with the microbiome that need further explored.

Whole transcriptome analysis for the effects of β-glucans on fish was carried out using an early microarray platform containing 1800 defined genes from salmonid fish (SFA2.0 immunochip) [76] where rainbow trout were fed a diet enriched with lentinan (a β-glucan from mushroom) followed by an immune challenge by injection of LPS to drive a proinflammatory response. Those fish fed the β-glucan diet showed a reduced number inflammatory response compared to fish fed a control diet, which may have helped prevent tissue damage, but general responses of immune genes showed that fish fed both diets had key defence genes activated, but this paper did show how the examination of large numbers of genes helped interpret the responses to the feeds.

Several more recent studies have used RNA-seq to identify the pathways which β-glucans alter by characterising distinct transcriptomic changes. The two key species that have been examined are the rainbow trout [77] and the Carp [67], where both common responses are observed but also species differences (Table 1). The trout experiment [77] was based on a 0.2% inclusion of β-glucans for 42 days, followed by an *Aeromonas salmonicida* challenge, the spleen being chosen as the key tissue for analysis at 4- and 6-days post-infection (dpi) to examine how the diets impacted the early pathogen response. At 4 dpi and 6 dpi 378 and 594 differentially expressed genes (DEGs) were identified respectively between control and β-glucan fed fish to establish how the dietary treatment impacted the post-infection transcriptional response DEGs were further analysed for enrichment of gene sets for functional analysis by both gene ontology (GO) and KEGG pathway analysis. These showed clear enrichment for processes involved in both immune function and metabolism between the fish on different diets. Complement proteins "complement and coagulation" were clearly increased to a greater extent at 4 dpi between diets but the effect was still observed at 6 dpi. Overall, 4 key pathways were identified by the gene set enrichment that was believed to be driven by β-glucans impacting the immune response, these were the complement and coagulation, PI3K-AKT signalling pathway, platelet activation, and T-cell receptor signalling. Several other immune groups were altered to a different magnitude, but responses were generally in the same direction.

The trout fed β-glucan enriched diet showed higher complement response transcriptionally than those on control diet at both 4 and 6 dpi [77]. The complement pathway is central in the innate immune response to eradicate foreign antigens and promote inflammation. C1q and C3 are involved in the activation of the classical and alternative complement cascades, respectively, and C5, C7, and C9 are involved in producing the membrane attack complex.

Toll-like receptor (*TRL5*) and its related signalling pathway with *myd88* and *relb* also increased in expression in the fish fed β glucan, suggesting β-glucans impact both the TLR and NFκB signalling pathways, possibly through crosstalk between the complement cascade and the TLR signalling pathways [77]. There was also evidence of increased T-cell activation, potentially related to increased binding of antigens to the MHC class I for presentation to T cells or dendritic cells. The impact of the β glucan on these immune pathways indicates dietary modulation is influencing the response to the pathogen, but the experimental model does not indicate if the main effect is on pre-infection changes or how the fish mount their immune response. Of interest, the RNA-seq approach was able to identify other metabolic pathways including the P13k-AKT signalling pathway and the mTOR signalling pathway suggesting changes in protein and energy metabolism. Additionally, apolipoproteins A-I, B-100, and C–I were higher expressed in the β-glucan group following infection confirming a role in cholesterol and fatty acid metabolism during infection. Previous research using microarrays indicated that *Aeromonas salmonicida* infections in Turbot [78] and Salmon [79] also resulted in upregulation of apolipoproteins and the role of these metabolic pathways during inflammation.

To define the mechanisms by which β-glucans directly impact immune cells, carp primary head kidney macrophages were directly stimulated with two different β-glucans followed by whole transcriptome analysis by RNA-seq [67] (Table 1). The two β-glucans used here were curdlan, a polymer made up of 1–3 β-glucan or Macrogard®, and cells were stimulated for 6 h. The transcriptional analysis shows there were 528 DEGs with Curdlan and 781 DEGs using Macrogard® of which 85% and 80% were upregulated, respectively. Analysis using GO enrichment and KEGG analysis showed concordant expression patterns. The DEG datasets annotated to zebrafish KEGG pathways showed 92 and 112 pathways for curdlan and Macrogard® respectively. Four pathways were significantly over-represented in both DEG datasets; cytokine-cytokine receptor interaction, apoptosis, NOD-like receptor signalling pathway, and ECM-receptor interaction, with a further nine unique pathways significantly over-represented in the Macrogard®-DEGs; phagosome, lysosome, herpes simplex infection, Toll-like receptor signalling pathway, VEGF signalling pathway, arachidonic acid metabolism, phosphatidylinositol signalling system, and the adipocytokine signalling pathway. These findings confirmed that β-glucan can regulate the CLR pathway for both curdlan and Macrogard®. C-type lectins are a superfamily of proteins that are expressed on the extracellular matrix or secreted as soluble molecules. C-type lectins can bind to a broad number of ligands including mannose and galactose carbohydrates on both self and non-self and as such play a role in several different cellular processes, homeostasis, immunity, antigen presentation. Furthermore, several DEGs were identified with a C-type lectin domain suggesting that β-glucans may bind to C-type lectins and trigger subsequent signalling for an immunological response. The candidate genes containing a WxH-motif in the CTLD were further examined concerning the abundance of transcript and expression regulation upon stimulation with β-glucans and finally conservation of synteny with the mammalian NK cell receptor cluster. Based upon these criteria the *CLEC4C* and *SCLRA* genes were proposed as targets for further research to identify which receptor binds to β-glucans through sugar-binding assays.

### 2.2. *Molecular responses of the host caused by the supplementation of oligosaccharides*

A second key group of prebiotics used as functional feeds in aquaculture includes the oligosaccharides which include Mannanoligosaccharides (MOS), Fructooligosaccharides (FOS), Galactooligosaccharides (GOS), and inulin as key components. They are complex carbohydrates made from repeating units of sugars and are usually found in yeast *(Saccharomyces cerevisiae)* or plant cell walls [80]. Like β-glucans the direct and indirect effects of the oligosaccharides are far from fully understood but the responses may depend upon the sugars that make up the oligosaccharide.

MOS are composed of glucose and mannose subunits originating from the cell walls in yeast. MOS can be recognised by the host via the mannan-binding receptor, a key pattern recognition receptor in

**Table 2**
Fish studies using transcriptomic technologies to characterize immunological responses to Oligosaccharides.

| Fish Species | Omics Technology | Dietary Manipulation | Source | Disease Challenge | Comparison and Sampling | Tissue Analysed | Main findings | Reference |
|---|---|---|---|---|---|---|---|---|
| Atlantic salmon *(Salmo salar)* | Microarray | Supplementation with several additives: including nucleotides, mannooligosaccharides, fructooliogsaccharides, vitamin C and vitamin E | BioMar, UK | - | Control diet vs Fish supplemented over 16 weeks | Liver and muscle | Supplemented diet reduced expression of genes encoding proteins involved in adaptive and innate immune responses | [13] |
| Rainbow trout *(Oncorhynchus mykiss)* | qPCR | Supplementation with immunogen | ICC Co, USA | *Aeromonas hydrophila* | Control vs 2 g/kg Immunogen supplementation for 5 weeks | Head kidneys | ↑ *TNFα, lysozyme* ↓*HSP70*<br>↑ survival to *A.hydrophila* with immunogen supplementation (64.44% vs 24.44%) | [100] |
| European sea bass (*Dicentrarchus labrax)* | qPCR | Supplementation with cMOS | Actigen®, Alltech, Inc., USA | - | Control vs 1.6 g/kg cMOS supplementation for 8 weeks | Liver and posterior intestine | *Ig, MHC-II, TCRβ* and *caspase 3 (*↑*)*<br>*COX2, CD4*$^{+}$*, CD8α*+*, MHC-I, Caspase 9, IL10, IL1β, IL8, IL6 and TNFα (*↔*)*<br>*TGFβ (*↓*).* | [84] |
| Rainbow trout *(Oncorhynchus mykiss)* | RNA-seq | Supplementation with MOS and *Saccharomyces cerevisiae* | Ewos® | - | Control vs 0.6% MOS vs 0.5% *S. Cerevisiae* vs 0.6% MOS +0.5% *S. Cerevisiae* | Anterior and posterior intestine | Fish fed MOS, ↑ of genes related to mucosa structure and stability and ECM functioning. ↓ of some immune related transcripts, perforin precursor and immunoglobulin lambda chain. ↑ of some complement genes and other innate chemokines, *Component factor D, and IL-6* signal transducer. | [87] |
| Zebrafish (*Danio rerio)* | qPCR | Supplementation with varying concentrations of galactooligosaccharide (GOS) | Vivinal-GOS®, Friesland foods Domo Company, The Netherlands | - | Control vs 0.5%, 1%, 2% GOS supplementation for 8 weeks | Whole body with heads or fins | 1 and 2% GOS supplementation caused increase in total immunoglobulin levels. IL1β was not affected by supplementation. *TNFa* ↑ in 0.5 and 1%. *Lyz* ↑ in all diets | [101] |
| Rainbow trout (*Oncorhynchus mykiss)* | qPCR | Supplementation with MOS, β-glucans, nucleotides | Active MOS extracted from yeast, Biorigin, São Paulo, Brazil/Beta-glucans, G5011, Sigma, Norway | LPS | RTgutGC cell line was stimulated with 1 mg/ml LPS, 10 mg/ml nucleotides, 20 mg/ml MOS and 2 mg/ml β-glucans | RTgutGC cell line | MOS ↑ albumin permeation, *IL1β*, *IL6*, *IL8*, *TNFa*, and *TGFb* expression, but ↓ ROS production, cell proliferation and *MYD88* expression. Nucleotides and β-glucans ↑ *IL1β* and*IL8*. LPS ↑*IL1β, IL6, IL8* and *TNFa* but had no effect on ROS. Barrier function related genes, all treatments up regulated the expression of *cldn3* and suppressed *cdh1* levels. Beta-glucans increased TEER levels and F-actin content. | [14] |
| Rainbow trout (*Oncorhynchus mykiss)* | qPCR | Supplementation containing multi-strain yeast fraction product | MsYF, Lallemand SAS, Blagnac, France | - | Control diet vs diet containing MsYF (1.5 g/kg of feed) (continuous) vs MsYF (2 week rotations with the control diet) | Posterior Intestine | ↑ *TLR2, IL1R1, IRAK4, and TOLLIP2* after 4 weeks<br>↑ *IL1β, IFNγ* and *IL12* after 8 weeks in fish fed continuously | [85] |

**Table 3**
Fish studies using transcriptomic technologies to characterize immunological responses to Glucoinsolates.

| Fish Species | Omics Technology | Dietary Manipulation | Source | Disease Challenge | Comparison and Sampling | Tissue Analysed | Main findings | Reference |
|---|---|---|---|---|---|---|---|---|
| Atlantic salmon (*Salmo salar*) | Microarray and qPCR | Supplementation with Glucosinolates | GL's, EWOS, Norway | *Lepeophtheirus salmonis* | Control vs 3.61% Glucosinolates (Low dose) supplemented vs 13% Glucosinolates (High dose) supplemented for 31–35 days | Skin | Atlantic salmon fed glucosinolates had significantly ↓ lice load. Microarray analysis showed induction of over 50 IFN related genes. After infection genes ↑ included type 1 pro-inflammatory factors, antimicrobial and acute phase proteins, extracellular matrix remodelling proteases and iron homeostasis regulators in fish fed glucosinolates. | [92] |
| Atlantic salmon (*Salmo salar*) | Microarray and qPCR | Supplementation with glucosinolates | GL's, EWOS, Norway | *Lepeophtheirus salmonis* | Control vs 3.61% Glucosinolates (Low dose) supplemented vs 13% Glucosinolates (High dose) supplemented for 31–35 days | Liver, muscle kidney | DEGs were highest in the liver (232), followed by the distal kidney (188) and the muscle (156). Extreme GLs dose caused a decrease in hepatic fat deposition and growth which suggested tissue remodelling and reduction of cellular proliferation in the skeletal muscle and liver. Prevalent activation of phase 2 detoxification genes in all diets. Increased iron sequestration from blood and modulation of iron metabolism both prior to and during lice infection. | [93] |

identifying foreign antigens [58]. The mannan-binding receptor and its homologs, Ladderlectin, are c-type lectins that detect carbohydrate structures on the surface of microorganisms and pathogens [81]. The mannose-binding receptors are endocytic receptors expressed on the surface of a variety of leucocytes allowing for the uptake of antigens, through endocytosis potentially resulting in the innate immune response and complement pathways being activated. The mannose receptor has an important role in several different immune functions: phagocytosis, antigen processing and presentation, cell migration, and intracellular signalling. The mannose-binding receptor has been studied in a variety of important aquaculture species from Sea Bream, Cod, and Salmonids [81–83]. MOS binds to the mannose-binding lectin resulting in immunomodulation and activation of the complement system in functional feeds as a prebiotic.

There have been many trials where targeted gene expression has been used to examine changes in gene expression in different tissue types after the addition of MOS to the diets in farmed fish and this is summarized in Table 2. In a sea bass trial using a diet containing MOS vs commercial fish feed for 8 weeks, the immune responses in both the liver and posterior intestine were altered [84]. *MHCII, TCRβ,* and *caspase 3* expression were increased suggesting MOS was driving a pro-inflammatory response and increasing antigen presentation. A study in rainbow trout using feed containing multi-strain yeast fraction product (*S. cerevisiae and C. jardinii)* vs commercial feed for 8 weeks showed significant modulation of the mucosal immune response [85] (Table 2). The mucosal immune response showed significant increases in *tlr2, il1r1, irak4, and tollip2* after 4 weeks, and in the inflammatory response genes, *il1β, ifnγ, and il12* after 8 weeks in the fish fed continuously. This suggests yeast fractions, which contain MOS and other oligosaccharides, could modulate the mucosal immune response through the toll-like receptor 2 signalling pathway leading to increased antigen presentation to mucosa-associated lymphoid tissue. A study by Agboola et al., [86], into salmon fry, using various yeasts (*Cyberlindnera jadinii, Blastobotrys adeninivorans, and Wickerhamomyces anomalus*) showed that the functionality of the yeast in lessening enteritis was dependent on the processing method and the type of yeast, with both *C. jadinii* and *W. anomalus* exhibiting encouraging effects in gut health.

Several studies have used transcriptomics (microarray or RNA-seq) to further examine the fish response to oligosaccharides. A trial using a feed containing MOS, FOS, nucleotides, and vitamins in Atlantic Salmon compared to a control diet over 16 weeks on the gene expression in liver and muscle suggested a general decrease in immune-related genes in the liver of the fish, with several binding lectins, *MHCII, prostaglandin D synthase* and *complement C4* were all reduced in expression in the fish fed the functional feed [13] (Table 2). The data from this trial suggested that fish fed the functional feed were able to decrease certain aspects of the immune response allowing the fish to enhance performance indicators such as growth and metabolism whilst maintaining the ability to upregulate the immune response when challenged with a pathogen.

Rainbow trout kept at either high or low density were examined for the intestinal transcriptomic response to functional feeds by RNA-seq, here one of the diets was a MOS diet chosen as the prebiotic [87]. Although density had a major effect on gene expression responses several genes related to mucosal structure, stability, and the extracellular matrix with the upregulation of tenascin precursor and fibronectin. Fish fed MOS in the low-density group showed some immune transcripts were upregulated including *MHCII beta chain precursor* whilst other immune transcripts such as immunoglobulin lambda chain, and the perforin precursor were down-regulated. When gene set enrichment was examined for the fish fed MOS at high or low density it was hard to identify processes in common, suggesting a complex interaction. At low-density stocking, the MOS increased genes in the PI3K-Akt signalling and Ras signalling, but HIF-1 signalling, leukocyte transendothelial transport, and cell adhesion pathways were decreased, however in high density few pathways were found significantly enriched with PI3K-Akt signalling being the only increased common pathway kept at low density. These were interesting observations as revealed by the high throughput transcriptomics which suggested that trout maintained at higher stocking density caused the suppression of immune-related transcripts in those fish fed a normal diet that was counteracted when fed the functional feed diets.

FOS are comprised of linear chains of fructose molecules, linked by β (2–1) glycosidic bonds and range between 2 and 60 molecules in length

[88]. FOS are digested to short-chain carboxylic acids, L-lactate, and other metabolites by intestinal bacteria. There is also emerging evidence FOS can directly modulate the immune response through interaction with host receptors. For example, in humans, FOS interacts with TLR2, on the surface membrane of macrophages, leucocytes, and dendritic cells [89]. Subsequent activation of the immune response can be triggered through the activation of the signal transduction pathway with the activation pattern dependent on the length of the fructan molecules.

The other oligosaccharides GOS, and inulin have been shown to modulate immune transcripts, but often these trials have been carried out in combination with probiotics [90], to date no high throughput transcriptomics have been carried out singularly on these functional feed components, but further research is needed for these molecules.

### 2.3. Molecular responses of the host caused by the supplementation of glucosinolates

Glucosinolates (GL's) are secondary metabolites, and phytochemicals and belong to a group of diverse compounds that are used by the Brassicaceae family of plants to detract from being eaten. GLs are broken down, by the enzyme myrosinase which hydrolyses GLs into isothiocyanates. Isothiocyanates can exert beneficial effects on the heath of invertebrates leading to an increase in the antioxidant, detoxifying and pro-inflammatory response type 1 pathways. GLs can be considered as prebiotics and may have the potential to reduce parasitic sea lice (*L. salmonis)* burden on Atlantic Salmon. Sea lice secrete a variety of compounds into the host which causes the immunosuppression of type 1 inflammatory responses as well as type 2 inflammatory responses in salmonids as demonstrated by qPCR [91]. Fish that elicit higher type 1 responses during the early stages of infection in the skin are negatively correlated to the number of infective stage lice [92]. The immunosuppression of Type-1 proinflammatory responses could be counteracted by the addition of glucosinolates to functional feeds to promote type-1 responses in salmonids leading to a decrease in the levels of sea lice infection.

The impact of dietary GL on the skin transcriptome and sea lice attachment was examined by microarray after 31 days of feeding either 3.6% or 13% GL inclusion to diet vs a control diet [92] (Table 3). Microarray analysis revealed that over 50 known IFN response related genes before infection with lice in fish fed the high-density GL, many of these genes are involved with the innate antiviral response. Both groups showed large gene expression differences following the lice challenge, with significant differences between the fish fed the different diets. qPCR validation confirmed an increase in the expression of several groups of proinflammatory cytokines, chemokines, and effectors including *IL17A*, *IFNy,* and *LECT2*. Fish fed GLs had significantly decreased lice load by 25% in the low-level GL group and 17% in the higher GL group. After infection with sea lice, upregulated genes included type 1 pro-inflammatory factors, antimicrobial and acute-phase proteins, extracellular matrix remodelling proteases and iron homeostasis regulators in fish fed glucosinolates at both dosages. Fish fed the control diet after infection showed higher expression of genes involved in lipid and glucose metabolism and muscle contraction.

Further research by Skugor et al., [93], examined the impact of GLs in the muscle, kidney, and liver transcriptome (again by microarray) of Atlantic salmon in response to lice infection (Table 3). In this experiment, a 13% GL supplemented diet was compared to a control diet. The scale of GL-induced expression changes between tissues was similar and with some differences between tissues identified through DEGs; DEGs were highest in the liver (232), followed by the distal kidney (188) and the muscle (156). Microarray data on the liver showed that pathways linked to chromatin organisation regulation and DNA replication and repair compromised a large part of the DEGs. Increased expression was shown in genes relating to the negative regulation of cell proliferation; *cullin 1b, btg1, abracl*, whilst genes related to DNA replication and mitosis were downregulated, *securin, condensin complex subunit 3 and cyclins G2/mitotic-specific cyclin-B1 and cyclin-A2*. Upregulation was also seen in genes involved in iron uptake; *hepcidin-1* and *cytochrome*-b which led to lower levels of iron in the plasma, with components of the cytosolic iron-sulphur protein assembly being downregulated, *cytosolic iron-sulphur protein assembly protein ciao 1*. Suppression of some dietary immune genes was observed in the extreme glucosinolate diets, whilst 4 complement genes were upregulated in the liver; *complement factor H precursor, properdin P Factor 2 and 3,* and *complement C1q-like protein 4*. Analysis of the distal kidney showed there was modulation of immune responses with *IFN-y* being upregulated. The kidney showed upregulation of the anti-fibrotic responses after overexposure to glucosinolates, alongside upregulation of the detoxifying pathways and antioxidant pathways. Upregulation of molecules that regulates the WNT-signalling pathway was also seen with *TGFβ-1 and wnt5b* both being upregulated to prevent renal fibrosis. Whilst the muscle tissue showed responses regarding proapoptotic and inhibitory effects on proliferation due to the upregulation of *actin-related protein 2/3 complex subunit 1B, Bax, and androgen-induced proliferation inhibitor*. The results from both trials suggest that GLs incorporated in the diet can modulate the gut immune factors resulting in a systemic response through the complement system and pro-inflammatory type 1 which may be beneficial in parasite prevention.

### 2.4. Direct responses to prebiotics as revealed by in-vitro cell culture experiments

Recent approaches to testing functional feeds use cells rather than tissues to identify if functional feeds have specific effects on immune, epithelial, or fibroblast cells. An experiment by Medina-Gali et al., [94] showed that the addition of 10 μg/ml of β-glucans could be used to stimulate Zebrafish Fibroblast (ZF4) cells alongside stimulation with spring viremia of carp virus (SVCV) virus (Table 1). These fibroblasts showed that with stimulation there was an enhanced response to SVCV with the interferon-related genes: *gig2, tlr2, ifnphi1,* and the inflammatory cytokines *IL-1β, IL-6, IL-8, IL-10,* and *TNFα* transcripts were also upregulated after 48hrs post-infection. In parallel experiments *in-vivo* zebrafish mortality was reduced after being primed with β-glucans with 73% surviving compared to 33% in the control group.

A recent study by Camino Ordás et al., [95] has shown that *in-vitro* stimulation of a macrophage like cell line RTS11 and RTgutGC cells with β-glucans and *A. salmonicida* separately induced a pro-inflammatory response (Table 1). RTS11 cells showed a significant upregulation when stimulated with 60 μg/ml of M β-glucan (particulate β-glucan with ~60% purity) in proinflammatory genes (TNFα, IL1β, IL6, IL8, COX2A, hepcidin, and VCAM) compared to the untreated cells. When stimulated with both β-glucans and *A. salmonicida,* the RTS11 cells showed a significant upregulation in these proinflammatory genes than compared to these stimuli alone in RTS11 cells. Whilst only IL1β and IL8 showed significant increases in the RTgutGC cell line when stimulated with both β-glucans and *A. salmonicida* compared to these stimuli individually.

Further in-vitro experiments have examined targeted gene expression responses of RTgutGC cell line stimulated with MOS which upregulates albumin permeation, *il1b, il6, il8, tnfa,* and *tgfb* expression and decrease ROS production, cell proliferation, and *myd88* expression [14] (Table 1).

## 3. General conclusions and future perspectives

This review has shown that recent advances in transcriptomics have facilitated the understanding of the immune response to prebiotics in salmonid fish. The differential effects of prebiotics have been highlighted with varying responses between species and functional feed additives. Prebiotic supplementation in functional feeds demonstrates a unique method by which the proinflammatory response can be modulated and can further be used in functional feeds to reduce stress caused to animals. Although there has been significant research on the impact of

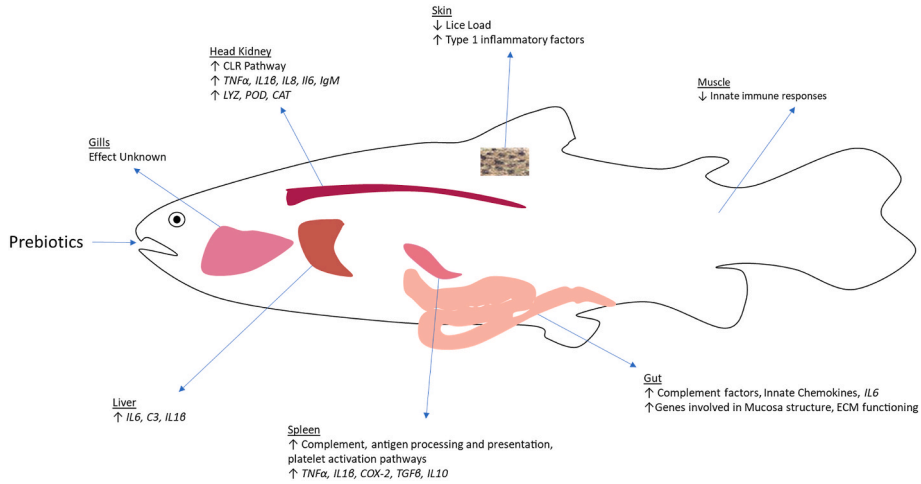


**Fig. 1.** Effect of prebiotics on different tissues in Farmed Salmonids.

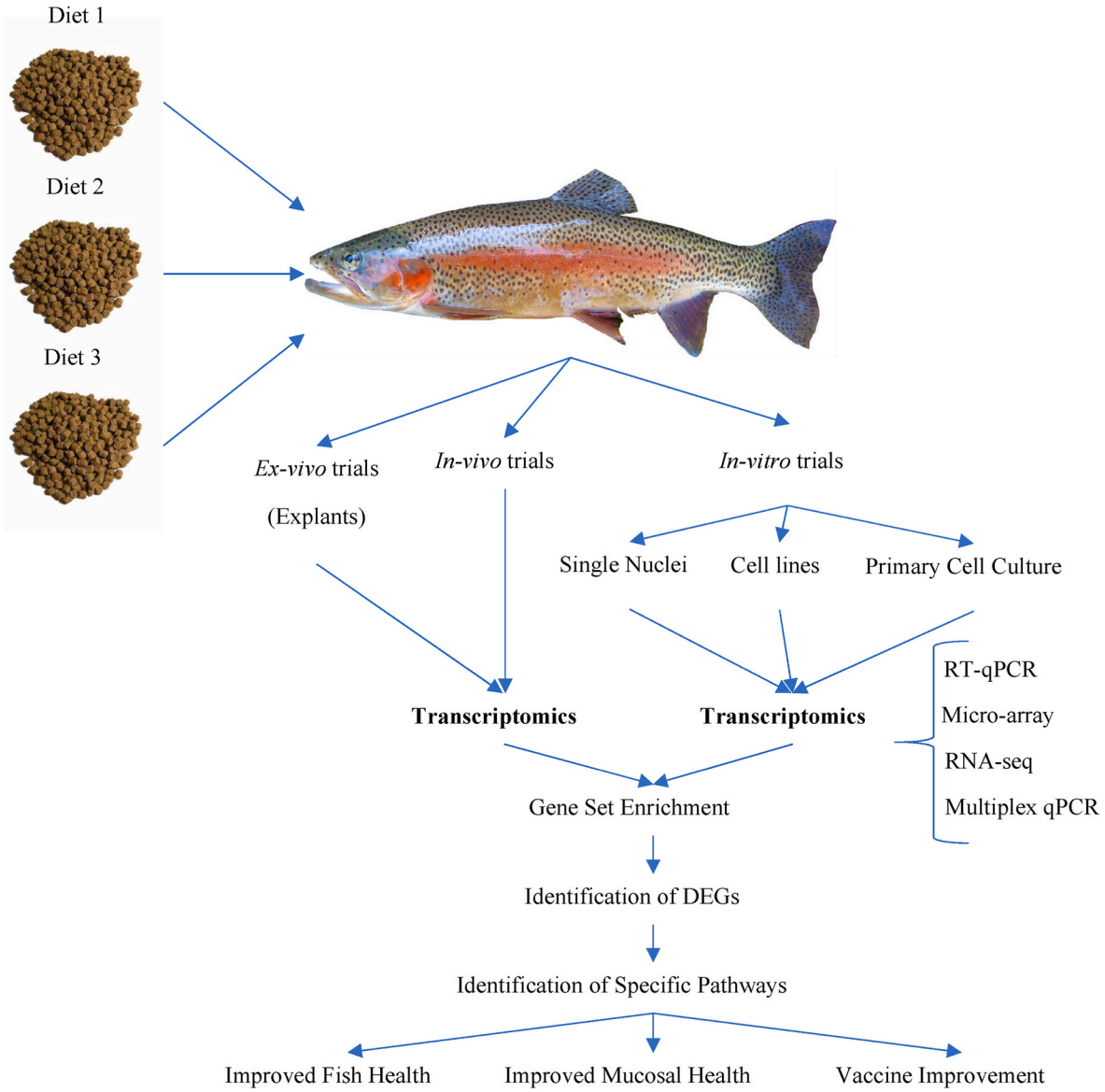


**Fig. 2.** Pipeline of experimental approaches using transcriptomic to identify immunomodulatory effects of prebiotics.

functional feeds on the immune response, the mechanisms by which these occur are still poorly understood, especially in the gut, as such there is a need for further research into the direct immune response at the nutrition-health interface.

Salmonid cell lines offer a quick and non-invasive method of examining direct immune responses to functional feed ingredients which also addresses the 3Rs in reducing whole animal experiments. Studies by Wang et al. [14], and Camino-Ordás et al., [95] have demonstrated the effectiveness of using the RTgutGC and RTS11 as a quick, non-invasive alternative to a feeding trial whilst still demonstrating the potency of prebiotics to modulate the immune response. However, whilst cell lines may reduce the need to use whole fish, the number of cell lines available is limited and the phenotype of these cells is likely to have changed from their original *in-vivo* state, especially in the intestine with the complex nature and multiple cell types present. Another cell culture alternative is using primary cell cultures to look at the immune response whilst maintaining the phenotypes and different cell types that are seen in the host. Gut-associated lymphoid tissues (GALT) leucocytes have been extracted and have shown to be a good health screen to PAMPs in previous studies by Attaya et al., [56] but their role as a viable alternative to traditional feeding trial methods is yet to be seen. Head kidney macrophages have also been used by Petit et al., [67], alongside RNA-seq experiments which showed the direct immune response to Macrogard®, a compound made up of β-glucans showed that the complement pathway was upregulated in response, and CLEC4C and SCLRA represent the best genes for future studies as potential β-glucan receptors. These insights into the direct response to immune challenges and functional diets demonstrate the unique ability of cell culture alternatives to the standard feeding trial approach in isolating the direct immune response to prebiotics.

A study by Peñaranda et al., [96], demonstrated the potential of explants from Gilthead Seabream to identify the immune responses in the gut to bacterial pathogens and prior effects of feeding with fishmeal or plant protein diets. This study demonstrated that gut explants from the Gilthead Seabream (Sparus *aurata, L.)* were used to determine mucosal sensitivity to two bacterial pathogens: *Vibrio alginolyticus* and *Photobacterium damselae* subsp. Piscicida triggering an immune and inflammatory response. Both bacteria caused a high positive correlation between the pro-inflammatory genes encoding interleukin 1β, interleukin 6, and cyclooxygenase 2. This study represents a potential unique method for testing other functional feed ingredients.

Multiplex PCR allows for simultaneous quantification of multiple transcripts and can rapidly speed up targeted qPCR analysis. A study by Caballero-Solares et al., [97], developed two multiplex PCR panels to target 40 genes incorporating 3 control genes and biomarkers that cover growth, metabolism (*srebp 1, elovl2*), oxidative stress (*txna, prdx1b*), and inflammation (*pgds, 5 loxa*), which were used to examine three different diets (Marine ingredients, animal by-products and vegetable oil, and a plant protein and vegetable oil). The study demonstrates the potential method of detecting immunomodulation by prebiotics if panels can be designed to identify a pro-inflammatory or complement response.

Advances in transcriptomics have meant that single nuclei can now be extracted to illustrate the transcriptomic and immunological profile of Atlantic Salmon Gill [98]. The method of single nuclei RNA-seq could lead to identifying the direct response of different immune cell types to different functional feed additives during a feeding trial and lead to the understanding of how prebiotics alter the immune response to infections particularly in response to different tissue types (see Fig. 1).

Fig. 2 illustrates the potential methods of experimental approach using transcriptomics to identify immunomodulatory effects of functional feeds to be able to reduce the 3 R s and categorise the direct immune response to prebiotics in the future. Lastly, future studies into the impacts on the microbiota both before and after supplementation with prebiotics alongside '*omics* technologies may provide a unique insight into the roles of specific bacteria that provide a symbiotic relationship with prebiotics. This data would be beneficial to identify specific metabolic, inflammatory, and immunological pathways that are modulated by the microbiome and functional feed interface.

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## CRediT authorship contribution statement

**D. Porter:** Writing – original draft, designed and wrote the original manuscript draft. **D. Peggs:** Conceptualization, helped conceptualization and edited the manuscript. **C. McGurk:** Conceptualization, helped conceptualization and edited the manuscript. **S.A.M. Martin:** Conceptualization, Supervision, Writing – review & editing, conceptualized the manuscript, supervised the writing and edited the manuscript.

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