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Review

Transcriptomic responses in the fish intestine

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ABSTRACT

The intestine, being a multifunctional organ central to both nutrient uptake, pathogen recognition and regulating the intestinal microbiome, has been subjected to intense research. This review will focus on the recent studies carried out using high-throughput gene expression approaches, such as microarray and RNA sequencing (RNA-seq). These techniques have advanced greatly in recent years, mainly as a result of the massive changes in sequencing methodologies. At the time of writing, there is a transition between relatively well characterised microarray platforms and the developing RNA-seq, with the prediction that within a few years as costs decrease and computation power increase, RNA-seq related approaches will supersede the microarrays. Comparisons between the approaches are made and specific examples of how the techniques have been used to examine intestinal responses to pathogens, dietary manipulations and osmoregulatory challenges are given.

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1. Introduction

The gastrointestinal tract of vertebrates along with its single layer of epithelial cells constitutes the largest and most important barrier against the external environment (Groschwitz and Hogan, 2009). The intestinal epithelium acts as a selectively permeable barrier for dietary nutrients, electrolytes and water, while maintaining an effective defence against pathogens and tolerance toward dietary antigens (Peterson and Artis, 2014). The epithelial cells are also crucial mediators of mucosal innate and adaptive immunity, important for distinguishing pathogens from commensal microbiota that live in the gut (Kinnebrew and Pamer, 2012; Donaldson et al., 2016). Fish and their immune system has received considerable attention from comparative immunologists, in part because of the unique position of this group to provide key insights into the evolution of immune systems (Trede et al., 2004; Cooper and Herrin, 2010; van Niekerk et al., 2015). While the innate immune mechanisms can be found in nearly all forms of life, the origins of mammalian-like (recombination-activating gene (RAG)-dependent) adaptive immunity reach back approximately 450 million years, coinciding with the emergence of the first jawed vertebrates (reviewed in Flajnik and Kasahara, 2010). The presence of convergently evolved system that is RAG-independent has been recently discovered in jawless vertebrates such as hagfish and lamprey (Pancer et al., 2004).

Fish are also known for their substantially higher exposure to pathogens than non-aquatic vertebrates, with typically a million of bacteria and 10 million of viruses per millilitre of seawater (Fuhrman, 1999). The pathogen exposure in fish starts immediately after hatching from their protective chorions, providing an interesting contrast to mammals protected during early development by maternal immunity (Trede et al., 2004). The exposure to pathogens is further enhanced during the mouth and gut opening stages and at the onset of exogenous feeding (Castro et al., 2015). However, the early life exposure to pathogens does not necessarily equip fish with the 'knowledge' of the microorganisms they may encounter in later life. Indeed, many fish species are exposed to different and unfamiliar pathogens when they switch between fresh and salt water environments (Jeffries et al., 2014). Evidence is also growing that some fish, including non-migratory species, are being exposed to novel pathogens as a result of climate change, because warmer environments are associated with an increase in the diversity of diseases, increased population growth rates of most microorganisms and increased vulnerability of coldwater fish (Crozier and Hutchings, 2014).

The transport of nutrients, solutes and pathogens across the epithelial barrier is controlled by two main mechanisms, either through the cells (transcellular transport) or between the cells (paracellular transport) (reviewed in Sundh and Sundell, 2015). Transcellular transport requires either active or passive transporters, intracellular trafficking and then excretion of the substances at the basolateral membrane of the cell, with amino acids, fatty acids and carbohydrates (mainly sugars) as the key substances being transported. Paracellular transport is controlled by cellular contact and the tightness of the contacts. The integrity and control of the intestinal barrier is often attenuated by both nutritional and immunological challenges in the fish.

Our knowledge of the fish immune system is advancing rapidly, with many of the cell types, humoral factors and regulatory molecules now identified (Collet, 2014; Castro and Tafalla, 2015) (Fig. 1). Within the intestine, immune activity is controlled by the gut associated lymphoid tissue (GALT) containing numerous immune cell types that are involved in both innate and adaptive responses (reviewed in Salinas and Parra, 2015). Of central importance is antigen sampling across the epithelial barrier, likely to involve antigen-sampling cells equivalent to mammalian microfold (M) cells and dendritic cells (DCs) (Fuglem et al., 2010). Although a specific DC subset has not yet been fully identified in fish, the presence of dendritic-like cells has been suggested in intestinal epithelium (Fuglem et al., 2010) and peripheral blood (Haugland et al., 2012) of Atlantic salmon as well as various non-intestinal tissues of rainbow trout (Johansson et al., 2012; Granja et al., 2015) and zebrafish (Lugo-Villarino et al., 2010). The intestinal dendritic-like cells are hypothesised to present luminal antigens to T and B cells ensuring the maintenance of the gut microbiome and identification of pathogens. B cells secrete different Ig molecules (Parra et al., 2013; Salinas, 2015) and are produced at high levels in the intestinal mucus to bind luminal antigens. The combination of B

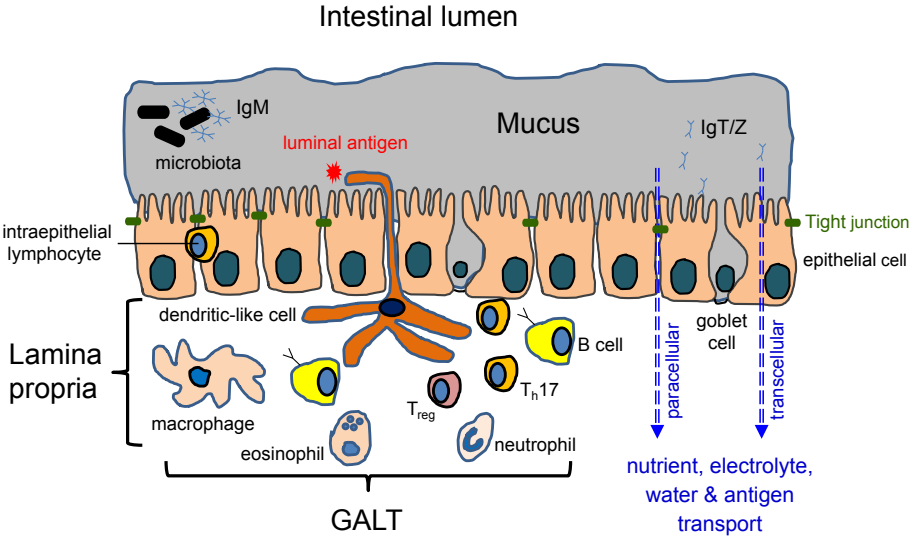

Fig. 1. Diagram of the intestinal mucosa in teleost fish. This complex tissue contains epithelial cells involved in the intestinal barrier function that control para- and transcellular transport, with associated genes regulating these processes. The gut-associated lymphoid tissue (GALT) is contained within the lamina propria and has a complex populations of immune cells. The presence of dendritic-like cells and T cell subtypes in fish has not yet been fully confirmed. Transcriptomics by its high-throughput nature, coupled with continually improving gene annotation, can reveal parallel changes in gut permeability and immune function.

and T cells oversee the immune status of the intestine and ensure its correct functioning from early development. Specifically, T_{reg} and Th17 cells in mammals maintain the intestinal balance between reacting in an inflammatory or non-inflammatory manner to sustain homeostasis, although the presence of these T cell subtypes in fish remains to be confirmed. Some aspects of the mucosal immunity are fish specific, such as immunoglobulin T (IgT), secreted from intraepithelial lymphocytes (Parra et al., 2013; Ballesteros et al., 2013).

The intestinal immunity of fish is of special interest for the fish farming industry for a number of reasons. Firstly, farmed fish kept at high stocking densities are susceptible to intestinal infections, with the gut being an important entry point for pathogens (Salinas and Parra, 2015). Secondly, farmed fish are typically fed commercial pelleted feeds, which opens up avenues for manipulating fish health through the incorporation of various feed additives, drugs and vaccines into the feed (reviewed in Caipang and Lazado, 2015). Thirdly, the gut immune system of teleost fish allows microbial colonization by symbionts (Ringø et al., 2014), and this microbial community is a potential platform to modulate fish pathogens. Finally, gut microbiota in fish are likely to respond to dietary manipulations (Merrifield and Rodiles, 2015; Llewellyn et al., 2014; Rurangwa et al., 2015). Hence, a comprehensive understanding of the diet-gut interactions and immunoregulatory properties of intestinal epithelium in fish could aid in the development of new strategies to prevent and treat their multiple infectious and inflammatory diseases.

Recent advances in high-throughput technologies to survey RNA, especially microarray profiling and RNA sequencing (RNA-seq), have revolutionized the discipline and enabled the study of fish intestine at the level of whole transcriptome rather than individual transcripts, typically targeted by Northern blot or quantitative PCR (Qian et al., 2014; Li and Li, 2014). The transcriptome refers to the complete set of transcripts in a specific cell, tissue or organism, including all protein-coding messenger RNAs (mRNAs) as well as non-coding RNAs (ncRNAs), which regulate gene expression and maintain cellular homeostasis (Lindberg and Lundeberg, 2010). Unlike the relatively stable genome, the transcriptome varies with developmental stage, physiological condition and external environment. Further complexity is also added by the presence of splicing isoforms, gene-fusion transcripts, post-translational modifications and epigenetic controls (Mastoridis et al., 2015). The large-scale analysis of transcriptome (commonly referred to as transcriptomics) has become a powerful tool for understanding complex interactions between genotype and phenotype, providing insights into molecular mechanisms that control cell fate, development and function, both in health and disease (Wang et al., 2009). Transcriptomics is also essential to guide and interpret subsequent analyses by proteomics, metabolomics and other emerging technologies.

In this review, we will present the current use of high-throughput transcriptomic approaches to investigate gut function and immunity in fish. Specifically, we will focus on microarray and RNA-seq approaches and discuss the merits of the different platforms. These technologies are constantly advancing, along with the development of genomic resources for increasing number of fish species. The importance of immune function of the intestine is fully recognised (Rombout et al., 2014), but the new tools can help understand its regulation and impacts from disease, nutrition and many other aspects of environmental stress.

2. Overview of transcriptomic technologies

The high-throughput technologies, such as microarray and RNA-seq, allow for the simultaneous measurements of thousands of transcripts, with the ultimate aim of understanding fish intestinal responses *via* differentially expressed genes, *i.e.*, genes that differ in the expression levels between conditions and treatments. Before specific examples are presented, a brief overview of the technologies is given, along with the requirements for genomic resources and also advantages and disadvantages of the respective approaches. Microarrays are based on hybridization, whereas RNA-seq utilises new ultrahigh throughput sequencing that became available in the recent years. The hybridization-based approaches typically involve incubating fluorescently-labelled complementary DNA (cDNA) with pre-defined sequences, such as PCR products or long oligonucleotides (mostly 60 mers), densely spotted onto a solid modified glass surface. In contrast to microarray methods, sequence-based approaches determine gene expression levels by directly sequencing cDNAs. Both approaches generate relative abundance of mRNAs, which reflect gene expression levels. The outcome of transcriptomic studies strongly depends on sequence availability, computational methods for gene annotation and gene set enrichment. The summary of the pipelines used for microarray and RNA-seq technologies are shown in Fig. 2.

The overall goal of transcriptomics is not to identify single genes that may be altered, but to define which biological pathways are being altered in a more holistic approach. We are now at transition period where microarrays have been used to the present time, but there is an increasing shift towards RNA-seq. Although the technical aspects of both technologies differ considerably (as described below), they both generate lists of differentially expressed genes and the biological interpretation of these genes is central to the biological interpretation of the experiment.

2.1. Microarray technology

Microarrays were first used in fish studies during the late 1990's (reviewed in Gracey and Cossins, 2003; Douglas, 2006; Goetz and MacKenzie, 2008), based on the sequencing of expressed sequence tags (ESTs). The ESTs were often generated from cDNA libraries that were enriched for genes associated with infection or developmental stages (O'Farrell et al., 2002; Taggart et al., 2008). The cDNA libraries were constructed by printing amplified PCR products usually derived from EST libraries onto specially prepared glass slides. Such arrays were generated for many fish species, including catfish (Ju et al., 2002), carp (Gracey et al., 2004), rainbow trout (Koskinen et al., 2004), Atlantic salmon (von Schalburg et al., 2005) and halibut (Byon et al., 2005) amongst others. The printing technology did not allow for high density slides to be generated and numbers of genes varied between 4000 and 16,000, reflecting the printing robotic ability. Most of the arrays were bespoke and generated by independent research groups or consortiums, such as GRASP and TRAITs for Atlantic salmon (von Schalburg et al., 2005; Tacchi et al., 2011a). Hybridization conditions and print quality were always an issue and often poor batches of slides were generated. Additionally, the laborious generation of PCR products soon changed the approach to oligonucleotide platforms. The conversion to oligo arrays for general non-model fish species was brought about by companies such as Agilent, providing online tools to assist oligo design. Since that time, the majority of microarrays have used the 60-mer oligo approach, with either 4 × 44K or 8 × 15K oligos printed per slide. Protocols for labelling of target mRNAs have improved and almost all labelling is now done by linear amplification of cDNAs. The main limitation of the microarray technology is that only the pre-determined genes can be analysed. However, as most fish species and other vertebrates have between 20,000 and 30,000 genes, a well-designed (using up-to-date annotated genomes) oligo microarray should be able to assess > 90% of the transcriptome. This, however, will not allow for

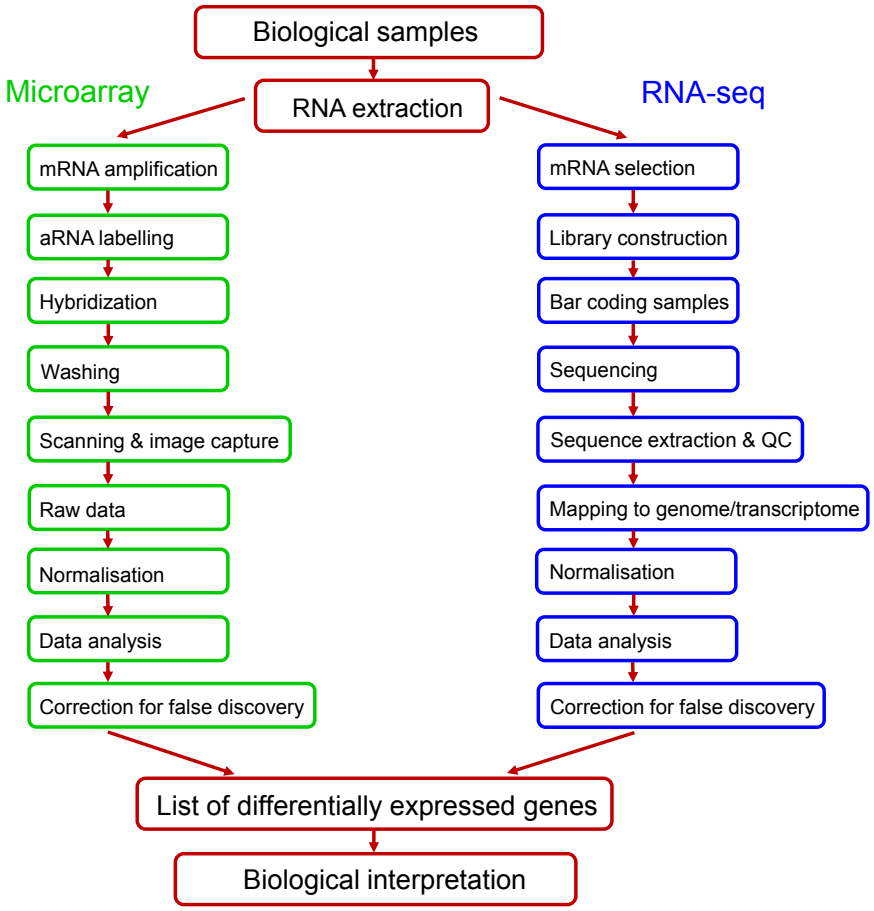

Fig. 2. Flow diagram of the key steps in a transcriptomic experiment using microarray and RNA-seq technologies. Biological samples and sufficient numbers of replicates are essential for both approaches. Following RNA extraction, different experimental and normalization methods are used to generate lists of differentially expressed genes. Once the gene lists are generated, similar approaches are used to interpret the data to gain functional significance, often using enrichments for biological pathways as described in text.

analysis of differential splicing or clear identification between paralogues of duplicated genes, which is of special interest for salmonid fish. There are also issues with very low and very high expressed genes, as discussed below.

2.2. RNA-seq technology

The advent of new sequencing technologies has come about by completely new chemistry and approaches to generate DNA sequences, with technologies such 454, Ion Torrent, AB SOLiD and Illumina being among the most important. The Roche 454 sequencers generated relatively long reads (up to 500 bases) and were used for generating transcriptomes (Salem et al., 2010; Micallef et al., 2012), similar to the previous EST approach, but substantially more efficient. However, this platform did not generate sufficient sequence depth for studying multiple samples for gene expressing. The Illumina platforms (currently MiSeq, Nextseq 500 and HiSeq 2000) all have the ability to sequence giga bases of DNA on a single run, and they are now often the platform of choice. The capacity of these platforms means that 20–50 million individual sequences can be generated per biological sample with individual reads up to 400 bases in length. For differential gene expression experiments, a "cDNA library" is generated from each biological replicate following enrichment for mRNAs, either by depletion of ribosomal RNAs or capture of mRNAs by polyA selection. The cDNA is fragmented to various lengths depending on the sequencing platform and adaptors ligated to the ends of cDNA for initial amplification. These adaptors often have sequence-specific "bar codes" to allow sorting of sequences derived from different samples (libraries), once the sequencing run has been completed. Decisions on library construction need to be taken at the early stage and also how the downstream analysis will be carried out. If long fragments are generated (for example 100–2000 bp), these can be sequenced in both directions (pair-end sequencing). Even though the sequence might not meet in the middle, each sequence can be used to help anchor the cDNA to the transcriptome or genome. Recent use of RNA-seq in fish has been reviewed by Qian et al. (2014), focussing on platforms and bioinformatic approaches.

2.3. Comparison of microarray and RNA-seq technologies

There are only few examples of both microarray and RNA-seq analyses using the same RNA material, especially for fish. One experiment that is relevant to highlight was using the mycobacterium infection model in zebrafish (Hegedus et al., 2009). The authors use an early RNA-seq approach called digital gene expression (DGE) to generate short sequence reads close to a restriction enzyme site in the 3′ end of cDNAs, which were then sequenced on the Illumina platform. Previously, several microarray platforms had defined a core set of 120 differentially expressed genes (Meijer et al., 2005; van der Sar et al., 2009). The DGE approach confirmed 100 of these genes as differentially expressed (Hegedus et al., 2009). This showed 83% correlation between the

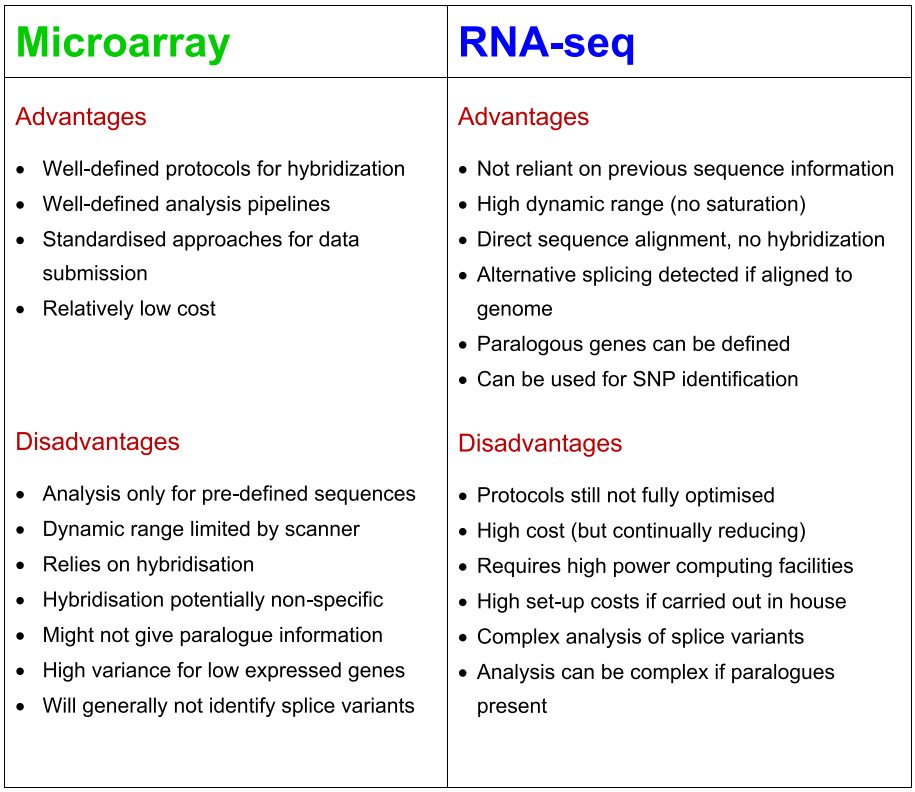

Fig. 3. Summary of advantages and disadvantages between microarray and RNA-seq approaches. All these aspects should be considered before embarking on a transcriptomic experiment.

platforms for this core gene set. However, when all differentially expressed genes were correlated between microarray and DGE, this was reduced to 60%. The authors identified key advantages of the DGE as not being limited to a pre-defined set of genes on the microarrays.

In addition to the above, there are a number of relevant publications available in other systems that have made in-depth comparative analysis between the two approaches. An experiment on rat liver to examine the dose-response to bromobenzine used RNA for Affimetrix oligo microarray (Thomas et al., 2013), and the same samples were reanalysed and compared using RNA-seq (Black et al., 2014). As a result, the normalised expression of all genes was correlated at 0.75 to 0.78 between microarray and RNA-seq, and the correlation increased to 0.95–1.0 when filtering for fold change (>1.5 or <−1.5) was included. However, when statistical tests were applied and the −Log *P*-values were used, the correlations between microarray and RNA-seq dropped dramatically to 0.33–0.46. This difference was found to result from the normalization methods followed by the statistical analysis, and was related to the dynamic range of the technologies used. The RNA-seq data showed much greater variation between individuals, which was not detectable during the microarray hybridization procedure. The methods for the normalization of RNA-seq data are still under development. More recently, Nault et al. (2015) compared Agilent microarray and RNA-seq for mouse liver following gavage with tetrachlorodibenzo-*p*-dioxin (TCDD). In this experiment, 1270 and 901 genes were differentially expressed for the microarray and RNA-seq approach, respectively. However, only 449 genes were common between the gene lists. Relaxing the "filtering" criteria increased the overlap, reflecting the differences in the statistical analyses between the two approaches. The authors concluded that RNA-seq outperformed the microarray with fewer false positives and negatives when candidate genes were examined by quantitative PCR, and suggested that the differences between the platforms were not as significant as previously reported and they were mainly due to the different approaches to the filtering the data for subsequent analyses. Other advantages of RNA-seq over the microarray (Zhao et al., 2014) include more robust expression profiles derived from RNA-seq especially for very low expressed genes, as such genes do not perform well under hybridization conditions (Fig. 3). Additionally, the RNA-seq analysis is not limited to the pre-defined genes and can also identify differential splice variants.

2.4. Biological interpretation of transcriptomic data

Both microarray and RNA-seq technologies generate lists of genes that are differentially expressed. However, the lists of genes are typically not very informative on their own. The great advantage of the transcriptomic approach is to be able to sort these genes under strict criteria and associate them with biological processes, molecular pathways and cellular sites of expression. When the genes are assigned to the functional groups, further statistical analysis is performed to find if such groupings are significantly enriched. Such enrichment is performed using many different approaches, the most common being Gene Ontology (GO) enrichment and KEGG pathway enrichment that can be carried out using programs such as DAVID (Huang et al., 2009) and Ingenuity Pathway Analysis (IPA). Some research groups have developed their own methods for transcriptomic analysis, *e.g.*, the Krasnov group in NOFIMA (Norway) using STARS (Salmon and Trout Annotated Reference Sequences) (Krasnov et al., 2011). In carrying out such enrichment analysis, the combination of genes means it is highly likely that there is a true effect occurring. Despite the growing

Table 1
Fish studies using high-throughput profiling to characterise gut transcriptome responses to pathogens, parasites and vaccines.

Fish species	Challenge	Comparison & sampling	Tissue analysed	Method & platform	References
Bacterial infection					
Channel catfish (*Ictalurus punctatus*)	*Edwardsiella ictaluri*, immersion	Infected *vs* control fish (0, 3, 24 h & 3 d)	Entire intestine	RNA-seq, Illumina HiSeqTM 2000	Li et al., 2012
Channel catfish (*Ictalurus punctatus*)	*Edwardsiella ictaluri*, immersion	Hsp40 multigene expression in infected *vs* control fish (0, 3, 24 h & 3 d)	Multiple, including intestine	Illumina-based RNA-seq datasets	Song et al., 2014
Channel catfish (*Ictalurus punctatus*)	*Edwardsiella ictaluri*, immersion	Cytochrome P450 multigene expression in infected *vs* control fish (0, 3, 24 h & 3 d)	Multiple, including intestine	Illumina-based RNA-seq datasets	Zhang et al., 2014
Channel catfish (*Ictalurus punctatus*)	*Edwardsiella ictaluri*, immersion	Claudin multigene expression in infected *vs* control fish (0, 3, 24 h & 3 d)	Intestine	Illumina-based RNA-seq datasets	Sun et al., 2015
Channel catfish (*Ictalurus punctatus*)	*Edwardsiella ictaluri*, immersion	Tumor suppressor multigene expression in infected *vs* control fish (0, 3, 24 h & 3 d)	Multiple, including intestine	Illumina-based RNA-seq datasets	Mu et al., 2015
Asian seabass (*Lates calcarifer*)	*Escherichia coli* LPS, intraperitoneally & *Vibrio harveyi*, intraperitoneally	Infected *vs* control fish (40 h)	Intestine	RNA-seq, 454 GS FLX Titanium (Roche)	Xia et al., 2013
Parasite infection					
Gilthead sea bream (*Sparus aurata*)	*Enteromyxum leei*, immersion	Exposed infected and non-infected *vs* control fish (113 d)	Multiple, including intestine	Microarray, GPL8467 (Gene Expression Omnibus)	Davey et al., 2011
Gilthead sea bream (*Sparus aurata*)	*Enteromyxum leei*, immersion	Infected fish fed vegetable (VO) or fish (FO) oil *vs* non-infected fish fed VO or FO (102 d)	Distal intestine	Microarray, GPL15203 (Gene Expression Omnibus)	Calduch-Giner et al., 2012
Turbot (*Scophthalmus maximus*)	*Enteromyxum scophthalmi*, oral intubation	Infected *vs* control fish (7, 24 & 42 d)	Multiple, including pyloric caeca	RNA-seq, Illumina HiSeqTM 2000	Robledo et al., 2014
Viral infection					
Grass carp (*Ctenopharyngodon idella*)	Reovirus (GCRV), immersion	Infected *vs* control fish (0, 2, 24, 48, 72, 96 & 120 h)	Multiple, including intestine	RNA-seq, Illumina HiSeqTM 2000	Shi et al., 2014
Vaccination					
European sea bass (*Dicentrarchus labrax*)	Vaccine against *Vibrio anguillarum*, orally	Vaccinated *vs* control fish (135 d)	Multiple, including hind gut	RNA-seq, 454 GS FLX Titanium (Roche)	Sarropoulou et al., 2012
Rainbow trout (*Oncorhynchus mykiss*)	DNA vaccine against infectious pancreatic necrosis virus (Ipnv), orally	Vaccinated *vs* control fish	Multiple, including pyloric caeca	Microarray, GPL14155 (Gene Expression Omnibus)	Ballesteros et al., 2012a
Immunostimulants					
Rainbow trout (*Oncorhynchus mykiss*)	2 diets with and without immunostimulants	Fish fed immunostimulant *vs* control diets (4 weeks)	Multiple, including intestine	Microarray, GPL6154 (Gene Expression Omnibus)	Doñate et al., 2010

number of tools available, the effective identification of functional groups of genes relevant to the underlying physiology across different conditions remains a challenge.

Caution should be exercised when interpreting results of transcriptomic studies because the differences in gene expression may not be reflected at the protein level. It has been common practice to use mRNA concentrations as proxies for the concentrations of the corresponding proteins, assuming that transcript abundance is the main determinant of protein abundance. However, recent technological advances have demonstrated a substantial role for regulatory processes occurring after mRNA is made (such as post-transcriptional, translational and protein degradation regulation) in controlling protein abundance (Dahan et al., 2011). Based on the limited data available for bacteria and some eukaryotes (mammalian cells, worms, flies and yeast), on average approximately 40% of the variation in protein concentration can be explained by the levels of mRNA, while the remaining 60% of the variation has been attributed to the post-transcriptional regulation and measurement errors (Vogel and Marcotte, 2012). Such correlations between mRNA and protein abundance are not available for fish, because of the lack of specific antibodies.

3. Overview of gut transcriptome studies in fish

3.1. Disease challenge and immune function

During disease response and immune stimulation studies, the intestinal tissues have not received as much attention as the well documented primary and secondary immune tissues such as spleen, head kidney and liver. This is now changing rapidly with the realisation that not only it is imperative that the gut responses to pathogens are fully elucidated, but also because of the emerging mechanisms by which oral vaccines and gut microbiota can affect gut immune system and function. There are relatively few studies that take the whole transcriptome approach to pathogen and immune modulation in fish intestine (Table 1). Despite the differences in the pathogens used and their routes of experimental infection, there are some clear messages that can be taken from these studies. However, it should also be noted that the intestine varies between species and their life history stages, and also that the different sections of the intestine were subjected to transcriptomic evaluation. Thus, caution should be taken when comparing experiments.

3.1.1. Parasitic infection

The intestinal myxosporean parasites *Enteromyxum leei* and *Enteromyxum scophthalmi* are major pests for sea bream (*Sparus aurata*) and turbot (*Scophthalmus maximus*), respectively. These parasites contribute to the development of gut inflammation (enteritis), impaired food intake and assimilation, thus causing major economic losses as there are currently no efficient treatments for the infected fish. Both fish species have been examined for the transcriptional response to the pathogen in the intestine. In the sea bream, the transcriptomic changes to the parasite infection were examined using a cDNA microarray (18,400 features), enriched for differentially expressed genes following SSH cloning (Davey et al., 2011). Three groups of fish were examined, non-exposed control fish, exposed and infected individuals, and the third group was exposed but not infected (*i.e.*, assumed to be resistant to the pathogen). The infection model lasted 113 days, as previously described (Sitjà-Bobadilla et al., 2008). The results showed contrasting outcomes from the fish groups. In the infected group, there were many more down-regulated rather than up-regulated genes in the intestine, suggesting a suppression of responses. In contrast, the resistant group had more genes up-regulated than down-regulated. Gene set enrichment by GO analysis using Blast2GO (Götz et al., 2008) revealed that digestive function in the infected group was potentially altered by reduced expression of genes associated with digestive proteases, including cathepsins, trypsins and chymotrypsins. This effect was paralleled by a clear decrease in expression of immune genes, including complement components, mannose-binding lectins and acute phase proteins amongst others. In contrast, those fish that were able to recognise and prevent parasite amplification (resistant fish) had enriched GO terms for immune activation signal, particularly interferon signalling and antigen presentation, suggesting an active immune role in the removal of the parasites. The resistant fish also showed MHC class II genes increased, possibly indicating an interferon-related and adaptive cell-mediated immune response. Together, the results suggested that the parasite depressed the immune responses to become established and replicate, but the host response varied between individuals with the resistant fish mounting an effective response. One gene of significance was the mannose-binding lectin that is central to the initiation of the lectin complement pathway, which has been often increased in expression during immune activation.

A follow-up experiment on sea bream infected with *Enteromyxum leei* included dietary manipulation, with fish fed diets containing either fish oil or vegetable oil (66VO) for 9 months prior to parasite exposure. Infected fish fed both diets showed signs of anorexia, but the 66VO diet fed fish had more severe disease symptoms and poorer outcome as a result of the parasite challenge (Estensoro et al., 2011). Parasite load was determined by PCR at 32, 53 and 88 days following exposure and individual fish grouped as being early infected (by 53 days) or late infected (88 days), with final sampling at 102 days. A customized sea bream oligo microarray platform was developed (Calduch-Giner et al., 2012), enriching the gene set on the previous cDNA microarray. Principal component analysis revealed two clear groups splitting the control and infected groups irrespective of diet. Secondly, a less dramatic grouping could define the fish fed fish oil at both early and late infection differing from the two infected groups fed the 66VO diet. In total, more than 2000 genes were differentially expressed, but using the filtering criteria only one gene was found altered between the uninfected diet groups. The genes responding to infection were grouped by k-means clustering that identified 4 clusters, strongly up-regulated in all infected groups, moderately up-regulated, strongly down-regulated and moderately down-regulated. The first cluster contained 88 genes, which surprisingly did not reveal any enriched GO categories. This potentially reflected a combination of poor annotation and a disparate group of genes with varying functions. The second cluster representing moderately up-regulated genes showed enrichment for several key metabolic processes including translation, RNA processing and cell cycle, but also included functions related to urea cycle. The overall depression of immune-related genes (as well as transcripts related to protein and lipid metabolism) confirmed the results from Davey et al. (2011), but with a clear effect of diet changing the magnitude of the response. Of interest was the alternative activation of macrophages relating to clearing of pathogens with arginase-1. Highly up-regulated were also genes encoding enzymes related to polyamine synthesis increase, such as ornithine decarboxylase and ornithine aminotransferase. The authors suggest that the increase in polyamines and the ornithine production from arginine *via* arginase-1 represses the synthesis of nitric oxide as found in their previous experiments (Estensoro et al., 2011).

In turbot, the pyloric caeca, head kidney and spleen were analysed by RNA-seq following an oral infection of *E. scophthalmi via* intestinal scrapings of previously infected fish (as described by Redondo et al., 2002), 42 days following infection. Three individuals were selected showing heavy infestation and “severe” lesions (Bermúdez et al., 2010) and compared to a time-matched control. Paired-end libraries were sequenced (approximately 15M reads per sample), mapped to the turbot genome (NCBI: PRJEB11743) and differentially expressed genes identified using EdgeR and filtered by Log2 fold change > 1 and *P*-value < 0.05, following correction for FDR (Robledo et al., 2014). The pyloric caeca showed the highest number of genes responding (562 increased and 851 decreased), possibly reflecting ongoing severe inflammation. In both head kidney and spleen, there was also a greater number of genes decreased in expression. Only 2.5% (117 genes) of the modulated genes were in common between tissues suggesting the tissue-specific responses. Within the commonly modulated group, interferon-related genes were decreased in expression, as were MHC I transcripts. To gain functional interpretation, genes were categorised by GO and grouped for immune and defence response, apoptosis and cell proliferation, iron metabolism, metabolism and digestive function and cytoskeleton/extracellular matrix. The intestine showed clear inflammatory responses, and genes considered to be markers for dendritic-like cells, CD83 and CD209, were increased in expression. Tissue repair and remodelling genes were also increased in the pyloric caeca, which may be associated with the overall degeneration of the gut function. Metabolic and digestive enzyme genes were also severely decreased in expression. Importantly, there were a number of common features between the enteromyxosis infections in sea bream and turbot, most clearly seen by the normal digestive function being decreased in the heavily infected fish. However, there were also some contrasting functions being altered. In the sea bream, there was a general depression of immune genes in intestine, whereas these genes had significantly increased expression in the turbot. In both cases, the adaptive immune response as seen in head kidney was repressed, especially in the infected fish. When comparing such studies, consideration needs to be given to the infection kinetics, species-specific host parasite interaction and tissue examined (in this case, pyloric caeca in turbot and unspecified region of intestine in sea bream).

3.1.2. Viral response

An example the intestinal transcriptome response to viral infection was studied in grass carp infected with grass carp reovirus (GCRV), taking an RNA-seq approach (Shi et al., 2014). Four different tissues were analysed in parallel: intestine, gill, liver and spleen. Juvenile carp were infected by bath challenge and samples

retained for RNA-seq at times 0, 2, 24, 48, 96 and 120 h post-infection, all challenged time points were compared to the uninfected group at time 0. The RNA-seq reads were aligned to the grass carp genome (NCBI Bioproject: PRJNA39737, Wang et al., 2015) with approximately 7M reads per sample on Illumina HiSeq with expression determined by RPKM method (Mortazavi et al., 2008). At the time when the experiment was performed, the genome was fully annotated and further Blast searches were used to give putative identifications to the differentially expressed genes with an *E*-value of 10^{-6} being used to define identity. Differentially expressed genes were filtered at *P*-value < 0.0001 (FDR) and fold change Log2 > 1. The highly stringent filtering is likely to reflect the massive changes in genes expression resulting from the challenge. The main outcome from the experiment was the highly conserved response in all the tissues examined, with majority of the differentially expressed genes shared between tissues. However, the temporal response varied between tissues, with both gill and intestines grouping closely together with limited numbers of genes altered at the early time points (2, 24 and 48 h), followed by much more substantial changes at later sampling points (96 and 120 h). The pattern obtained here is of interest as the sites of pathogen invasion were likely to include both intestine and gill. However, there was a clear major immune response from the primary and secondary immune organs. This virus is known to cause haemorrhaging in muscle and gill, and enteritis in intestine, which was further confirmed by the experiment. The GO enrichment analysis indicated the large numbers of functional terms related to metabolic processes and immune function, as well as associated processes contained within these categories. Much focus was given to energy metabolism particularly *via* gluconeogenesis. One pathway of interest in the intestine was the adiocytokine pathway, which can be stimulated by pro-inflammatory cytokines such as TNFα and the hormone leptin. The peptide hormone neuropeptide Y was only found in the intestine and was decreased in expression following the viral infection. However, the reason behind that alteration is unclear because the infected fish are unlikely to ingest any food, and this result may be a reflection of altered behaviour during the infection. Genes associated with classic, lectin and alternative complement pathways were altered in gill, liver and spleen, whereas in liver only the alternative complement pathway genes were enriched, showing the organ-specific responses. Antigen presentation *via* MHC I was increased in all tissues as would be expected following an antiviral response and type I interferon-stimulated genes.

3.1.3. Bacterial response

The bacterial pathogen *Edwardsiella ictaluri* that causes enteric septicaemia in the channel catfish (*Ictalurus punctatus*) has been used as model for intestinal responses to bacterial infection by RNA-seq approach (Li et al., 2012). This pathogen can cause varying pathology from chronic long-term infection to acute disease. The experimental samples were generated following a bath challenge of 4×10^8 CFU/ml for 2 h using the MS-S97-773 bacterial isolate, and fish were sampled at times 0, 3, 24 h and 3 days post-infection. For these experiments, whole intestine was used and pools of 10 fish per time-point were generated, with single libraries constructed for each time point. Although this approach limited the power of the experiment, the authors acknowledged this potential limitation and generated lists of differentially expressed genes, many of which were confirmed by quantitative PCR. A two-stage approach was taken for the experimentation, with the generation of a comprehensive *de novo* transcriptome followed by identification of differentially expressed genes. Different *de novo* assemblers were used to assess the quality of assemblies that were generated from the Illumina reads. These included five assembly programs: ABySS (Simpson et al., 2009), TransABySS (Robertson et al., 2010), Velvet (Zerbino et al., 2009), Assembly assembler and CLC genomics workbench. The authors concluded that TransABySS gave the best coverage of large contigs, which were annotated following Blast searches. At 3, 24 h and 3 days, 693, 918 and 1035 genes were altered, respectively. At both 3 and 24 h, similar numbers of genes were up and down-regulated. However, at 3 days there were considerably more genes decreased in expression than increased. Six main functional groups were found to respond, including cytoskeleton, cell junctions, lysosome/phagosome, inflammation, pathogen recognition and endocrine factors. The increase in cytoskeleton-related genes was interpreted as a bacterial entry method into the cells, possibly by inducing "ruffles" as described for cellular salmonella invasion (Hallstrom and McCormick, 2011), and could also be interpreted as altering permeability of apical junctions and hence pathogen entry. Genes encoding the apical junction complex (tight junctions and desmosomes) and claudins suggested reduced cellular connectivity and an increase in paracellular leakage. Many immune and inflammatory genes, including nattectin, CD209 and C1q-like genes, were markedly decreased in expression, suggesting mechanisms of immune evasion driven by *Edwardsiella ictaluri* secreted effectors. There was also a minimal response of TLR5, which recognises flagellin, further suggesting the inhibition of immune response. The conclusions from this paper show parallels found in the intestine of fish following dietary change with dysfunction of the cellular junction and trans- and paracellular transport related genes changing in expression. The data set generated by Li et al. (2012) has been followed up using meta-analysis to examine specific gene families in the channel catfish, showing the wealth of information generated by a single deep sequencing experiment. Specifically, the HSP40 gene family (Song et al., 2014), cytochrome P450 genes (Zhang et al., 2014), claudin genes (Sun et al., 2015) and tumor suppressor genes (Mu et al., 2015) were investigated. In each case, the full repertoire of the channel catfish genes has been resolved and their response to the *E. ictaluri* infection in the intestine evaluated.

3.1.4. Immunostimulants

In addition to the direct effects of pathogens on the intestinal transcriptome, the modifying effects of immunostimulants and functional feeds are most likely to have their effect on the intestine as well. Rainbow trout fed a commercially relevant immunostimulatory diet (Gama Quakistar 26, BioMar, aimed at producing a PAMP-PPR host response following feeding) was examined using an early cDNA microarray platform (SFA2.0) containing 1.8K features (Jørgensen et al., 2008). The trial lasted for four weeks and both gill and mid intestine were used for transcriptome analysis (Doñate et al., 2010). There was little in common in the responses between tissues as many of the alterations in gill would have been secondary effects, whereas direct alteration by dietary components (and microbiota) would be observed in intestine. Only two genes were found to be altered in a common direction between the two tissues, a metalloprotease 9 and a cyclin D2 gene. Of interest, both tissues showed a general decrease in immune-related genes, both by direct observation, but also following GO enrichment analysis. In the intestine, genes associated with general inflammatory response, response to biotic stress and response to bacteria were depressed, however several genes associated with NF-kappa B cascade were found enriched. Similar decreases in immune activity were also demonstrated in liver of salmon fed immunostimulant diets (Tacchi et al., 2011b), which suggests that these diets may not act through the induction of localized inflammatory response.

3.1.5. Vaccination

The last example in this section is the intestinal response to oral

vaccination. This area of research is likely to increase dramatically in the future as the vaccine stability will be strongly affected by gut function and host gene expression. Some bacterial pathogens can be controlled by oral vaccination such as *Yersinia* (Ghosh et al., 2016) and oral vaccines to viral pathogens are under intense research (Rivas-Aravena et al., 2015). Examples of transcriptomics being used to define the effect on intestinal response can be found for both European seabass (Sarropoulou et al., 2012) and rainbow trout (Ballesteros et al., 2012a). In seabass, a commercial oral vaccine for protection against *Vibrio anguillarum* (Aquavac Vibrio Oral) was used to explore the nature of the cellular response by examining the mRNA profiles of both the intestine and the head kidney. An initial vaccination followed by a booster four months after the primary vaccination gave protection of 57% and 33% at 30 and 80 days post-challenge, respectively, suggesting a protective response being initiated. The samples for transcriptome analysis were taken one day following the end of the booster vaccination (156 days from primary vaccination) and RNA was subjected to 454 sequencing. A comprehensive transcriptome of 71,676 clusters was obtained for the intestine and 49,089 for the head kidney, from which 336 genes were found differentially expressed in the gut. However, in neither tissue there was a convincing immunological response observed between vaccinated and non-vaccinated fish. The results from this paper significantly added to the sequence data available for sea bass at the time, but also revealed the need to have more comprehensive biological replicates. The authors also commented on the issues associated with low abundance of transcripts, and the presence of potential isoforms for the same gene. It was likely that the depth of sequencing by the 454 platform did not allow for the robust statistical analyses available now for the Illumina approaches.

A microarray approach was taken for rainbow trout that were given an oral DNA vaccination in microspheres containing a recombinant plasmid to express the VP2 gene of IPNV or the empty plasmid, and fish were sampled following 7 days (Ballesteros et al., 2012a). This vaccination had previously been found to elicit protection (Ballesteros et al., 2012b). This early time point of 7 days was expected to reveal immunological responses to the vaccine, but would not have generated a full acquired response in this species. An Agilent 8 × 15K oligo array was designed to be highly enriched for immune genes taken from publically available resources at the time (Ballesteros et al., 2012a). In the intestine, more genes were down-regulated than increased in expression, whereas the opposite was found in head kidney, showing tissue-specific response, as was shown previously for infection studies. Both tissues showed a significant type I interferon response and many interferon-induced genes being increased in expression, confirming antiviral response. There were differences in complement gene responses between tissues with classic complement activation in head kidney, but only genes suggesting alternative complement pathway were altered in the pyloric caeca. This targeted immune array was unable to take a fully global view of the responses and many key metabolic pathways that also change during the vaccination would have been missed, but the experiment did identify immune factors altered during antigen uptake in the intestine and then the systemic response in the head kidney. Further work could define the expression location of the plasmids that might help to interpret the tissue-specific transcriptional responses.

3.2. Search for alternative plant materials in aquaculture feeds

Farmed carnivorous fish, such as salmonids, were historically fed diets containing high levels of fish meal as protein source and fish oil as lipid source to mimic their natural feeding habits (Naylor et al., 2009). With the rapid expansion of aquaculture and the limited availability of wild-caught fish, further reliance on fish meal and fish oil has become unsustainable. Instead, the future of aquaculture depends to a large extent on alternative plant proteins and vegetable oils that would effectively replace limited marine ingredients in the diets. However, the use of plant materials to feed carnivorous fish does not come without its own challenges, such as a wide range of anti-nutritional factors (ANFs) produced by plants to defend themselves from predation by animals. When ingested, these ANFs typically interfere with digestion, absorption and utilisation of nutrients and have numerous adverse effects on intestinal physiology as well as animal health and performance (Krogdahl and Bakke, 2015). Furthermore, plant materials may substantially differ from marine ingredients in their amino acid and fatty acid profiles (Tocher and Glencross, 2015). Understanding the effects of plant materials on fish health has been greatly advanced by combining feeding trails with large-scale analysis of gut

Table 2
Fish studies using high-throughput profiling to characterise gut transcriptome responses to dietary plant materials.

Fish species	Dietary manipulation	Comparison & sampling	Tissue analysed	Method & platform	References
Plant proteins					
Atlantic salmon (*Salmo salar*)	2 diets with 20% soybean meal (SBM) and fish meal (FM)	Fish fed SBM *vs* FM diets (1, 2, 3, 5 and 7 d)	Distal intestine	Microarray, cGRASP 44K salmonid platform	Sahlmann et al., 2013
Atlantic salmon (*Salmo salar*)	4 diets with 0, 10, 20 and 30% soybean meal (SBM)	Fish fed 10, 20 and 30% SBM *vs* 0% SBM (12 weeks)	Multiple, including distal intestine	Microarray, A-MEXP-2065 (ArrayExpress)	De Santis et al., 2015a
Atlantic salmon (*Salmo salar*)	6 plant protein (PP) diets including soybean meal and 1 diet with fish meal (FM)	Fish fed PP *vs* FM diets (56 d)	Distal intestine	Microarray, A-MEXP-2065 (ArrayExpress)	Król et al., 2016
Atlantic salmon (*Salmo salar*)	2 diets with soy protein concentrate (SPC) and fish meal (FM)	Fish fed SPC *vs* FM diets (77 d)	Multiple, including mid intestine	Microarray, A-MEXP-2065 (ArrayExpress)	Tacchi et al., 2012
Atlantic salmon (*Salmo salar*)	5 plant protein (PP) diets with soyasaponin supplementation and 5 PP non-supplemented diets	Fish fed supplemented *vs* non-supplemented diets (80 d)	Distal intestine	Microarray, GPL10706 (Gene Expression Omnibus)	Kortner et al., 2012
Atlantic salmon (*Salmo salar*)	2 diets with genetically modified (GM) and non-GM maize	Fish fed GM *vs* non-GM diets (82 d)	Multiple, including distal intestine	Microarray, cGRASP 44K salmonid platform	Sissener et al., 2011
Zebrafish (*Danio rerio*)	2 diets with high (HNPM) and low (LNPM) novel protein meal	Fish fed HNPM *vs* LNPM diets (21 d post fertilization)	Intestine	RNA-seq, Illumina HiSeq™ 2000	Rurangwa et al., 2015
Vegetable oils					
Atlantic salmon (*Salmo salar*)	2 diets with vegetable (VO) and fish (FO) oil	Lean and fat fish fed VO *vs* FO diets (55 weeks)	Pyloric caeca	Microarray, A-MEXP-1930 (ArrayExpress)	Morais et al., 2012a
Atlantic cod (*Gadus morhua*)	2 diets with vegetable (VO) and fish (FO) oil	Fish fed VO *vs* FO diets (12 weeks)	Intestine (midgut)	Microarray, A-MEXP-2053 (ArrayExpress)	Morais et al., 2012b
Atlantic salmon (*Salmo salar*)	3 diets with fish oil (FO), wild-type *Camelina* oil (WCO) and engineered *Camelina* oil (ECO)	Fish fed FO *vs* WCO *vs* ECO diets (7 weeks)	Pyloric caeca	Microarray, A-MEXP-2065 (ArrayExpress)	Betancor et al., 2015b

transcriptomes (Table 2).

3.2.1. Plant proteins

In recent years, significant progress has been made towards replacing fish meal with plant protein meals and concentrates made from legumes such as beans, peas and lupins, which are rich in digestible proteins and have favourable amino acid profiles (Hardy, 2010). Legumes are generally high in ANFs, but their specific profile varies from plant to plant and depends on the method used to extract proteins (Champ, 2002). When salmonids are fed diets containing full-fat or solvent extracted soybean meal (SBM), they develop an inflammatory condition in distal intestine, called gut inflammation or enteritis, commonly characterised by shortening of mucosal folds, infiltration of the lamina propria with inflammatory cells, increased numbers of goblet cells and decreased numbers of absorptive vacuoles in the enterocytes (Baeverfjord and Krogdahl, 1996).

Global gut gene expression profiling has provided important insights into mechanisms underlying SBM-induced enteritis by focussing so far on early progression of the disease (Sahlmann et al., 2013), increasing levels of SBM in the diet (De Santis et al., 2015a) and the specificity of SBM effects (Król et al., 2016). All these studies were performed on Atlantic salmon, with fish being sampled either during the first week of exposure to SBM diet (Sahlmann et al., 2013) or at the end of 8–12 week feeding trials (De Santis et al., 2015a; Król et al., 2016). Switching diet from fish meal to SBM resulted in rapid changes in the gut transcriptome, indicating the initiation of an immune response followed by dysfunction of intestinal barrier and gut (Sahlmann et al., 2013). The most prominent gene expression changes were observed on days 3 and 5 of dietary manipulation, with the immuno-related transcripts dominating during the first 5 days of exposure and the genes linked to the gut function dominating from day 5 onwards. Among the most up-regulated genes associated with the immune response were GTPase IMAP family members, NF-κB-related genes and regulators of T cell and B cell function. Subsequent down-regulation of transcripts related to endocytosis, exocytosis, detoxification, transport and metabolic processes suggested an impairment of intestinal barrier and suboptimal gut function. Furthermore, comparison of the gut immune responses associated with the short (Sahlmann et al., 2013) *versus* long (De Santis et al., 2015a; Król et al., 2016) exposure to SBM diet indicated the presence of active inflammation that was independent of the duration of dietary manipulation, with no obvious signs of resolving the condition. Indeed, the gut transcriptome responses to chronic SBM treatment were characterised by: 1) activation of T cell mediated processes *via* up-regulation of the CD86 antigen, cytotoxic T lymphocyte-associated protein 4, interleukin-18 (IL-18) and IL-22, 2) increased expression of T cell receptors, and 3) activation of TNF- and NF-κB-mediated responses and up-regulation of components of the respiratory burst complex *via* TNF signalling pathway (De Santis et al., 2015a). The nature of these alterations might reflect the ongoing excessive translocation of luminal bacteria, viruses and antigens across the intestinal epithelium, which was further supported by the enrichment of the pathways related to endocytosis, such as clathrin-mediated endocytosis signalling, macropinocytosis signalling and virus entry *via* endocytic pathways (Król et al., 2016). Finally, the latter study reinforced the idea about microbial translocation contributing to SBM-induced enteritis by identifying pathways that were directly linked to the intestinal barrier function (e.g., remodelling of epithelial adherens junctions and epithelial adherens junction signalling) and significantly modified by the SBM treatment.

Further processing of SBM to produce soy protein concentrate (SPC) typically uses an aqueous alcohol wash, which removes alcohol-soluble ANFs, such as saponins. Since feeding salmon diets containing SPC does not induce major changes in gut histology or transcriptome that resemble the SBM-induced enteritis (Tacchi et al., 2012; Król et al., 2016), development of this condition has been linked to the presence of saponins (Knudsen et al., 2007; Krogdahl et al., 2015). However, when gut transcriptome profiling was combined with the use of purified soy saponins to supplement salmon diets containing different plant proteins, it became clear that some supplemented diets were safe to use (corn gluten, sunflower, rapeseed and horsebean), while combination of saponins with pea protein concentrate (PPC) caused enteritis and major changes in gut transcriptome (Kortner et al., 2012). These changes included up-regulation of cytokines, NF-κB- and TNF-related genes and regulators of T cell function, while the IFN-axis was suppressed. Furthermore, the induction of lectins, complement, metalloproteinases and the respiratory burst complex paralleled a down-regulation of genes for free radical scavengers and iron-binding proteins. The important implication of this study is to demonstrate that saponins do not necessarily cause enteritis on their own or without being potentiated by other ANFs. Instead, they may act to increase gut permeability and therefore expose the local immune system to antigens that would not normally cross the intestinal epithelial barrier or would cross it at lower rates (Knudsen et al., 2008; Penn et al., 2011; Chikwati et al., 2012; Krogdahl et al., 2015). Despite intensive research, the antigens responsible for triggering the SBM-induced inflammatory reaction in salmon gut have not yet been identified (Couto et al., 2014).

There is growing evidence that gut inflammation in salmon may also be induced by high dietary inclusions of plant proteins that are naturally low in saponins. These include bean protein concentrate (BPC) made from the faba bean (*Vicia faba*), produced by fine grinding of dehulled seeds into flour, followed by air classification (De Santis et al., 2015b; Penn et al., 2011). The ANF profile of BPC is characterised by high levels of condensed tannins and the presence of faba bean-specific glucosides such as vicine and convicine (Helsper et al., 1993). The analysis of gut transcriptome responses to diets containing 36% SBM and 45% BPC revealed that both diets generated substantially different and unique gene expression profiles, with relatively few transcriptomic alterations common for both treatments (Król et al., 2016). The nature of these common responses (especially alterations of ILK signalling and germ cell-Sertoli cell junction signalling pathways) suggests that although the mechanisms by which different plant proteins affect gut health may be different, they are all likely to contribute to the overall loss of intestinal integrity that promotes inflammation. The overlapping gut transcriptomic responses to SBM and BPC diets (Fig. 4A) are of special interest for aquaculture as they may harbour biomarkers that characterise all types of gut inflammatory diseases, independent of the their origin and causes.

Finally, gut transcriptome profiling has contributed to understanding the impacts of single *versus* mix plant proteins on fish health and performance (Król et al., 2016). When different plant proteins (SPC and BPC) were mixed, they generated less extensive alterations of the gut transcriptome relative to single plant protein diets with either 45% SPC or 45% BPC, probably due to reduced levels of individual ANFs (Fig. 4B). Importantly, the mixed plant protein diets were associated with an improved body composition of fish, suggesting a potential link between the magnitude of changes in the gut transcriptome and whole-animal performance. Furthermore, fish with histologically more advanced gut inflammation (moderate enteritis induced by 36% SBM) had more extensive alterations of gut transcriptome than fish with mild enteritis induced by 45% BPC. The results of the faba bean (Król et al., 2016) and other plant protein studies presented in Table 2 clearly indicate that gut transcriptome profiling provides a useful tool for testing the applicability of alternative protein sources for

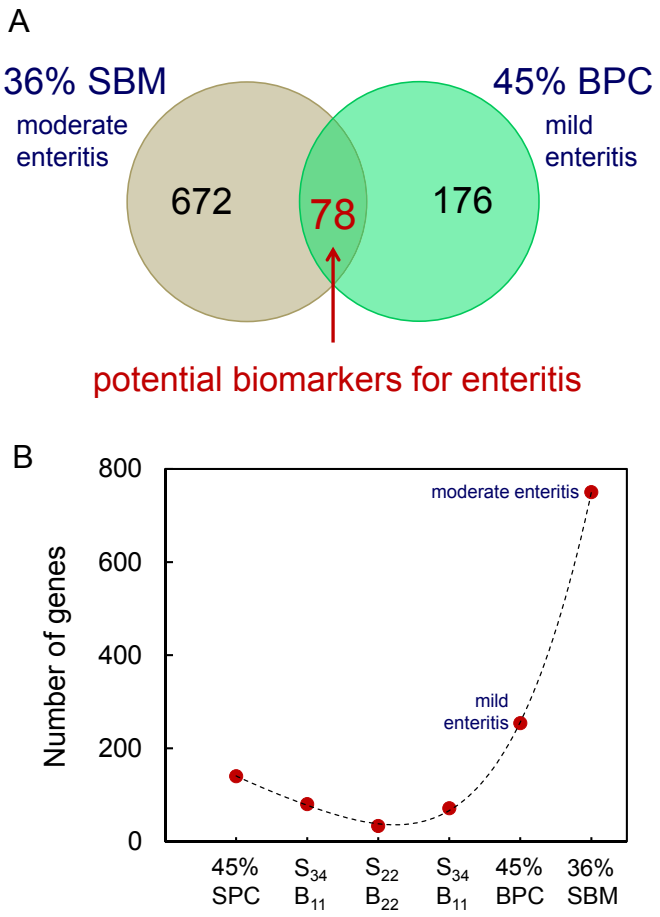

Fig. 4. Results from a microarray experiment characterising the gut transcriptome responses to dietary plant proteins in Atlantic salmon (modified from Król et al., 2016). (A) Venn diagrams show the numbers of common and unique genes altered in gut transcriptome by single plant protein diets, enriched with either 36% soybean meal (SBM) or 45% bean protein concentrate (BPC) (B) Number of differentially expressed genes in the gut of fish fed single (45% SPC and 45% BPC) *versus* mixed ($S_{34}B_{11}$, $S_{22}B_{22}$, $S_{11}B_{44}$) plant protein diets and 36% SBM.

aquaculture feeds and for designing diets with the reduced impact of ANFs on fish health.

3.2.2. Vegetable oils

The major obstacle to effective replacement of fish oil with vegetable oil is the difference in their fatty acid profiles, especially in the content of omega-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFA) such as eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids (Hixson, 2014). These fatty acids are essential or conditionally essential for vertebrates and have well-established health effects in humans, but they are not synthesised by terrestrial plants (Calder, 2013). As a result, feeding farmed fish with vegetable oil in an uncontrolled manner may have detrimental effects on fish health and compromise the health benefits of humans that consume these fish (Gil et al., 2012). In recent years, significant progress has been made in understanding the effects of vegetable oil on fish health by gene expression profiling of pyloric caeca and midgut.

The effects of vegetable oil (a blend of rapeseed, palm and *Camelina* oils) and fish oil on the pyloric caeca transcriptome in Atlantic salmon depended on the genetic background of fish (lean *versus* fat) (Morais et al., 2012a). At the completion of a 55-week feeding trial, the lean fish showed an enhanced response to the low content of n-3 LC-PUFA in the vegetable oil, based on their magnitude of changes in the expression levels of Δ5fad, Δ6fad, elovl5b and elovl2 genes. Furthermore, the vegetable oil increased lipogenesis in lean fish (as assessed by expression of FAS), while β-oxidation appeared unaffected, although transcripts involved in mitochondrial respiratory or electron transport chains were down-regulated. Both lean and fat fish fed vegetable oil were characterised by relatively high expression levels of genes involved in xenobiotic metabolism (CYP1A and EPHX2), antioxidant defence (CAT, HPX and PRDX1) and apoptosis (Casp3B), probably reflecting the presence of contaminants such as polycyclic aromatic hydrocarbons in the vegetable oil.

The applicability of different inclusion levels of *Camelina* oil to replace fish oil was evaluated in a 12-week feeding trial performed on juvenile Atlantic cod, with focus on the gene expression profile of midgut (Morais et al., 2012b). The microarray analysis identified a total of 289 genes that were significantly altered by 66% *Camelina* oil *versus* fish oil. These genes were linked to translation (18% of all genes), cell proliferation, differentiation and apoptosis (14%) as well as a structural molecule activity (12%), followed by transporter activity (9%) and immune response (7%). In contrast, metabolism appeared to be less affected, with 6% of the genes involved in proteolysis, 5% involved in energy metabolism and 4% in lipid metabolism. Other minor categories included regulation of transcription (4%), signalling (4%) and protein folding (3%). Overall, the gene expression profile of midgut in fish fed 66% *Camelina* oil was consistent with the altered balance between cell proliferation and death, leading potentially to the different rates of tissue regeneration and/or repair.

One approach that has recently received considerable attention in the context of fish oil replacement in aquaculture feeds is the use of metabolically engineered *Camelina sativa* to synthesize n-3 LC-PUFA in seeds (Betancor et al., 2015a). To achieve this goal, transgenic *Camelina sativa* was equipped with a suite of five microalgal genes to produce seed oil that contained 20% of total fatty acids as EPA. The feeding trial was performed on Atlantic salmon and lasted 7 weeks, during which fish were fed diets containing engineered *Camelina* oil (ECO) with EPA, wild-type *Camelina* oil (WCO) or fish oil (Betancor et al., 2015b). The microarray analysis identified a total of 2298 differentially expressed genes in the pyloric caeca between fish fed ECO and fish oil diets, while 1152 genes with differential expression were found between fish fed ECO and WCO diets. The analysis showed that both ECO and WCO diets induced a similar transcriptomic response in the pyloric caeca in comparison to fish oil diet. Thus, when the transcriptomes of pyloric caeca from fish fed ECO and WCO diets were compared to fish fed fish oil diet, the same cell processes were similarly affected, with the major categories being metabolism, signalling and immune response. Within metabolism, the pathways most affected were that of lipid metabolism. However, all these changes were subtle and provided evidence that the oil from genetically modified *C. sativa* did not have any unexpected or potentially detrimental effects on gut transcriptome and function. These results along with the result of the two previous studies (Morais et al., 2012a,b) clearly indicate that gut transcriptome profiling provides a useful tool for testing the applicability of vegetable oils to substitute fish oil in aquaculture feeds.

3.3. Environmental stress and developmental factors

Stress can encompass many different aspects of health, nutrition and environmental change and thus may lead to dysfunction in the intestine, potentially with a common signature in the transcriptome response (Table 3). Xia et al. (2013) subjected Asian seabass (*Lates calcarifer*) to four different stress challenges (LPS

Table 3
Fish studies using high-throughput profiling to characterise gut transcriptome responses to environmental stress, gut microbiota and transgenic manipulations.

Fish species	Type of manipulation	Comparison & sampling	Tissue analysed	Method & platform	References
Environmental stress					
European eel (*Anguilla anguilla*)[a]	Experimental transfer from fresh (FW) to salt (SW) water	SW *vs* FW fish (6 h, 2 and 7 d, then 5 months)	Multiple, including intestine	Microarray, E-MAXD-24 (ArrayExpress)	Kalujnaia et al., 2007a,b
Brown trout (*Salmo trutta*)[a]	Chronic metal exposure	Fish from rivers with high *vs* low metal content	Multiple, including intestine	RNA-seq, Illumina GAIIx Genome Analyzer	Uren Webster et al., 2013
Asian seabass (*Lates calcarifer*)[b]	High salinity (33 ppt)	Stressed *vs* control fish (8 d)	Intestine	RNA-seq, 454 GS FLX Titanium (Roche)	Xia et al., 2013
Japanese medaka (*Oryzias latipes*)[b]	Exposure to space environment	Space *vs* ground fish (56 −60 d)	Multiple, including intestine	RNA-seq, Illumina HiSeq™ 2500	Murata et al., 2015
Gut microbiota manipulation					
Zebrafish (*Danio rerio*)	Germ-free (GF), conventionalized (CONV) and conventionally raised (CONR) fish	GF *vs* CONV *vs* CONR fish (6 d post fertilization)	Intestine	Microarray, 16K zebrafish platform	Rawls et al., 2004
Transgenic model of immunodeficiency					
Zebrafish (*Danio rerio*)	Rag1^{-}/$^{-}$, recombination-activating gene 1 knockout	Rag1^{-}/$^{-}$ *vs* Rag1^{+}/$^{-}$ fish	Multiple, including intestine	Microarray, GPL7244 (Gene Expression Omnibus)	Jima et al., 2009

[a] Experiment performed on wild fish.
[b] Experiment performed on farmed fish.

injection, *Vibrio* infection, salinity changes and fasting) and used an Illumina RNA-seq approach to explore the transcriptome responses. These stressors were very different in nature and for each one the majority of responses were unique. However, there were common intestinal responses with 59 genes being altered on all occasions. As expected, common genes were found between the LPS and bacterial challenges, but the response in intestine was mostly a decrease in gene expression, potentially reflecting the route of infection. Although the authors were attempting to define a comprehensive intestinal response to stressors, the challenges were probably too diverse to gain a complete picture of responses.

An extreme example of fish stress was examined in medaka (Murata et al., 2015), where these fish were flown to the International Space Station, following which tissues were subjected to RNA-seq in comparison to non-flying controls. The interest here is that the intestine showed the greatest response to zero gravity when compared to eye, brain, liver and gonads, and the enrichment for GO terms in the intestine included antigen presentation by MHC I and apoptotic processes. The relevance of this experiment here is debatable, but has been included for completeness.

3.3.1. Role of intestine in osmoregulatory function

The intestinal wall is one of the key organs with the gills and kidney that regulate plasma ions in fish (Marshall and Grosell, 2006), particularly in the species that move between fresh and salt water. Although much work has been carried out on the physiology of the intestine in salmonids relating to ion transport (Sundell and Sundh, 2012), there is still no in-depth transcriptomic reports on how the gene expression signature changes in the intestine between fresh and salt water. Tilapia species are known for their varying tolerance and performance in different salinities, which is clearly seen in *Oreochromis niloticus* and *Oreochromis mossambicus*, with *Oreochromis niloticus* performing poorly in salt water. To examine the differing intestinal transcriptomic responses to salinity, fish were either maintained in fresh water or acclimated to full strength sea water (3.5% salinity) over a period of one week (Ronkin et al., 2015). After four weeks, RNA was extracted from both anterior and posterior intestine and RNA-seq analysis performed on Illumina HiSeq 2000, with reads mapped against a reference tilapia transcriptome. Transcripts with >3.5 fold change in response were selected following FDR correction; these genes would represent an adaptive osmoregulatory response rather than acute response. The *O. niloticus* species showed a marked increase in numbers of genes responding particularly in the anterior intestine, but both species appeared to have similar numbers of genes altered in the posterior intestine. As anticipated, many ion channels and transporters were altered, with NKA, NKCC2 and VHA being increased and NCC being decreased in response to salinity. As with salmon gill (Nilsen et al., 2007), the tilapia intestine also showed differential expression of several NKA α1 subunits, but these differences were associated with the different sections of the intestine being sampled. An unexpected finding was that the intestinal region had the larger effect on the gene expression than species or salinity, making it difficult to compare experiments and draw general conclusions. Indeed, it should be noted that tilapia have particularly long intestine up to 8 times their body length (Smith et al., 2000), leading to great differences between fish species intestine function. Unfortunately, the data were not used for advanced gene set enrichment, only a basic GO analysis was performed at high level, but it did confirm increased transporter activity to be a major grouping.

The European eel (*Anguilla anguilla*), a species that migrates back to the ocean for spawning, was examined for intestinal transcriptome responses. Here, a 6144 feature cDNA array was used to identity differentially expressed genes between 6 h and five months following transfer (Kalujnaia et al., 2007a,b). In the intestine, major groups of genes that changed were associated with ion transport, energy metabolism and immune function. However, there was no clear pattern that emerged from the analysis of these changes other than immune genes appeared to be altered more during early time points. At the time that the experiments were carried out, the annotation of the microarray was not extensive and only limited functional processes could be determined.

4. Future perspectives

The intestine is a highly complex organ that plays a crucial role in the immune system. As demonstrated in this review, there are numerous factors that can impact both immune function and digestive performance of the intestine, which can be evaluated using the transcriptomic approach. To gain greater interpretation of the transcriptomic experiments will require improved genome annotation across many species, and currently this could be viewed as one of the major obstacles in the field. Defining the cell types involved in either GALT or barrier function will help to reveal

crosstalk between cells and also with the systemic immune system. Such work will require firstly cell culture systems for intestine to be developed, but also other approaches including laser capture of specific cell types, linked with in-depth transcriptome analysis. Model species such as zebrafish are being used more to examine intestinal function as they offer the possibility of transgenic manipulation of the key pathways related to gut performance and immunity. Insights into the epigenomic landscape of the intestine, which is an emerging area of investigation in fish immunity, will help to define how early life experience regulates gene expression in later life. Gene editing using approaches such as Crispr/Cas9 will play an important role in future research and potentially in aquaculture. Finally, the relationship between the microbiome and host gene responses will be explored to improve nutrient uptake, fish performance as well as vaccine efficacy and effectiveness. Together, the emerging technologies will be highly beneficial to further understanding and manipulating intestinal function for both disease control and improved performance.

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