Transcriptomic Assessment of Dietary Fish Meal Partial Replacement By Soybean Meal and Prebiotic Inclusion In The Liver of Juvenile Pacific Yellowtail (*Seriola Lalandi*)

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Abstract

Seriola lalandi is an important species for aquaculture, due to its rapid growth, adaptation to captivity and formulated diets, and high commercial value. Due to the rise in fish meal (FM) price, efforts are made to replace it partially or entirely with vegetable meals in diets for carnivorous fish. The use of prebiotics when feeding vegetable meals has improved fish health. In this study, four experimental diets were assessed in juveniles, the control diet consisted of FM as the main protein source, the second diet included 2% GroBiotic®-A (FM-P), the third included FM with 25% replacement by soybean meal (SM25), and the fourth consisted in SM25 with 2% of GroBiotic®-A (SM25-P). RNA-seq of the liver tissue was performed, followed by differential expression analysis and functional annotation to identify genes affected by the diets. Growth was not affected by this level of FM replacement, but it was improved by the prebiotic. Annotation was achieved for 78,826 transcripts, of which 135 were up-regulated and 78 down-regulated among all treatments compared to the control. Transcriptomic profiles of control samples were clustered with those from fishes fed SM25 and SM25-P diets, and the most differentiated group was that one fed the FM-P diet. SM25-P showed the least amount of differentially expressed genes, maintaining general transcriptomic profiles of control diet in the liver, therefore this diet is a potential economic alternative to the FM diet, for *S. lalandi* juveniles. Assessment of the effects of this diet along the digestive tract is recommended.

Introduction

The Pacific yellowtail *Seriola lalandi* is a pelagic marine fish of the perciform order of the Carangidae family, which is distributed worldwide in temperate and subtropical waters [1] ranging from 18 to 24° C [2–4]. It feeds mainly on mackerel, anchovy, sardine, and squid [2] and can grow up to 2.5 m long and weigh up to 70 kg [5]. It is an important species for commercial and recreational fishing in Japan, Australia, and New Zealand [6]. *S. lalandi* is a commercially important species for aquaculture because it grows rapidly, adapts well to captivity, accepts formulated diets, and has an excellent acceptance in the market and high commercial value due to the quality of its meat [7]. One of the key aspects for the successful culture of this species is adequate feeding and nutrition, which depends on the quality and source of the ingredients that make up the diet [2]. Fish meal is the primary source of protein in diets for carnivorous fish. This ingredient has a high digestibility and an excellent amino acid profile [8]. However, because it is a limited resource, in the last years the costs have increased dramatically, and its availability has decreased since its global production has not increased to meet the demands of the aquaculture industry [9]. Replacing fish meal with alternative protein sources helps to optimize production costs. In this sense, several studies have been performed to replace fish meal with vegetable meals [10, 11]. Soybean meal (SBM) has a good balance of essential amino acids and an excellent nutritional composition at a reasonable price, it is widely used to replace fish meal [12]. However, it should be considered that SBM has several antinutritional factors (ANFs) [13]. Besides, when fed to carnivorous fish SBM can cause intestinal damage leading to enteritis [10] and the ANFs produced by plants as a defense

mechanism against animals can alter the biochemical, physiological, or immunological response of the animals fed with SBM [14–16].

The use of prebiotics in the diets of fish has emerged as an alternative to reduce the adverse effects of alternative proteins such as SBM-based formulated diets [17–19]. Some commercial products containing prebiotics have recently been evaluated with interesting results in fish such as Previda ™, Bio-MOS®, and GroBiotic®-A [20–22]. Prebiotics are indigestible carbohydrates used as food ingredients that beneficially affect the host, by stimulating the growth and activating the metabolism of the health-promoting bacteria in the digestive tract [23]. GroBiotic®-A is a widely used prebiotic in aquaculture, which consists of a mixture of brewer's yeast and dry fermentation products. It is composed of several polysaccharides, one of which, β-glucan is known to induce positive immunological responses in fish [24]. It has been reported that in juvenile hybrid striped bass (*Morone chrysops* × *Morone saxatilis*) the inclusion of GroBiotic®-A in the diet, improved respiratory burst and resistance against infection with *Streptococcus iniae* and showed a significant increase in feed efficiency [18]. In another work, [19] authors reported an improved growth performance in hybrid striped bass fed a diet enriched with GroBiotic®-A and a general improvement in survival. Likewise, it was reported that when GroBiotic®-A was included in the diet, the apparent digestibility coefficients of protein and carbohydrate increased for red drum (*Sciaenops occelatus*) [17]. Other works have shown that prebiotics affect lipid and glucose metabolism in mammals, reducing hepatic lipogenesis, triglycerides levels, and cholesterol in serum and liver while improving glucose tolerance [25, 26].

Differences in diet composition promote changes in the levels of enzyme activities involved in the intermediary metabolism in the fish liver [27]. Metabolic factors, such as metabolite and enzymatic activity, are currently used to determine the capacity for metabolic adaptation to dietary supply in fish. In this regard, key liver enzyme activities of intermediary metabolism have been shown to match well with nutritional status and the growth rate of fish [28]. The liver is the primary metabolic organ of fish, being the center of the intermediary metabolism [28, 29]. It is involved in the use of nutrients, plays an essential role in carbohydrate and lipid metabolism, synthesis of bile salts and most of the plasma proteins and hormones, and is the main energy storage. These features make the liver a reliable indicator in the evaluation of the nutritional condition, including energy metabolism, and an indicator of the state of the fish immune system [30–33]. Moreover, the liver has been the target organ to assess the effect of experimental diets on the physiological performance of fish [28, 29, 31, 34–43]. However, very few studies have evaluated the effect of diets on fish liver physiology at gene expression and transcriptomic levels [29, 42].

In this study, the effects of three experimental diets on the liver physiology of juveniles were evaluated using a transcriptomic approach by implementing the RNA-seq technology. The present work aims to identify which genes were affected in their expression levels due to the inclusion in the diet of SBM, the prebiotic, and their combination, making emphasis on key biological processes like carbohydrate metabolism, lipid metabolism, growth, and the immune response.

Material And Methods

2.1. Experiment design and feeding trial

The juveniles of Pacific yellowtail *S. lalandi* used in this work came from Ejido Eréndira, Ensenada, B.C., Mexico, which were donated from the company Ocean Baja Labs. The feeding trial was conducted in the aquaculture department of the Center for Scientific Research and Higher Education of Ensenada (CICESE). Before the experiment, the fish were placed for 17 days in 3m^3 raceway ponds, then reared for 58 days in a 9 m^3 pond where they reached the size of 97.9 ± 8.5 g. For the experimental trial, 180 fish were randomly taken and distributed in 12 tanks at a rate of 15 organisms per tank. The tanks consisted of circular fiberglass ponds with a capacity of 500 L, with a flat bottom, a biofilter with Kapdes type aeration, with a volume of 350 L, a compensation tank of 275 L, and two water pumps (Sweetwater High efficiency She 3.0 Aquatic Eco-Systems and an Aqua Logic heat pump model 2TWB0018A1000AB). In experimental tanks, dissolved oxygen was maintained between 5-7 mg/L, the temperature at 23.5 ± 1 °C, and total ammoniacal nitrogen below 0.05 mg/L. The fish were in a 12-day acclimatization period in the new culture system, where they fed the commercial diet for marine fish (Skretting: 55% protein and 15% lipids), three times a day (8:00, 12:00, and 16:00 h). The feeding trial was conducted to evaluate the effect of including a commercial prebiotic, GroBiotic®-A, and the replacement of fish meal with SBM [44]. Four experimental diets were formulated (Table 1), the control diet consisted of fish meal as the main protein source (FM), the second diet was formulated with fish meal as the main protein source with the inclusion of 2% GroBiotic®-A (FM-P), the third diet included fish meal with 25% replacement by SBM (SM25), and the fourth diet, fish meal with 25% replacement by SBM and the inclusion of 2% of GroBiotic®-A (SM25-P). Each diet was supplied in three tanks (45 fish per diet); therefore, we had a 2x2 factorial design including two levels of FM replacement (0 and 25%) and two levels of prebiotic (0 and 2%), with three replicates (three tanks). The fishes were fed daily to apparent satiation, at each feeding period, all the uneaten feed in each tank was removed after one hour from feeding and was dry weighed (dw). Consumed feed was = total feed offered dw – uneaten feed dw, in that feeding period. The consumed feed was divided by the number of alive fish per diet.

For growth analysis, all the fishes alive were measured on days 14, 28, 42, and 56. The thermal growth coefficient (TGC) [45] and feed conversion rate (FCR = ingested food dry weight/fish wet weight gained) were estimated for every interval between sampling dates. Then at the end of the study, these parameters were averaged to obtain mean values for the whole study and analyzed by two-way ANOVA with a statistical significance of $P < 0.05$, using STATISTICA v.8.0. After 56 days of the experiment, the fish were first anesthetized by immersion in seawater with tricaine methanesulphonate (MS-222) (90 mg/L) and then euthanized through brain puncture. Then the livers were extracted and embedded in RNA later at -80° C until used for analysis.

2.2. RNA extraction and sequencing

For RNA sequencing, three biological replicates per diet were used for a total of twelve liver samples. Total RNA was extracted from 10 mg of every liver sample using the commercial kit Pure Link RNA mini kit (Invitrogen) following the manufacturer's instructions. The concentration and purity of the RNA were quantified using Qubit (Thermo Fisher Scientific, Waltham, USA). The integrity of the RNA was calculated with the 2100 Bioanalyzer capillary electrophoresis system (Agilent Technologies, Santa Clara, CA, USA). The synthesis of complementary DNA (cDNA) to build the sequencing libraries was performed for each sample using the Illumina TruSeq RNA Sample Preparation Kit v2, according to the manufacturer's instructions. Paired-end RNA sequencing was performed using the Illumina Hiseq 4000 platform at Roy J. Carver Biotechnology Center (University of Illinois, Urbana, IL, USA).

2.3. Transcriptome assembly

The quality of the sequencing reads was analyzed using the FastQC software, then adaptors, ambiguous nucleotides, and low-quality sequences were eliminated with the Trimmomatic-0.36 software using a sliding window of 5 nucleotides and a minimum average quality of 28 in the Phred scale [46]. Finally, high-quality reads from all the libraries were assembled *de novo* using the Trinity v2.4.0 program with default parameters [47].

2.4. Differential expression analysis

The differential gene expression analysis was carried out using Bowtie2 [48] for the alignment of the reads and abundance estimation of the transcripts in each treatment, using the assembled transcriptome as reference. The quantification and normalization were performed using transcripts per million (TPM) through the RSEM program [49]. The expression analysis was performed using the TPM matrix of each treatment through DESeq2, comparing each treatment against the control diet [50] in R software [51]. In this analysis, the parameters of false discovery rate (FDR) were set below 0.01 with a fold change above 2. The results of the differential expression analysis were represented with heatmaps which were generated following the R scripts included in Trinity software [47]. Finally, the transcripts with differential expression between treatments versus the control were classified with Venn diagrams, using the Venny 2.1 platform (bioinfogp.cnb.csic.es/tools/venny/) [52].

2.5. Functional annotation

The functional annotation of the assembled transcriptome was carried out through the search of homologs with BLASTx [53] using the public databases UniProt (release 2019_2). The E value filter for this analysis was set at E-5. On the other hand, the annotation with terms of gene ontology (GO) was made with the Blast2GO program [54]; each GO was classified into the categories of biological processes, cellular components, and molecular functions. The enrichment analysis for down and up-regulated genes of each treatment versus the control was performed through Fisher's exact test (FDR <0.05) using the Blast2GO program. As well, the metabolic pathways from the Kyoto Encyclopedia of Genes and Genomes database (KEGG) were downloaded through DAVID 6.8 [55], using the up and down-regulated genes of each experimental diet. Additional enrichment analysis of biological processes and KEGG pathways was

carried out for the reference transcriptome in DAVID 6.8 (P-value < 0.01). UniProt IDs from enriched KEGG pathways were analyzed in STRING software [56, 57], to visualize the protein-protein interactions of each pathway, using *Homo sapiens* and *Danio rerio* as models.

Results

3.1. Growth performance

The weight gained, and percent weight gained, resulted significantly higher due to the prebiotic. Besides, the thermal growth coefficient (TGC) was significantly higher in those fish fed with prebiotic. Additionally, no interaction was found between both factors (diet/prebiotic) and no significant differences were observed in the food consumed between treatments (Table 2).

3.2. Sequencing and transcriptome assembly

A total of 712,373,692 high-quality paired reads of 100 bp were obtained including all libraries, and a total of 155,247 contigs were assembled with an average length of 972 bp, an N50 of 1,722 bp, and a GC content of 47%, in total 150,952,398 bases were assembled.

3.3. Differential gene expression

In the differential expression analysis, the three experimental treatments against the control diet were compared. Results showed that a total of 135 genes were up-regulated in all treatments compared to the control. From these, 83 transcripts were up-regulated in the FM-P treatment, 30 up-regulated in the SM25-P treatment, and 57 up-regulated in the SM25 diet. Of which 55 were exclusive to the FM-P treatment, 18 were exclusive to the SM25-P treatment, and 31 to the SM25 treatment. From all these transcripts, 4 were up-regulated in all the experimental treatments when compared to the control (Fig. 1A). A total of 78 transcripts were down-regulated in all the treatments, and none was shared among them. Of which, 35 were down-regulated in the FM-P treatment, 14 down-regulated in the SM25-P treatment, and 35 transcripts in the SM25 treatment. Where 33 were exclusive of the FM-P treatment, 9 transcripts were exclusive of the SM25-P treatment and 30 transcripts were exclusive of the SM25 treatment (Fig. 1B).

The biological replicates from all the diets were clustered based on the normalized expression values (TPM) of the up-regulated transcripts in the experimental diets, the up-regulated transcripts in the control diet, and selected transcripts with gene ontologies corresponding to carbohydrate metabolism, lipid metabolism, growth, and immune system. The heatmap of up-regulated transcripts in the treatments showed two main clusters, the first one containing the three replicates of the control diet in the same branch, and the sample SM25_1 in an adjacent branch; the second cluster contained the rest of the samples grouping those from SM25 and SM25-P with FM-P_1, and in an adjacent branch FM-P_2 and FM-P_3 were grouped (Fig. 2). The heatmap of up-regulated transcripts in the control showed three main clusters, the first one grouped two control samples with SM25-P_2; another grouped one control sample with two SM25 samples and SM25-P_3; and a third cluster grouped all three FM-P samples in the same

branch, with SM25-P_1 and SM25_3 in adjacent branches (Fig. 3). Finally, the heatmap including transcripts with relevant functional annotation showed two main clusters, the first one grouped all control samples in the same branch with two SM25 and two SM25-P in adjacent branches; the second cluster grouped the three FM-P samples with SM25_3 and SM25-P_1 (Fig 4). All heatmaps clustered the control samples with samples of fish fed diets including SBM with and without prebiotic. Also, all heatmaps separated the control samples from FM-P samples. Transcriptomic profiles of control samples were more similar to those of fishes fed diets including SBM, with and without prebiotic and the most differentiated group was that one fed the FM-P diet. Annotations of relevant transcripts selected for cluster analysis are shown in Table 3.

3.4. Functional annotation

In the functional annotation through BLASTx, homologs were searched for the 155,247 contigs, and 58,167 matches were found with significant homology in the UniProt protein database, of which 51,609 had a gene ontology and functional annotation. The species with the highest number of homologies was *Homo sapiens* (28,787), followed by *Mus musculus* (15,788) and *Danio rerio* (9,183). Other fish species with significant matches were *Takifugu rubripes* (432), *Oncorhynchus mykiss* (388), and *Salmo salar* (279). Based on the results of functional annotation, gene ontology (GO) terms of cellular component, molecular function, and biological processes were assigned. The most abundant terms in the cellular component category corresponded to *intracellular part* (38,744), *intracellular organelle* (32,680), and *membrane-bounded organelle* (29,361). In the category of molecular function, the top terms were *protein binding* (23,390), *organic cyclic compound binding* (15,094), and *ion binding* (14,913). Finally, in the category of biological processes, the most abundant terms were *cellular metabolic process* (28,175), *metabolic process of nitrogen compounds* (25,837), *cellular component organization* (15,651), and *biosynthetic process* (15,013).

3.5. Enrichment analysis for biological process and metabolic pathways

According to the functional annotations, important metabolic processes were enriched for the liver transcriptome like *phospholipid biosynthetic process*, *steroid metabolic process,* and *protein transport,* as well as the *immune system process* (Table 4). KEGG pathways were also enriched including *phosphatidylinositol signaling system*, *inositol phosphate metabolism*, *aldosterone synthesis and secretion*, *mTOR signaling pathway*, *B cell receptor signaling pathway*, *thyroid hormone signaling pathway*, *glucagon signaling pathway*, and *insulin signaling pathway* among others (Table 5). Protein-protein interactions involved in the signaling pathways enriched in liver transcriptome are shown in Supplementary Figure S1 A-G.

In the FM-P treatment, metabolic GO terms like *negative regulation of protein catabolic process* and *glucose metabolic process* were enriched by upregulated-transcripts; by contrast, down-regulated transcripts enriched processes like *cholesterol transport* and signaling processes related to *apoptosis* and the *Wnt signaling pathway*. In the SM25-P treatment metabolic terms like *tyrosine catabolic process*, *L-phenylalanine catabolic process,* and *fatty acid biosynthetic process* were enriched by up-regulated

transcripts; on the other hand, down-regulated transcripts enriched processes related to *cholesterol transport, phospholipid homeostasis, phospholipid catabolic process,* and the *Wnt signaling pathway*. Finally, in the SM25 treatment GO terms like *gene silencing*, *response to bacterium*, and *protein dephosphorylation* were enriched by up-regulated transcripts; inversely, down-regulated transcripts enriched processes like *positive regulation of transcription, DNA-templated*, and *cell division* (Table 6).

Based on the KEGG database, differentially expressed transcripts enriched the *metabolic pathway* category in FM-P. Besides, down-regulated transcripts in SM25 enriched the *Rac 1 cell motility signaling pathway* (Table 7).

Discussion

In this study, we analyzed the liver gene expression of the Pacific yellowtail *S. lalandi* juveniles fed with diets containing different protein sources, with and without a prebiotic. The study was focused on the liver because it is the main organ involved in the use of nutrients and is the center of intermediary metabolism in animals [32]. Moreover, it is important for fatty acids metabolism, lipid storage and is the main responsible for glucose homeostasis by regulating both gluconeogenesis and glycolysis processes [58, 59].

Regarding the experimental diets, the level of FM replacement by SBM tested in this study (25%) was high, considering that up to 20% is the recommended FM replacement level using SBM or soy protein concentrate for juveniles [60, 61] and that inclusion of 30% or higher of SBM in diets have resulted detrimental for this species [62, 63]. Although the level of FM replacement was high, it did not affect fish growth. Interestingly, the inclusion of prebiotic in diets with and without FM replacement improved the growth. Therefore, this replacement level using SBM when combined with GroBiotic®-A is a good balance between cost and benefit for *S. lalandi* juveniles.

In the transcriptomic analysis, we distinguished differentially expressed genes (DEGs) potentially involved in metabolic pathways, growth, and the immune system. DEGs were more abundant in fish fed with FM-P diet, followed by SM25. On the other hand, SM25-P showed the least amount of DEGs. This suggests that the prebiotic in SM25-P compensated the general effect of SBM on the transcriptome, by keeping the expression of most of the genes at the same level as the control diets. Moreover, the cluster analyses based on the general transcriptomic profile grouped fish fed both diets including SBM with those fed the control diet, suggesting a similar physiologic performance of the liver in fish fed SM25, SM25-P, and control diets. The effect of diets on specific metabolic processes is discussed below.

Growth

Regarding the effect of the diets on growth, fishes fed with FM-P and SM25-P diets resulted in the highest percentage of weight gain, the greatest weight gain in grams and TGC [44]. Pacific yellowtail is a fast-growing fish as was observed with these results, which coincide with those obtained for *S. lalandi* juveniles fed diets formulated with vegetable ingredients [64]. In our analysis, we observed an up-

regulation of the *TGFBR3* gene, in the FM-P diet. This gene expression pattern showed a significant association with the FCR in chickens [65]. Moreover, this gene has a great interest in aquaculture because is important for muscle growth in rainbow trout *Oncorhynchus mykiss* [66]. On the other hand, in a genome-wide association study to identify molecular markers associated with growth in the Atlantic salmon *Salmo salar,* genes including the *TGFBR3* showed strong correlation with growth [67]. Another up-regulated gene in the FM-P treatment was the epidermal growth factor receptor kinase substrate 8 (*EPS8*). According to [68], the transcriptomic profiles of red and white carps were compared to identify differential gene expression and found up-regulated genes associated with muscle development, including the *EPS8.* By contrast, this gene was down-regulated in SM25, versus the control diet. However, the *mTOR* gene, which is also related to growth, was up-regulated in the SM25 diet in our study. This gene, both in rainbow trout and in mammals, promotes protein accumulation in skeletal muscle by the effect of adequate feeding [69]. Integrating physiological data measured in [44] with the transcriptomic data analyzed herein, it is considered that the diets including prebiotic (FM-P and SM25-P) can improve growth, as both diets induced a higher weight gain and more genes related to growth.

Carbohydrate metabolism

One of the main metabolic processes affected by the experimental diets was carbohydrate metabolism. Carbohydrate requirement in fish varies according to the feeding habits of the species, ranging from herbivorous to strict carnivores; despite this, glycolysis is an important metabolic pathway to provide energy to maintain cellular activities [70]. The capability of the liver to maintain the balance between the intake and release of glucose plays an essential role in the maintenance of the homeostasis of glucose in the blood [71]. Regarding carbohydrate metabolism, no differences were found in the amylase activity between diets [44]; however, in our transcriptomic analysis, both diets with SBM, showed high expression of the *H6PD* gene, and in the SM25 diet the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 4 (*PFKFB4*) gene was up-regulated. The enzyme encoded by this gene can reduce hepatic glucose production by increasing glycolysis and inhibiting gluconeogenesis, thereby lowering blood glucose [71]. On the other hand, in the European seabream *Sparus aurata*, it was found that fishes fed diets with high carbohydrate content, resulted in the up-regulated expression of the *PFKFB4* gene [72]. Authors conclude that the expression of *PFKFB4* helps carnivorous fish to adapt to diets with the inclusion of plant meals. In the case of the FM-P diet, it was evident that the glucose metabolism was up-regulated with high expression of *G6PD* y *H6PD* genes, both participating in glucose oxidation [73, 74]. Additionally, the protein phosphatase 1 regulatory subunit 3C (ppp1r3cb), which limits the breakdown of glycogen [75] was down-regulated. Moreover, protein catabolism was also down-regulated, suggesting that this diet enhanced glycolysis, and the energy supply obtained through this pathway was enough to reduce gluconeogenesis, improving protein weight gain. By contrast, in SM25-P, fatty acid biosynthesis was up-regulated, suggesting that in this diet the observed weight gain could be also related to lipid accumulation.

Lipid metabolism

A better understanding of the physiological mechanisms of lipid metabolism of fishes is critical to replace fish meal and fish oil in formulated diets, and if combined with gene expression studies would allow the identification of genes and potential markers for fish selection. According to our annotation and enrichment analysis, many genes involved in lipid metabolism were identified, as the *ATP8A2* gene, which is up-regulated in human adipocytes [76]. This gene (*ATP8A2*) participates in the asymmetric distribution and translocation of phospholipids and seems to be involved in the formation of vesicles and the uptake of lipid signaling molecules [77]. In our study, *ATP8A2* was up-regulated in diets including SBM (SM25-P and SM25), which can tell us about an effort by fish to maintain balance in the distribution of lipids due to SBM inclusion in the diet. On the other hand, no differences were found in the amount of lipid accumulated in the muscle of the fish [44]. High expression of *ATP8A2* can be suggested as a molecular marker of fishes adapting to vegetable diets. Other genes with differential expression in the experimental diets were *LIPG* and *LPIN2*. *LIPG* gene, which participates in the hydrolysis of high-density lipoproteins [78] was down-regulated in fish fed SM25-P diet; and *LPIN2*, which plays an important role in the control of the metabolism of fatty acids at different levels [79], was up-regulated in the FM-P diet. The transcriptomic analysis of the intestinal mucosa of the sea bass (*Dicentrarchus labrax*) fed with low fishmeal diets showed that *LPIN2* was up-regulated in fishes fed diet with the highest percentage of fishmeal [80]. However, in this study, this was observed only when the prebiotic was added to the fishmeal since the *LPIN2* gene had the highest expression level in the FM-P diet. Considering the mentioned above, experimental diets induced changes in the expression levels of genes related to lipid metabolism in the liver. However, it is necessary to evaluate the expression and activity of pancreatic and intestinal lipases in future work, to have a clearer view of the effects of diets with the inclusion of SBM and prebiotics.

Immune response

Finally, another relevant process affected by the experimental diets was the immune response. The immune system in fish is an important mechanism and the first line for protection against pathogens [81]. It should be noted that in this species, the leukocytes had a significant decrease in fish fed diets that contain prebiotic [44]. In this work, we found differential expression of genes with potential participation in the immune response. For example, the B-cell receptor *CD22* gene (*CD22*) was up-regulated in the FM-P diet, this gene seems to be involved in the regulation of B-cell antigen receptor signaling, mediates B-cell-B-cell interactions, and may be involved in the localization of B-cells in lymphoid tissues [82, 83]. In a transcriptomic analysis of the grass carp (*Ctenopharyngodon idellus*) infected with *Aeromonas hydrophila* the *CD22* was up-regulated [84]. Another up-regulated gene in the FM-P diet was serine/threonine-protein kinase *RIO3* (*RIOK3*), which plays a critical role in the innate immune response against DNA and RNA viruses [85]. In our work we found *RIOK3* up-regulated in fish fed with the FM-P diet. On the other hand, Caspase-1 (*CASP1*) was down-regulated in fish fed with the SM25 diet. *CASP1* is important for defense against pathogens and is involved in a variety of inflammatory processes. In a nutritional experiment with gilthead seabream (*Sparus aurata*), gene expression of *CASP1* increased in fishes fed with the diets that include the probiotic bacterium *Bacillus subtilis* [86]. Another important gene for the immune response, the complement component C9 (C9) was found up-regulated in SM25-P. This gene plays a key role in the innate and adaptive immune response by forming pores in the plasma

membrane of target cells [87]. The expression of C9 was detected in the spleen, stomach, intestine, and head kidney, with the highest levels detected in the liver of the southern catfish (*Silurus meridionalis*) [88]. On the other hand, evaluations in *Larimichthys crocea* under a *Vibrio alginolyticus* challenge, showed that two components of the terminal complement system: C7 and C9, were expressed in many tissues in adult healthy fish, with the highest levels detected in liver [89]. Moreover, SM25 and SM25-P showed up-regulation of the C3 gene, involved in inflammatory processes and response to bacteria [90], but it is also involved with glucose transport related to diabetes [91, 92].

Recommendations

Based on the overall results, the FM-P diet was the best, since fish fed this diet showed a better physiological response, especially considering the growth rate, as well as the expression pattern of metabolic and immune genes in the liver. However, this diet may increase production costs. Although 25% of fish meal replacement by SBM did not affect growth, the SM25 diet was the one with less up-regulation of growth-related genes and more down-regulated genes related to the immune system. Interestingly, the prebiotic inclusion in diets with and without SBM improved the growth rate and physiologic performance of the liver. In the SM25-P diet, the prebiotic seems to compensate for the effect of SBM on the liver at the transcriptomic level since the overall gene expression in fish fed this diet was maintained at similar levels as those of the control diet.

The results of this work and together with those of [44], suggest that up to 25% of fish meal can be substituted with SBM combined with the prebiotic. However, it is necessary to evaluate the response of the fish for a longer period, to test long-term effects in growth, physiologic performance, immune response, and meal quality in adult organisms, and evaluate the effect of this diet on other tissues of the digestive tract.

Declarations

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Conflicts of interest/Competing interests

Authors declare that no competing interests or conflicts of interest exist.

Authors' contribution

OEJ: Conceptualization, Formal analysis, Visualization, Writing – original draft.

RDV: Data curation, Formal analysis, Visualization, Writing – original draft.

FLDC: Conceptualization, Methodology, Resources, Supervision, Writing – review and editing.

JPL: Conceptualization, Methodology, Investigation, Resources, Funding acquisition, Supervision, Writing – review and editing.

ELL: Data curation, Formal analysis, Writing – review and editing.

DTR: Conceptualization, Methodology, Supervision, Writing – review and editing.

CEGS: Conceptualization, Methodology, Resources, Funding acquisition, Supervision, Writing – review and editing.

Ethics approval

This research complied with the Guidelines of the European Union Council (2010/63/EU) and the Mexican Government (NOM-062—ZOO-1999) for the production, care, and use of experimental animals, and with the ARRIVE guidelines.

Consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

All the RNA-Seq raw reads were deposited into the Sequencing Read Archive (SRA) of NCBI with the accession number SRR10211853 to SRR10211864. The BioProject ID of our data is PRJNA575250 and the BioSample accession is SAMN12816772.

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Tables

Table 1. Proximal composition and formulation of experimental diets.

Proximal composition	FM	FM-P	SM25	SM25-P
Proteins	45.4±1.2	47.0±0.5	48.8±0.5	49.6±0.5
Lipids	18.6±0.4	19.5±0.3	20.1±0.3	21.2±0.6
Humidity	0.3±0.02	0.6±0.05	0.7±0.02	2.0±0.07
Ashes	13.2±0.10	13.2±0.19	12.9±0.13	13.0±0.07
Ingredients				
Soybean meal	0	0	15	15
Chicken meal	9	9	9	9
Fish meal	55	55	45	45
Fish Oil	11.3	11.9	13	13
Starch	14	11.4	5.3	3.3
Jelly	2	2	2	2
Rovimix	5	5	5	5
Stay C	1.5	1.5	1.5	1.5
Lecithin	1	1	1	1
Taurine	1	1	3	3
Sodium benzoate	0.2	0.2	0.2	0.2
GroBiotic®-A	0	2	0	2
BHT	0.01	0.01	0.01	0.01

FM = fish meal as main protein source, FM-P = FM with 2% prebiotic, SM25-P = 25% FM replacement by soybean meal and 2% prebiotic, SM25 = 25% FM replacement by soybean meal. The values of ingredients are expressed in percentages. Taken and modified from [44].

Table 2. Growth results for each treatment.

	FM	FM-P	SM25	SM25-P	M	P	MxP
Initial weight (g)	97.5±3.5	96.0±1.5	99.6±1.3	98.5±0.8	NS	NS	NS
Gained weight (g)	124.9±18.5	161.4±33.6	140.2±6.4	165.6±10.7	NS	***	NS
Gained weight (%)	127.9±15.9	167.9±32.3	140.7±4.7	168.1±9.4	NS	***	NS
TGC	1.11±0.10	1.35±0.18	1.20±0.02	1.36±0.09	NS	***	NS
FCR	1.53±0.12	1.36±0.13	1.49±0.08	1.33±0.02	NS	***	NS

FM = fish meal as main protein source, FM-P = FM with 2% prebiotic, SM25-P = 25% FM replacement by soybean meal and 2% prebiotic, SM25 = 25% FM replacement by soybean meal. TGC = Thermal growth coefficient. FCR = feed conversion rate. M = Meal. P = Prebiotic. NS = no significant difference. *** = $P < 0.05$. Taken and modified from [44].

Table 3. Selected differentially expressed genes for cluster analysis.

Transcript	BLASTx hit	Process	Gene	Protein name
TRINITY_DN27611_c4_g1_i9	KCC2G_HUMAN	C	CAMK2G	Calcium/calmodulin-dependent protein kinase type II subunit gamma
TRINITY_DN26269_c2_g1_i2	ENOB_SALSA	C	ENO3	Beta-enolase
TRINITY_DN27760_c1_g2_i11	G6PD_TAKRU	C	G6PD	Glucose-6-phosphate 1-dehydrogenase
TRINITY_DN28668_c0_g1_i5	G6PE_MOUSE	C	H6PD	GDH/6PGL endoplasmic bifunctional protein
TRINITY_DN28824_c0_g1_i10	F264_HUMAN	C	PFKFB4	6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 4
TRINITY_DN27130_c1_g1_i3	PR3CB_DANRE	C	ppp1r3cb	Protein phosphatase 1 regulatory subunit 3C-B
TRINITY_DN29572_c3_g1_i23	SRBS1_HUMAN	C	SORBS1	Sorbin and SH3 domain-containing protein 1
TRINITY_DN26067_c0_g1_i1	EPS8_MOUSE	G	EPS8	Epidermal growth factor receptor kinase substrate 8
TRINITY_DN23956_c0_g1_i2	MTOR_MOUSE	G	MTOR	Serine/threonine-protein kinase mTOR
TRINITY_DN26179_c0_g1_i18	RPTOR_HUMAN	G	RPTOR	Regulatory-associated protein of mTOR
TRINITY_DN25891_c0_g1_i2	SCUB3_MOUSE	G	SCUBE3	Signal peptide, CUB and EGF-like domain-containing protein 3
TRINITY_DN30106_c1_g2_i10	SMAD4_RAT	G	SMAD4	Mothers against decapentaplegic homolog 4
TRINITY_DN18300_c0_g1_i2	TGBR3_MOUSE	G	TGFBR3	Transforming growth factor-beta receptor type 3
TRINITY_DN22340_c0_g1_i1	ZFN2B_HUMAN	G	ZFAND2B	AN1-type zinc finger protein 2B
TRINITY_DN26104_c0_g1_i2	C1QB_MOUSE	I	C1qb	Complement C1q subcomponent subunit B
TRINITY_DN27901_c1_g2_i11	CO9_TAKRU	I	C9	Complement component C9

TRINITY_DN23812_c0_g1_i1	CASP1_PIG	I	CASP1	Caspase-1
TRINITY_DN30179_c2_g2_i13	CD22_HUMAN	I	CD22	B-cell receptor CD22
TRINITY_DN29613_c2_g1_i6	MY18A_HUMAN	I	MYO18A	Unconventional myosin-XVIIIa
TRINITY_DN27406_c2_g1_i5	NOTC2_HUMAN	I	NOTCH2	Neurogenic locus notch homolog protein 2
TRINITY_DN29910_c2_g2_i6	P85A_HUMAN	I	P85A	Phosphatidylinositol 3-kinase regulatory subunit alpha
TRINITY_DN25444_c0_g1_i5	RIOK3_HUMAN	I	RIOK3	Serine/threonine-protein kinase RIO3
TRINITY_DN27700_c0_g1_i6	TCAIM_HUMAN	I	TCAIM	T-cell activation inhibitor, mitochondrial
TRINITY_DN18468_c0_g1_i4	TRAF3_MOUSE	I	TRAF3	TNF receptor-associated factor 3
TRINITY_DN29039_c3_g2_i1	ABH2A_DANRE	L	abhd2a	Monoacylglycerol lipase ABHD2
TRINITY_DN26059_c0_g1_i2	ACSF3_MOUSE	L	ACSF3	Acyl-CoA synthetase family member 3
TRINITY_DN27331_c0_g1_i10	ANGL3_MOUSE	L	ANGPTL3	Angiopoietin-related protein 3
TRINITY_DN26503_c1_g1_i3	AT8A2_MOUSE	L	ATP8A2	Phospholipid-transporting ATPase IB
TRINITY_DN28443_c0_g1_i16	LIPE_HUMAN	L	LIPG	Endothelial lipase
TRINITY_DN27990_c1_g1_i12	LPIN2_MOUSE	L	LPIN2	Phosphatidate phosphatase LPIN2
TRINITY_DN29764_c0_g2_i4	LRP5_HUMAN	L	LRP5	Low-density lipoprotein receptor-related protein 5
TRINITY_DN29140_c3_g3_i2	LSR_MOUSE	L	LSR	Lipolysis-stimulated lipoprotein receptor
TRINITY_DN29650_c0_g1_i4	NF2L1_HUMAN	L	NFE2L1	Endoplasmic reticulum membrane sensor NFE2L1
TRINITY_DN23944_c1_g1_i1	STAR3_DANRE	L	STARD3	StAR-related lipid transfer protein 3

G = Growth, L = lipid metabolism, C = carbohydrate metabolism/homeostasis, I = immune system.

Table 4. Top 20 enriched biological processes for the reference liver transcriptome of *S. lalandi* juveniles

GO	Term	Count	P-Value	Fold Enrichment
GO:0030030	cell projection organization	33	7.12E-21	6.362
GO:0007093	mitotic cell cycle checkpoint	8	6.68E-05	6.169
GO:0008380	RNA splicing	42	1.95E-23	5.655
GO:0000266	mitochondrial fission	10	1.10E-05	5.655
GO:0051028	mRNA transport	17	2.16E-09	5.546
GO:0016569	covalent chromatin modification	54	6.37E-29	5.453
GO:0007067	mitotic nuclear division	48	1.53E-23	5.027
GO:0006974	cellular response to DNA damage stimulus	64	2.41E-30	4.891
GO:0006397	mRNA processing	63	1.19E-29	4.858
GO:0006446	regulation of translational initiation	12	6.72E-06	4.847
GO:0001731	formation of translation preinitiation complex	13	2.67E-06	4.794
GO:0031047	gene silencing by RNA	14	1.07E-06	4.750
GO:0007049	cell cycle	89	7.44E-40	4.660
GO:0008654	phospholipid biosynthetic process	14	1.90E-06	4.567
GO:0051301	cell division	57	5.35E-25	4.561
GO:0008202	steroid metabolic process	12	2.06E-05	4.426
GO:0006417	regulation of translation	20	3.85E-08	4.138
GO:0008045	motor neuron axon guidance	16	2.29E-06	3.992
GO:0015031	protein transport	91	1.12E-	3.979

			33	
GO:0002376	immune system process	16	1.21E-05	3.572

Table 5. Top 20 enriched KEGG pathways for the reference liver transcriptome of *S. lalandi* juveniles

KEGG pathway	Term	Count	P-Value	Fold Enrichment
hsa00500	Starch and sucrose metabolism	20	0.001656	1.927
hsa02010	ABC transporters	26	4.20E-04	1.878
hsa04070	Phosphatidylinositol signaling system	57	1.08E-07	1.849
hsa00562	Inositol phosphate metabolism	41	1.13E-05	1.836
hsa04520	Adherens junction	40	3.22E-05	1.791
hsa04666	Fc gamma R-mediated phagocytosis	46	1.92E-05	1.741
hsa04925	Aldosterone synthesis and secretion	42	2.35E-04	1.648
hsa04150	mTOR signaling pathway	30	0.002368	1.644
hsa05220	Chronic myeloid leukemia	37	7.45E-04	1.634
hsa05100	Bacterial invasion of epithelial cells	40	4.56E-04	1.630
hsa04360	Axon guidance	64	1.28E-05	1.602
hsa04911	Insulin secretion	42	8.42E-04	1.571
hsa04662	B cell receptor signaling pathway	34	0.003109	1.566
hsa04611	Platelet activation	64	3.25E-05	1.565
hsa04919	Thyroid hormone signaling pathway	55	3.24E-04	1.520
hsa05231	Choline metabolism in cancer	48	9.85E-04	1.511
hsa04015	Rap1 signaling pathway	99	2.01E-06	1.499
hsa04931	Insulin resistance	50	0.001542	1.472
hsa04922	Glucagon signaling pathway	45	0.004222	1.445
hsa04910	Insulin signaling pathway	62	9.96E-04	1.428

Table 6. Enriched biological processes in each treatment vs the control diet (Fisher exact test).

Up-regulated genes	P-Value	Down-regulated genes	P-Value
FM-P			
Negative regulation of I-kappaB kinase/NF-kappaB signaling	0.00009	tRNA modification	0.00080
Cellular response to leukemia inhibitory factor	0.00027	RNA splicing	0.03796
Negative regulation of protein catabolic process	0.00046	Endoplasmic reticulum organization	0.03893
Actin filament bundle assembly	0.00200	Negative regulation of actin filament polymerization	0.03958
Regulation of cell shape	0.00272	Intrinsic apoptotic signaling pathway in response to DNA damage	0.04045
Negative regulation of cell migration	0.00318	Myeloid cell development	0.04239
Actomyosin structure organization	0.00362	Cell aging	0.04282
Protein localization to plasma membrane	0.00832	Positive regulation of canonical Wnt signaling pathway	0.04434
Glucose metabolic process	0.03426	Cholesterol transport	0.04628
		Cytoplasmic translation	0.04778
SM25-P			
Tyrosine catabolic process	0.00006	Cholesterol homeostasis	0.00000
L-phenylalanine catabolic process	0.00012	Positive regulation of high-density lipoprotein particle clearance	0.00000
Retrograde transport, endosome to Golgi	0.00106	High-density lipoprotein particle remodeling	0.00001
Negative regulation of apoptotic process	0.00296	Phospholipid homeostasis	0.00002
Positive regulation of NF-kappaB transcription factor activity	0.04573	Reverse cholesterol transport	0.00002
Lysosomal transport	0.04757	Positive regulation of cholesterol transport	0.00003
Cellular response to lipopolysaccharide	0.04794	Phospholipid catabolic process	0.00006
Fatty acid biosynthetic process	0.04886	Regulation of cell shape	0.04537
		Muscle contraction	0.04571
		Canonical Wnt signaling pathway	0.04942

SM25			
Gene silencing	0.02245	Regulation of transcription by RNA polymerase II	0.00438
Cell junction organization	0.03829	Positive regulation of transcription, DNA-templated	0.00717
Response to bacterium	0.04536	Protein modification by small protein conjugation or removal	0.02074
Cilium assembly	0.04660	Positive regulation of cell migration	0.02816
Negative regulation of protein phosphorylation	0.04821	Cell division	0.04160
Protein dephosphorylation	0.04839	Cellular response to cytokine stimulus	0.04280
		Pathogenesis	0.04649
		Positive regulation of protein kinase B signaling	0.04714
		Vascular endothelial growth factor receptor signaling pathway	0.04886
		Regulation of stress fiber assembly	0.04972

Table 7. Enriched metabolic pathways in treatments versus the control diet.

FM-P Up-regulated	P-Value	Fold Enrichment	Genes	Protein name
Metabolic pathways	0.019	4.55	METK2	S-adenosylmethionine synthase 2
			HUTU	Urocanate hydratase
			G6PE	GDH/6PGL endoplasmic bifunctional protein
			PI4KA	Phosphatidylinositol 4-kinase alpha
			LPIN2	Phosphatidate phosphatase
Synaptic Proteins at the Synaptic Junction	0.047	32.83	ANK1	Ankyrin-1
			EPB41	Protein 4.1
FM-P Down-regulated				
Metabolic pathways	0.034	8.53	CD38	ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase 1
			PNPH	Purine nucleoside phosphorylase
			PCY1B	Choline-phosphate cytidylyltransferase B
SM25 Down-regulated				
Rac 1 cell motility signaling pathway	0.024	54.72	MYLK	Myosin light chain kinase, smooth muscle
			P85A	Phosphatidylinositol 3-kinase regulatory subunit alpha

Figures

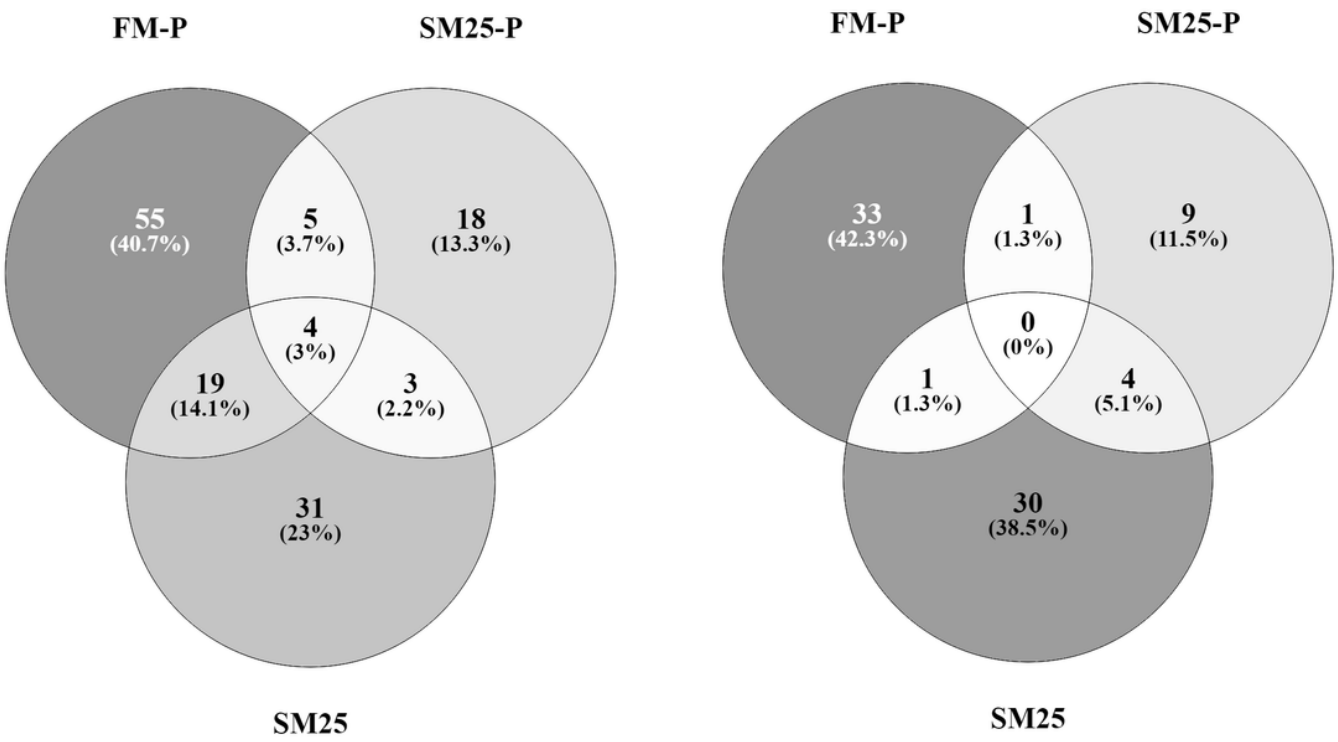

Figure 1

A) Left, up-regulated transcripts in treatments vs the control diet. B) Right, down-regulated transcripts in treatments vs the control diet. FM-P=fish meal with 2% prebiotic, SM25=25% fish meal replacement with soybean meal, SM25-P=25% fish meal replacement with soybean meal and 2% prebiotic.

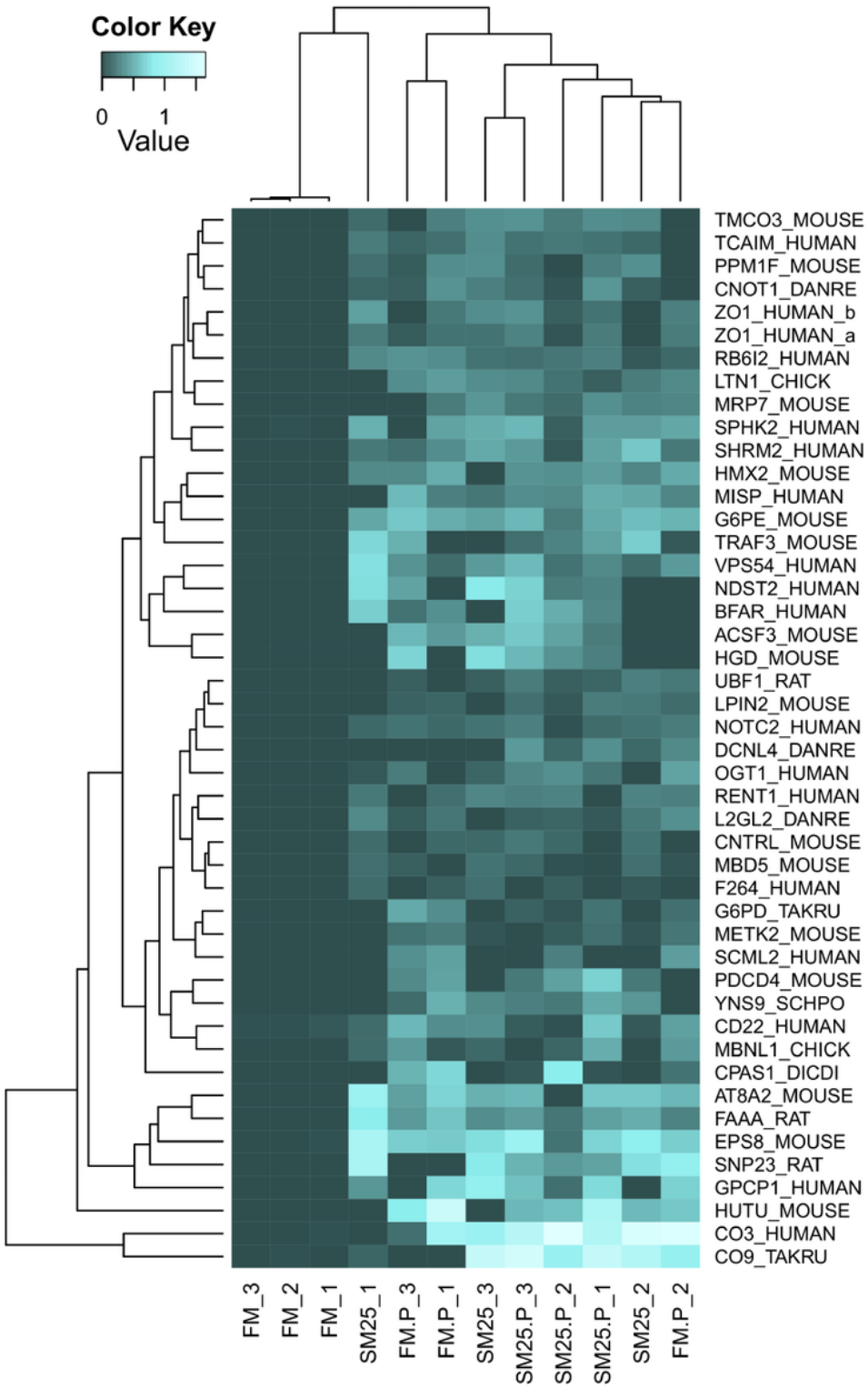

Figure 2

Sample clustering based on expression levels of up-regulated transcripts in treatments vs control diet, UniProt ID of the encoded protein is shown for each transcript. FM=control diet based on fish meal, FM-P=fish meal with 2% prebiotic, SM25=25% fish meal replacement with soybean meal, SM25-P=25% fish meal replacement with soybean meal and 2% prebiotic. Values = log10(TPM).

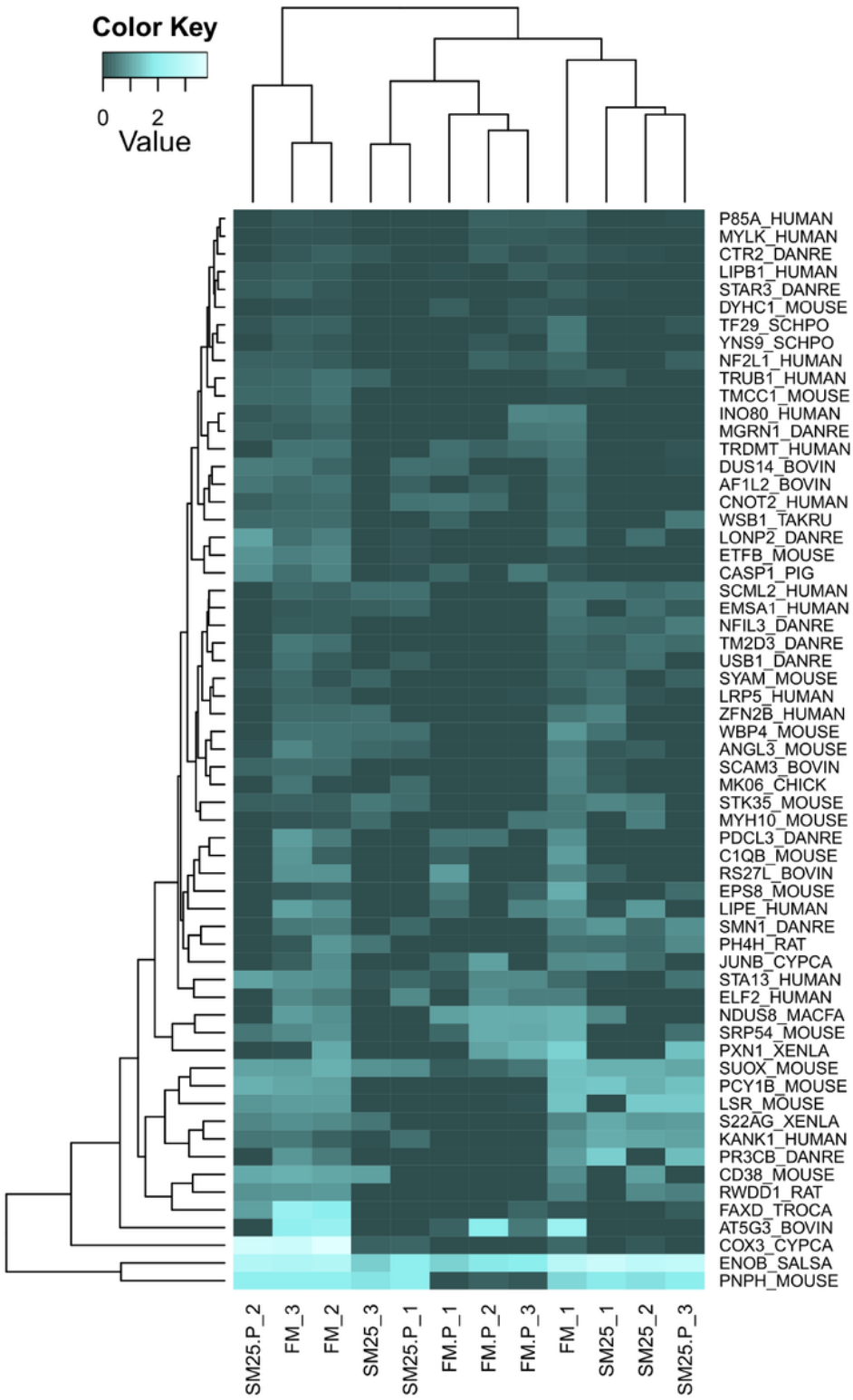

Figure 3

Sample clustering based on expression levels of down-regulated transcripts in treatments vs control diet, UniProt ID of the encoded protein is shown for each transcript. FM=control diet based on fish meal, FM-P=fish meal with 2% prebiotic, SM25=25% fish meal replacement with soybean meal, SM25-P=25% fish meal replacement with soybean meal and 2% prebiotic. Values = log10(TPM).

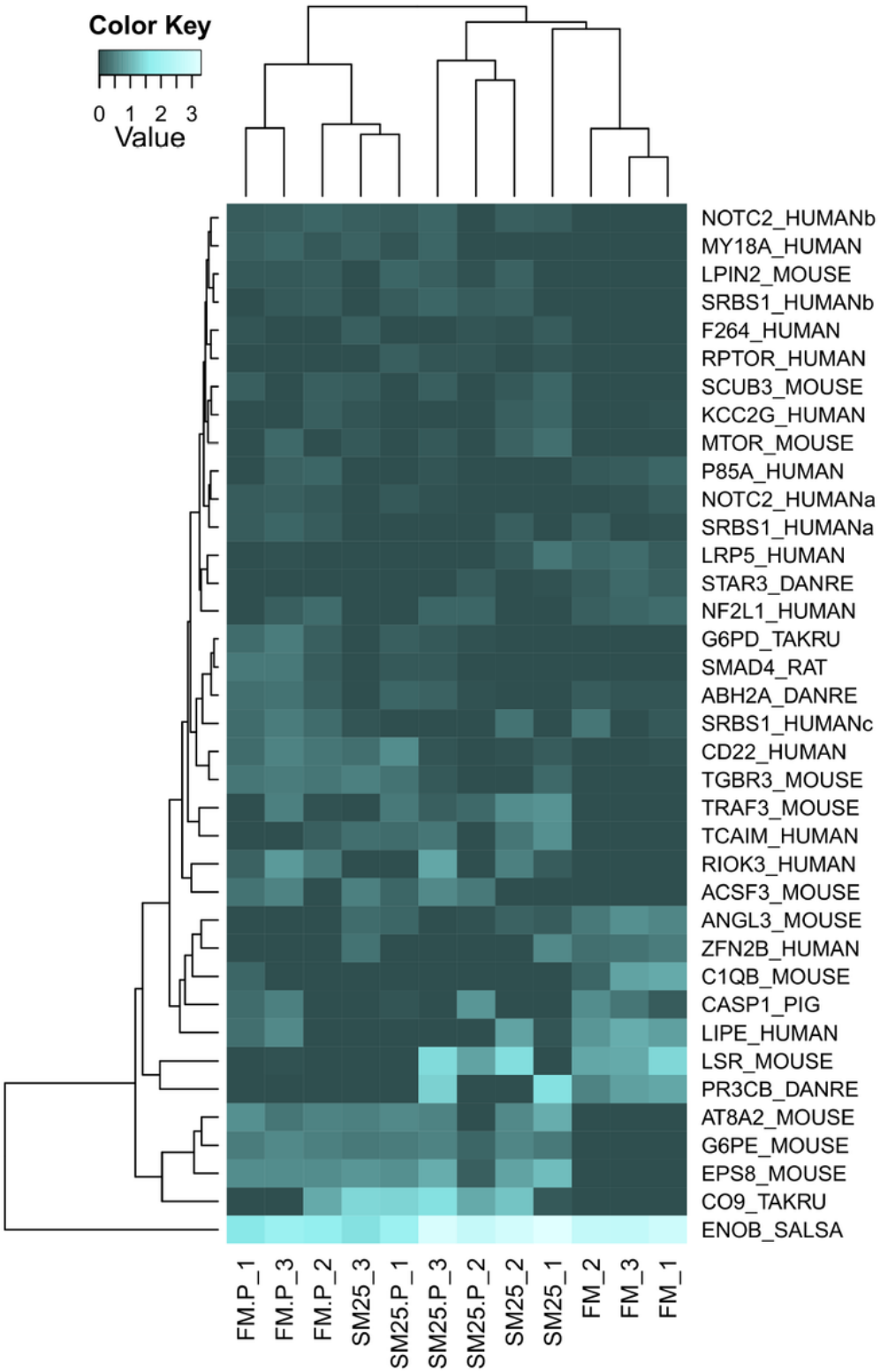

Figure 4

Sample clustering based on selected differentially expressed transcripts, UniProt ID of the encoded protein is shown for each transcript. FM=control diet based on fish meal, FM-P=fish meal with 2% prebiotic, SM25=25% fish meal replacement with soybean meal, SM25-P=25% fish meal replacement with soybean meal and 2% prebiotic. Values = log10(TPM).

Supplementary Files

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