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Omics analysis of red seabream (*Pagrus major*) fed a soybean meal-based diet

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Abstract: In response to the increasing demand for seafood, aquaculture production has been expanding worldwide in recent years. On the other hand, the high demand and unstable supply of fishmeal (FM) has made feed manufacturers difficult to use FM as the main ingredient in aquaculture feeds. Soybean meal (SBM) is one of the most promising substitutes for FM in aquaculture feeds because of its economic advantages and nutritional properties. Although SBM has a relatively high protein content and balanced amino acid composition, it has been reported that feeding of a large amount of SBM has adverse effects on the physiology and growth of fish. To facilitate the development of more effective aquaculture feeds containing SBM, it is required to elucidate the effects of dietary SBM on fish nutritional metabolism in detail. In this study, comprehensive approaches, i.e., transcriptomics and metabolomics analyses, were employed to investigate the effects of SBM on nutritional metabolism in red seabream (*Pagrus major*). Red seabream fingerlings were fed an SBM-based diet (SBMD) and FM-based control diet (FMD) for eight weeks and the hepatopancreas collected at 2, 4, and 8 weeks were used for transcriptome analysis. Growth retardation, and several abnormal physiological conditions such as lower serum cholesterol content and tissue degeneration in the distal intestine, were observed in the SBMD group. Transcriptome analysis (RNA-Seq) identified differentially expressed genes between the SBMD and FMD groups and showed increases in the expression levels of genes involved in the terpenoid and steroid biosynthesis pathways in the former group, suggesting that hepatic cholesterol biosynthesis was up-regulated in the SBMD group. The hepatopancreas of fish fed each diet for 8 weeks was also subjected to metabolome analysis and the results, coupled with the transcriptome analysis, suggest that SBMD affects the glutathione and glycine metabolism. Additionally, transcriptome of the distal intestine at 8 weeks revealed the changes in the expression levels of genes related to the cell cycle control and cholesterol metabolism. This study provides valuable information that enhances the understanding of the effects of dietary SBM on red seabream metabolism, which will lead to the development of a novel technology that relieves the abnormal physiological conditions. Findings obtained from omics analysis could be used to identify the indices of negative effects caused by respective alternative ingredients and thereby lead to the improvement of the quality of future aquaculture feeds.

Key words: *Pagrus major*, transcriptome, metabolome, soybean meal

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Introduction

In recent years, there has been a global trend to reduce the use of fishmeal (FM) in aquaculture feeds. One of the reasons is the increasing demand and price of FM along with the worldwide expansion of aquaculture production (FAO 2020). FM has been used as the main protein source in aquaculture feeds for various fish species (Olsen and Hasan 2012), and aquaculture feeds are still heavily relying on FM. This situation leads to further increase in the demand and price of FM, which has been a serious problem in the aquaculture industry. Additionally, as far as FM production depends on natural fish stock, unstable and limited supply of FM will continue to be inevitable for the aquaculture industry (Olsen and Hasan 2012). Increasing demand of natural fish stock for human foods should also be considered (Naylor *et al*. 2000).

To reduce the use of FM in aquaculture feeds, various ingredients are proposed as FM substitutes (Hussain *et al.* 2024). Protein sources originated from plant, such as corn and soybean, have already been used practically in aquaculture feeds. Animal proteins from livestock by-products are also included in some cases. Poultry by-product meal, meat and bone meal, blood meal and feather meal are representative animal ingredients. However, their use in aquaculture feeds is limited due largely to sanitary and ethical concerns. As novel ingredients from single cell proteins derived from bacteria, yeasts and microalgae, and insect proteins from black soldier fly, meal worms and crickets are still under consideration for aquaculture feeds (Samsing *et al*. 2024; Gao *et al*. 2024; Agboola *et al*. 2021; Alfiko *et al*. 2022). Soybean meal (SBM), a by-product of oil extraction from soybean, is one of the most promising ingredients as a substitute for FM (Gatlin *et al.* 2007). The advantages of SBM are its relatively low price, high availability, and favorable protein content. However, SBM has some issues to be considered for use as a main protein source in aquaculture feeds: deficiency of nutrients such as methionine and taurine, poor palatability, and presence of several anti-nutritional factors. Negative effects of SBM on fish physiology such as growth retardation, tissue degeneration, disfunction of digestive organs, and bile acid depletion in the gallbladder of salmonids have been reported (Romarheim *et al.* 2008; van den Ingh *et al*. 1991; Yamamoto *et al*. 2007). In red seabream *Pagrus major*, Murashita *et al*. (2018) reported that an SBM based diet caused delayed growth, lower gallbladder weight, lower activity and gene expression of digestive enzyme. To facilitate the development of more practical aquaculture feeds comprising of FM substitutes, it is required to investigate the effects of respective ingredients on fish nutritional metabolism in detail.

In fish physiological and nutritional studies, omics analysis could provide various novel insights (Wang *et al*. 2022; Nazari *et al*. 2021; Schock *et al*. 2012). For example, a transcriptome study suggests that dietary eicosapentaenoic acid and docosahexaenoic acid levels influence the expression levels of genes related to lipid and glucose metabolism, redox homeostasis and immune function in Atlantic salmon *Salmo salar* (Xue *et al*. 2020). Another trans-omics study using transcriptome and metabolome analyses in leopard coral grouper *Plectropomus leopardus* suggests that branched-chain amino acids are used for energy supply in fasting, and circadian changes of these metabolic processes were observed (Mekuchi *et al*. 2017). In the present study, to obtain deeper insights into the physiological effects of dietary SBM, we performed the histological and omics analyses in red seabream, an important cultured species in Japan (Yoshinaga *et al*. 2023).

Materials and methods

One hundred and five red seabream fingerlings (initial body weight, 15.7 ± 0.8 g), purchased from a local hatchery, were introduced into each of six 500 L tanks (3 replicates per diet). An FM-based diet (FMD) and SBM-based diet (SBMD) were prepared and fed to the fish twice daily for 8 weeks at 23.4 ± 0.9°C. At start, 1, 2, 3, 4, 6, and 8 weeks, samples for histological and other biological analyses were collected. The hepatopancreas samples were taken for transcriptome analysis (RNA-Seq) at 2, 4 and 8 weeks, and for metabolome analyses at 4 and 8 weeks. The feeding condition, method of histological observation, and procedures of transcriptome and metabolome analyses of the hepatopancreas were described in Yoshinaga *et al*. (2023). The distal intestinal samples (only at 8 weeks) were also collected for transcriptome analysis. Total RNA was extracted from the distal intestinal sample using a commercial kit (RNeasy mini kit, Qiagen) and RNA-Seq was performed at Novogene Co., Ltd. (Beijing, China) for the transcriptome analysis. The RNA-Seq reads were mapped to the red seabream reference genome and analyzed using the same method as described in Yoshinaga *et al*. (2023).

Results and discussion

Effects of SBMD on physiology and histology in red seabream

Compared with the FMD group, the body weight of the

SBMD group had already been significantly lower at 2 weeks and greatly retarded after 6 weeks. Specific growth rate and feed efficiency also significantly decreased after 6 weeks. In the SBMD group, the hepatosomatic index, serum cholesterol concentration, and biliary bile acid concentration became significantly lower at 1 to 3 weeks, suggesting that the fish physiology had already been damaged in early half of the experimental term (Yoshinaga *et al.* 2023). The hepatocytes were atrophied in the SBMD group from 1 week. Matsunari *et al.* (2015) also reported that an SBM based diet caused growth retardation and histological degeneration of the hepatopancreas in red seabream, and the negative effects on growth and hepatopancreas histology were relieved by replacing SBM with soy protein concentrate and ethanol-washed soy protein isolate. These results suggest that the ethanol-soluble fraction of SBM is causative of the physiological disorder in red seabream.

The relationship between SBM intake and histological change of the distal intestine was reported in several fish species, such as Atlantic salmon (Uran *et al.* 2009), rainbow trout *Oncorhynchus mykiss* (Romarheim *et al.* 2008; Iwashita *et al.* 2008) and gilthead seabream *Sparus aurata* (Bonaldo *et al.* 2008). We also confirmed that the distal intestine of red seabream fed SBMD had morphological changes. The distal intestine of fish in the FMD group showed normal features throughout the experimental term: abundant supranuclear vacuoles (SNV), well-developed microvilli, and scattered goblet cells (Fig.1). In contrast, the SBMD group showed a decrease of SNV from 1 week onward and showed severe abnormality at 6 and 8 weeks: disappearance of SNV and microvilli, and decrease of goblet cells. Iwashita *et al.* (2007) reported that saponin and other ANFs contained in SBM caused the liver and distal intestinal histological changes in rainbow trout. Moreover, deterioration caused by a prolonged period of feeding an SBM based diet was reported in Atlantic salmon; the higher the inclusion level of dietary SBM and the longer the experimental period, the severer the histological condition in the distal intestine was damaged (Uran *et al.* 2009).

Analysis of transcriptome and metabolome in the hepatopancreas

Transcriptome analysis of red seabream hepatopancreas was conducted at 2, 4, and 8 weeks after the start of SBMD feeding. The sequence data were used to detect differentially expressed genes (DEGs), which showed significant increase or decrease in the SBMD group compared with the FMD group. Then, 454, 353, and 566 DEGs at 2, 4, and 8 weeks, respectively, were subjected to enrichment analysis to identify the metabolic

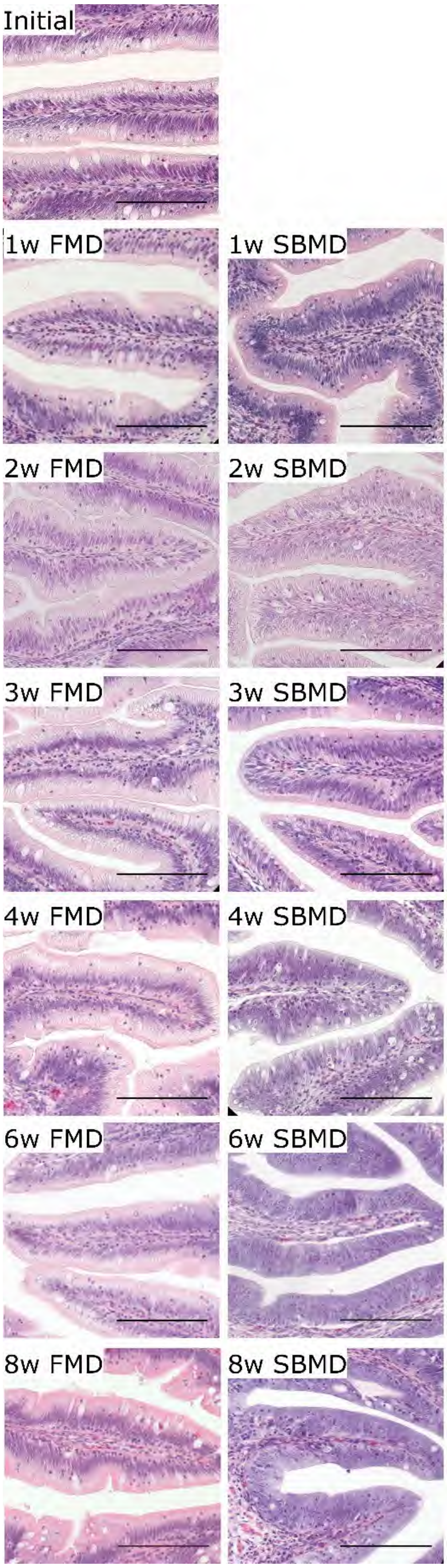

Fig.1 Morphology of the distal intestine of red seabream fed a fish meal diet (FMD) and soybean meal diet (SBMD) for 8 weeks

Scale bars represent 50 µm.

process affected by the diets. The enrichment analysis of DEGs revealed that the genes involved in steroid metabolic process and isoprenoid biosynthetic process were significantly abundant in the SBMD group throughout the feeding trial (Yoshinaga *et al*. 2023). Fig.2 shows the changes of expression levels of genes related to the cholesterol biosynthesis in the hepatopancreas of the SBMD group. The results suggest that biosynthesis of cholesterol is promoted by SBMD. Our result coincides with several previous studies that suggest that SBM intake affects the cholesterol metabolism in fish (Zhu *et al*. 2018: Kortner *et al*. 2013; Kemski *et al*. 2020; Takagi *et al*. 2002). Cholesterol plays a variety of biological roles, such as a constitution in the biological membrane, hormone biosynthesis, and bile acid production (Babin and Vernier 1989; Sheridan 1988). SBM-induced abnormality in fish may result from the changes in the cholesterol metabolic process.

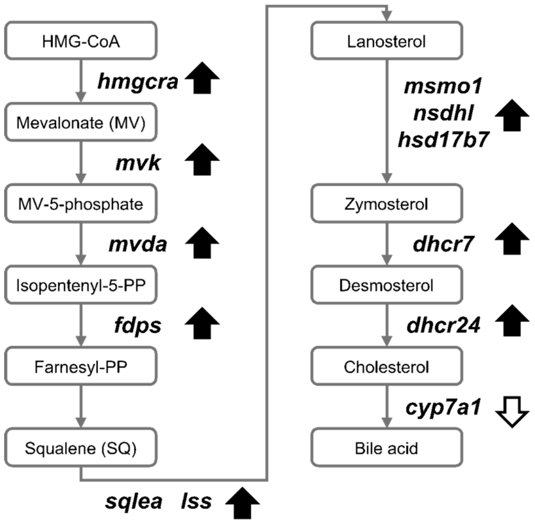

Fig.2 Expression levels of genes related to cholesterol metabolism in the hepatopancreas of red seabream fed a soybean meal diet

Black and white arrows represent the up-regulated and down-regulated genes, respectively. This figure is referenced from Yoshinaga *et al*. (2023).

In the metabolome analysis, the metabolites that showed quantitatively significant changes between diet groups were selected and subjected to the pathway analysis. The results of metabolome analysis, taken together with those of the transcriptome analysis, revealed significant changes in the glycine, serine, and threonine metabolism. Furthermore, metabolism of glutathione, which is a metabolite related to the oxidative stress response in fish (Sukhovskaya *et al*. 2017), was also affected. These results imply that SBMD induced oxidative stress in red seabream and impaired the stress response, which might have resulted in the growth retardation.

Analysis of transcriptome in the distal intestine

The distal intestinal samples of red seabream fed SBMD for 8 weeks were also used for transcriptome analysis. The raw read datasets were deposited in the DDBJ sequence Read Archive under accession nos. DRR656721-DRR656732 and

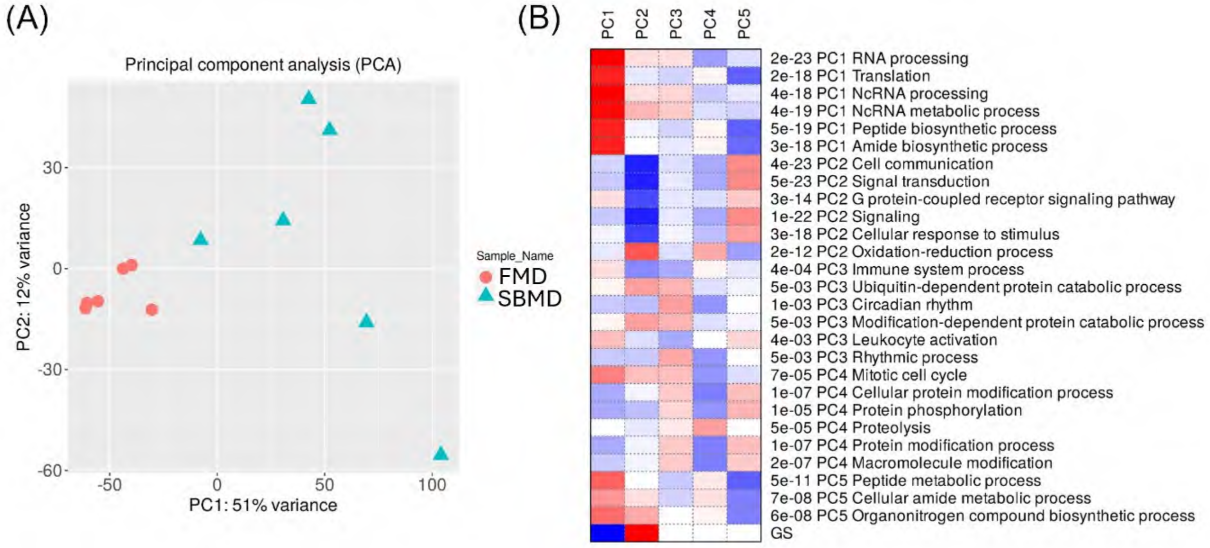

Fig.3 Results of principal component analysis of the transcriptome in the distal intestine of red seabream fed a soybean meal diet

(A), score plot in principal component (PC) 1 and 2; (B), loading factor.

the result of principal component analysis is shown in Fig.3. Compared with the FMD group, 1629 and 1228 genes were identified as up-regulated and down-regulated DEGs at 8 weeks, respectively, and these DEGs were subjected to enrichment analysis (Fig.4). Several genes related to cholesterol metabolism, such as methylsterol monooxygenase 1 (*msmo1*), hydroxysteroid 17-beta dehydrogenase 7 (*hsd17b7*), NAD (P) dependent steroid dehydrogenase like (*nsdhl*), squalene epoxidase a (*sqlea*) and lanosterol synthase (*lss*), were also up-regulated as found in the hepatopancreas. The genes related to cellular lipid metabolism, cellular catabolic process, and lysosomal function were down-regulated. The expression levels of genes involved in DNA replication including MCM complex, ORCs, and cyclin-dependent kinases, increased significantly. At the same time, expressions of genes related to cell cycle regulation, such as DNA-dependent protein kinase, cyclin-dependent kinase inhibitor 1, and 14-3-3 protein, were up-regulated in the SBMD group. In the eukaryotic cells, the cell cycle is regulated by the synthesis and degradation of cyclins and CDKs (Alberts *et al*. 2008), and the progression of cell cycle is controlled by monitoring the cell environment and DNA replication status. Previous studies suggested that intake of SBM-based diets affects the cell cycle and cell stress in the intestinal tract of Atlantic salmon (Sanden *et al*. 2005; Bakke-McKellep *et al*. 2007). These findings imply that SBM promotes the cell division for regeneration of the distal intestinal enterocytes, but meanwhile the cell division is halted due to the DNA damage. The changes observed in the transcriptome in the distal intestine of red seabream fed SBMD could partly be attributed to the growth retardation.

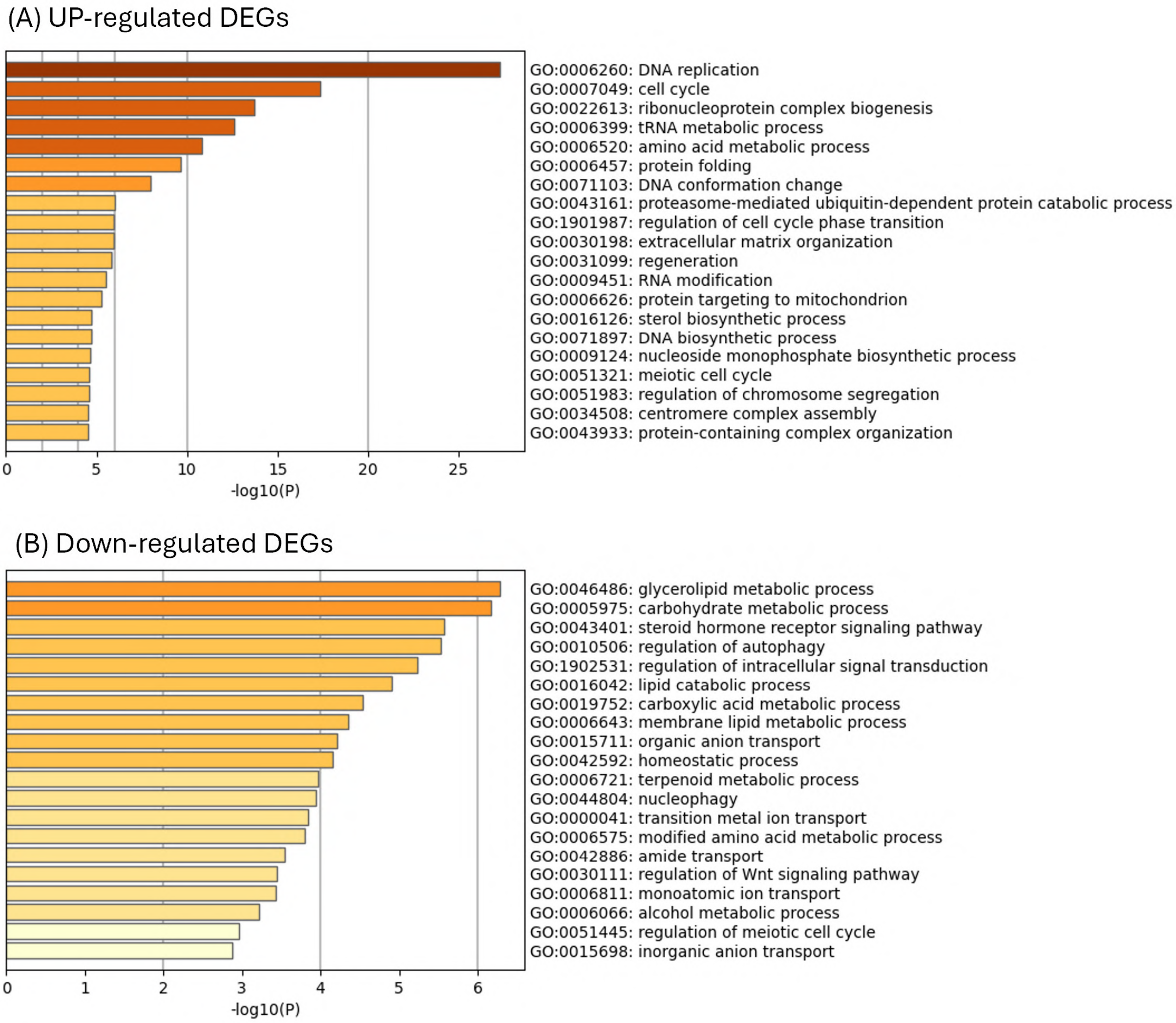

Fig.4 Heatmaps of enriched process and pathway (gene ontology term related to biological process) in the DEGs of the distal intestine of red seabream

DEGs were detected between soybean meal diet group and fish meal diet group. Up-regulated DEGs (A) and down-regulated DEGs (B) in the soybean meal diet group compared with the fishmeal diet group were used for the analysis.

Evaluation of feed ingredients by omics analysis

This study has shown that SBMD gradually induced various physiological changes; hepatocyte atrophy, degeneration of the distal intestine, decreases of serum cholesterol and biliary bile acid concentrations gradually progressed in red seabream fed SBMD. Additionally, transcriptome and metabolome analyses also imply that SBMD affected the metabolic processes, such as enhancement of cholesterol biosynthesis and abnormality of oxidative stress response. These results obtained through the omics analysis revealed the effects of a particular ingredient on the metabolism of a given fish, and will contribute to the development of a new technology that relieves the soybean-induced abnormal physiology. Moreover, as demonstrated in this study, omics analyses will help us find out suitable indices to evaluate the physiological effects of novel feed ingredients.

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Annotated Bibliography of Key Works

(1) Lu F, Haga Y, Satoh S (2015) Effects of replacing fish meal with rendered animal protein and plant protein sources on growth response, biological indices and amino acid availability for rainbow trout *Oncorhynchus mykiss*. *Fish. Sci.*, **81**, 95-105.

In this study, rainbow trout were fed six diets, a fish meal diet and 5 other diets in which fishmeal was replaced by one or in combination of poultry by-product meal, feather meal, blood meal, soybean meal and corn gluten meal. Growth performance was assessed by a feeding trial of 12 weeks. The result of biological indices and amino acid availability showed that a combination of some protein sources could effectively be substituted for fishmeal. This report shows the necessity of using multiple alternative protein sources instead of a single protein source as a substitute for fishmeal.

(2) Murashita K, Matsunari H, Furuita H, Rønnestad I, Oku H, Yamamoto T (2018) Effects of dietary soybean meal on the digestive physiology of red seabream *Pagrus major*. *Aquaculture*, **493**, 219-228.

The authors reported acute and chronic effects of dietary soybean meal on the digestive physiology of red seabream. Red seabream were fed fishmeal and soybean meal-based diets, and the authors investigated the gastric transit, digestive enzyme activity and expression levels of genes encoding digestive enzymes. In the acute trial, trypsin activity in the intestinal content was lower, suggesting that the soybean meal diet reduces the trypsin secretion. In the chronic trial, in addition to the growth retardation, changes in the gastric transit rate, lower gallbladder weight, and changes in the expression levels of hepatopancreatic enzyme genes were observed in fish fed the soybean meal diet. This paper is one of the reports that clearly shows the SBM effects on fish nutritional physiology in detail.

(3) Roques S, Deborde C, Richard N, Skiba-Cassy S, Moing A, Fauconneau B (2020) Metabolomics and fish nutrition: a review in the context of sustainable feed development. *Rev. Aquacult.*, **12**, 261-282.

The authors reviewed the usefulness of metabolomic approaches for the development of aquaculture feeds. It is crucial for aquaculture sectors to improve the quality of aquaculture feeds using plant resources and other raw materials as substitutes for fish meal and fish oil. In this review, previous studies related to fish nutritional metabolism using metabolomic analysis are summarized. Appropriate sample for metabolome analysis, future applications of metabolomics in fish nutritional study and development of aquaculture feed, evaluation of fish fillet quality, and non-invasive monitoring of fish state were discussed.

(4) Yoshinaga H, Yasuike M, Mekuchi M, Soma S, Yamamoto T, Murashita K, Matsunari H, Oku H, Furuita H (2023) Multi-omics analysis of hepatopancreas of red seabream (*Pagrus major*) fed a soybean meal-based diet. *Aquaculture*, **574**, 739631.

The authors analyzed the hepatopancreatic transcriptome and metabolome in red seabream fed a soybean meal diet for 8 weeks. Fish fed the diet showed delayed growth and physiological abnormalities relative to fish fed a fishmeal-based diet. The results of omics analyses suggest the soybean meal diet affects the metabolism of cholesterol, glutathione, and glycine.