Studies on the effect of taurine supplementation to low or non fishmeal diet on growth, nutrient digestibility, intestinal morphology and cytokines gene expression in juvenile red seabream, Pagrus major

Doctoral Dissertation

STUDIES ON THE EFFECT OF TAURINE SUPPLEMENTATION TO LOW OR NON FISHMEAL DIET ON GROWTH, NUTRIENT DIGESTIBILITY, INTESTINAL MORPHOLOGY AND CYTOKINES GENE EXPRESSION IN JUVENILE RED SEABREAM, *Pagrus major*

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FENGYU LI

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By

FENGYU LI

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Content

CONTENT 3

LIST OF TABLES 6

LIST OF FIGURES 8

CHAPTER 1. GENERAL INTRODUCTION..... 12

CHAPTER 2. LITERATURE REVIEW..... 20

2.1. AQUACULTURE OF RED SEA BREAM 21

2.2. PROTEIN SOURCES USED IN AQUAFEED INDUSTRY 21

2.3. USE OF PLANT PROTEINS IN RED SEABREAM DIETS 24

2.4. TAURINE SUPPLEMENT 26

2.5. INFLAMMATION AND CYTOKINES 27

REFERENCES 31

CHAPTER 3. EFFECT OF PLANT PROTEIN BASED LOW FISHMEAL DIET ON GROWTH PERFORMANCE, HISTOLOGY AND GENE EXPRESSION OF INFLAMMATORY CYTOKINES OF JUVENILE RED SEA BREAM (*PAGRUS MAJOR*)..... 36

3.1. INTRODUCTION 38

3.2. MATERIALS AND METHODS 40

3.2.1. Experimental diets 40

3.2.2. Experimental fish, experimental conditions and feeding 43

3.2.3. Carcass sampling 44

3.2.4. Chemical analysis 45

3.2.5. Histology analysis 46

3.2.6. Gene expression analysis 47

3.2.6. Statistical analysis 49

3.3. RESULTS 50

3.3.1. Growth performances and feed utilization efficiencies....50
3.3.2. Whole body proximate composition....50
3.3.3. Histology study....51
3.3.4. Relative gene expression....54
3.4. Discussion....57
3.5. Conclusion....60
References....62
CHAPTER 4. NON FISHMEAL DIET SUPPLEMENTED WITH TAURINE: EFFECTS ON GROWTH PERFORMANCE, FEED UTILIZATION, MORPHOLOGY AND CYTOKINE GENE EXPRESSION IN INTESTINE OF JUVENILE RED SEABREAM....68
4.1. Introduction....70
4.2. Materials and Methods....72
4.2.1. Experimental diets....72
4.2.2. Experimental fish and growth trial....74
4.2.3. Sample collection....75
4.2.4. Chemical analysis....76
4.2.5. Histological examination....77
4.2.6. RNA isolation and gene expression analysis....77
4.2.7. Digestibility trial....79
4.2.8. Statistical analysis....79
4.3. Result....80
4.3.1. Growth performances, feed utilization efficiencies and protein digestibility....80
4.3.2. Proximate composition of whole body....81
4.3.3. Intestinal morphology....81
4.3.4. Relative gene expression of IL-1β, IL-8, TNF-α and TGF-β1....84

4.4. DISCUSSION 85
REFERENCES 90
CHAPTER 5. EFFECT OF GRADED LEVELS OF TAURINE SUPPLEMENTATION TO NON FISHMEAL DIET ON GROWTH PERFORMANCE, FEED UTILIZATION AND NUTRIENT DIGESTIBILITY OF JUVENILE RED SEABREAM 96
5.1. INTRODUCTION 98
5.2. MATERIALS AND METHODS 100
5.2.1. Experimental diets 100
5.3.2. Experimental fish and growth trial 103
5.2.3. Sample collection 104
5.2.4. Chemical analysis 105
5.2.5. Digestibility trial 105
5.2.6. Statistical analysis 106
5.3. RESULTS 106
5.3.1. Growth performances and feed utilization 106
5.3.2. Liver observation 107
5.3.3. Digestibility 107
5.4. DISCUSSION 109
5.5. CONCLUSIONS 111
REFERENCE 112
CHAPTER 6. GENERAL CONCLUSION 115

List of tables

Table 1 Common anti-nutritional substances and effect on fish.....26

Table 2. Common cytokines in fish and their effect on fish.....30

Table 3. Ingredient composition (%) of experimental diets fed juvenile red sea bream, *Pagrus major* 41

Table 4. Proximate composition of the test diets (%) fed juvenile red sea bream, *Pagrus major*.....42

Table 5. Total amino acid content of the test diets (g/100 g, d.b.) fed juvenile red sea bream, *Pagrus major* 42

Table 6. Experimental condition for feeding trial of juvenile red sea bream, *Pagrus major* 44

Table 7. Primers sequence used for qPCR.....49

Table 8. Growth performance and feed utilization efficiencies of whole fish body of Red sea bream fed experimental diets for 12 weeks. 50

Table 9. Proximate compositions of whole fish body of Red sea bream (% wet basis, n=3).....51

Table 10. Pathological change in Red sea bream after 12 weeks feeding trial.....54

Table 11. Ingredient composition of experimental diets fed red seabream 73

Table 12. Proximate composition (% dry matter basis, n = 3) 74

Table 13. Total amino acid content of experimental diets for red sea bream, *Pagrus major*. (g/100g dry matter, n = 3) 74

Table 14. Primers sequence used for qPCR.....78

Table 15. Growth parameters, feed utilization and protein digestibility of red seabream fed experimental diets for 10 weeks. 80

Table 16. Proximate composition of whole body of red seabream fed experimental diets for 10 weeks. 81

Table 17. Morphological changes in terms of neutrophil infiltration of the distal intestine of red seabream fed the experimental diets for 10 weeks 82

Table 18. Ingredient composition (%) of experimental diets fed juvenile red sea bream, *Pagrus major* 101

Table 19. Proximate composition of the test diets (%) fed juvenile red sea bream, *Pagrus major*...102

Table 20. Essential and Conditionally essential amino acid content of the test diets (g/100 g, d.b.) fed juvenile red sea bream, *Pagrus major* 102

Table 21. Experimental condition for feeding trial of juvenile red sea bream, *Pagrus major* 104

Table 22. Growth performance and feed utilization of Red sea bream fed experimental diets for 12 weeks.....107

Table 23. Apparent digestibility coefficients of protein, lipid and dry matter of red seabream fed experimental diet.....108

List of figures

Figure 1. Histology procedure 47

Figure 2. Hematoxylin and eosin stained sections of liver of Red sea bream fed with experimental diets: FM (A), TELFM (B) or LFM (C, D). (a) indicates atrophic hepatocyte; (b) indicated hydropic degeneration; (c) indicates nuclear dislocation. (Scale bar = 50 μm).....52

Figure 3.Histological characteristics of intestinal mucosa from red seabream fed a fishmeal based control diet and three LFM diets. (A) FM (control); (B, C, D) LFM. The black arrowheads (a, b) represent the infiltrated neutrophils. (c). Inflammatory response. Scale bar = 50 μm 53

Figure 4. mRNA expression of 4 cytokine genes associated with gut inflammation in red seabream: IL-1β (A); IL-8 (B); TNF-α (C); TGF-β1 (D). Relative mRNA levels were determined by the comparative Ct method using β-actin gene as the control. Relative mRNA expression was evaluated by real-time quantitative PCR. Values are means ± S.D. (n = 4). Bars of the same gene bearing with different letters are significantly different by Tukey HSD test ($p < 0.05$).....56

Figure 5. Histological characteristics of distal intestinal mucosa from red seabream fed a fishmeal based control diet and three NFM diets. (a) FM (control); (b) NFM+1.0T; (c) NFM+1.5T; (c) NFM+2.0T. SNV, supranuclear vacuoles; GC, goblet cells; LP, lamina propria. The yellow arrowheads in (b) represent the infiltrated neutrophils. Scale bar = 50 μm.....83

Figure 6. mRNA expression of 4 cytokine genes associated with gut inflammation in red seabream: IL-1β (A); IL-8 (B); TNF-α (C); TGF-β1 (D). Relative mRNA levels were determined by the comparative Ct method using β-actin gene as the control. Relative mRNA expression was evaluated by real-time quantitative PCR. Values are means ± S.D. (n = 4). Bars of the same gene bearing with different letters are significantly different by Tukey HSD test ($p < 0.05$).....84

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［課程博士・論文博士共通］

博士学位論文内容要旨
Abstract

専攻 Major	Applied Marine Bioscience	氏名 Name	Fengyu Li
論文題目 Title	Studies on the effect of taurine supplementation to low or non fishmeal diet on growth, nutrient digestibility, intestinal morphology and cytokines gene expression in juvenile red seabream, *Pagrus major*（マダイ稚魚の成長、消化率，消化管形態，サイトカイン遺伝子の発現に対する低･無魚粉飼料へのタウリンの添加効果に関する研究）		

Plant protein (PP) sources have been receiving considerable attention over the past few decades as a partial or total fishmeal (FM) alternatives in aquafeed industry. And among the ingredients investigated, the products, soybean meal (SBM) and corn gluten meal (CGM) are the most promising. However, the presence of anti-nutritional factors (ANFs), the imbalances in amino acids, especially the low content of taurine in SBM and CGM have adverse impact on fish growth performance, feed utilization and health. Intestinal health is vital for body health. Once normal intestinal function is damaged, it will cause many diseases, such as loss of appetite, low food intake, growth retardation and the decline of digestive and absorptive capacity of nutrients. In fish, feed composition, especially the plant protein can always cause oxidative damage and structural damage of intestinal tract of fish, and then induce enteritis. In many inflammatory diseases, the increase of the cytokine levels can be detected. PPs have very low inclusion of taurine and FM is rich in taurine. Consequently, the removal of fishmeal may create several taurine deficiency symptoms. However, these physiological defects can be overcome by dietary taurine supplementation. Meanwhile, several studies have suggested that enzyme complex and some other additives supplementation help recovery from negative impact caused by PP. Therefore three studies were designed to, i) evaluate the supplement effect of taurine and enzyme complex in plant protein based low FM diet on growth performance and body health of red sea bream with, ii) evaluate the effect of complete replacement of FM with PP and taurine supplement on growth performance, feed utilization, morphology and cytokine gene expression in intestine of juvenile red seabream and iii) evaluate the effect of graded levels of taurine supplementation to non FM diet on growth performance, feed utilization and nutrient digestibility of juvenile red seabream.

In the first experiment, a 12-week feeding trial of juvenile red seabream with initial weight of 7.6g was conducted. In this trial, four iso-nitrogenous (47% protein) diets were employed: FM-based control diet (FM); low FM diet (60% of fish meal was replaced by PP), designated LFM; the diet supplemented with 0.5% taurine to LFM, designated as TLFM and the diet supplemented with 0.5% taurine and 0.05% enzyme complex to LFM, designated as TELFM. After 12 weeks, fish fed LFM showed significantly lower weight gain (%WG) and specific growth ratio (%SGR) than control group while TLFM and TELFM groups showed similar growth performance with control. Feed efficiency in terms of feed efficiency ratio (FCR) and protein efficiency ratio (PER)in LFM group was significantly lower than control and TELFM groups. Daily feed intake (DFI) showed no difference among diet groups. Typical pathological change (Neutrophil infiltration and blood congestion) mainly appeared in intestine submucosa of fish fed LFM. The expression of inflammation-relative genes (IL-1β, IL-8, TNF-α and TGF-β1) were up-regulated in intestine of fish fed low FM diets. And in low FM groups, fish have significantly higher expression

level than the control. However, in liver, the cytokine genes expression levels showed no difference among diet groups. Results in this experiment suggested that fish fed LFM indicated poorer growth, feed utilization and severer inflammatory response while taurine and/or enzyme complex supplementation ameliorated soybean-induced adverse impacts.

Second experiment was conducted to evaluate the effect of complete replacement of FM with PP and taurine supplement on taurine on growth performance, feed utilization, morphology and cytokine gene expression in intestine of juvenile red seabream. Four iso-nitrogenous diets with 47% protein were prepared. Red sea bream (5.4g) were fed one of the diets formulated with 50% FM diet (FM, control) or soy and corn protein based non FM diets supplemented with increasing level of 1.0-2.0% taurine (NFM+T1.0, NFM+T1.5 and NFM+T2.0) for 10 weeks. Fish fed NFM diets performed equally well final weight and DFI compared with the control group. Growth parameters in terms of %WG and SGR in FM group were significantly higher than those in NFM groups. Among non FM groups, WG and SGR showed no significant difference with increasing levels of taurine supplementation. Fish fed NFM+1.0T performed significantly lower FCR than control. And PER in fish fed NFM+1.0T was significantly lower than control. Among non FM groups, PER gradually increased with increasing level of taurine and PER of NFM+2.0 was significantly higher than NTF+1.0. Typical pathological change for soybean enteropathy such as neutrophil infiltration was observed in intestinal submucosa of fish fed NFM. Inflammatory cytokine genes were significantly up-regulated in intestine of NFM groups than FM group. Although there was no significant difference in gene expression level, gene expression levels decreased with increasing taurine supplementation levels. These results demonstrated that complete replacement of FM in red seabream diet by PP caused pathological changes and high expression of cytokine genes in intestine, but these changes can be improved by taurine supplementation.

A 12-week feeding trial was conducted as the third experiment (5.8g initial weight) to evaluate the effect of graded levels of taurine supplementation to non FM diet on growth performance, feed utilization and nutrient digestibility of juvenile red seabream. Diet formulations in this study were almost same with the experiment 2, except two non FM diets with 0 and 0.5% taurine were added. Six diets were designated as FM (control), NFM, NFM+T0.5, NFM+T1.0, NFM+T1.5 and NFM+T2.0. Fish fed NFM diets performed equally well final weight compared with the control group. % WG and SGR in non FM groups with less inclusion of taurine were significantly lower than the other groups. For DFI, there was no difference among diet groups. Fish fed NFM and NFM+0.5T performed significantly lower feed utilization efficiencies in terms of feed efficiency and PER than the other groups. Among non FM groups, growth parameters and feed utilization efficiency were improved with graded levels of taurine supplementation to diets. Apparent digestibility coefficient (ADC) of dry matter, protein and lipid were significantly higher in FM group than NFM, NFM+T0.5, NFM+T1.0 group. Comparing the ADCs among non FM groups, values were gradually increased as elevation of taurine supplement. ADCs of dry matter and lipid in NFM+T2.0 were significantly higher than NFM groups. Among the non FM groups, the ADC values were tended to elevate with increasing taurine supplementation, even though there was no significant difference.

In conclusion, dietary taurine improved fish growth performance and feed utilization and ameliorates pathological changes of distal intestine such as intestinal inflammation. FM in red seabream diet can be completely replaced without negative effect on growth with sufficient level of taurine supplementation. However, from the viewpoint of intestinal health, FM complete replacement along with PP was not feasible.

Chapter 1. General introduction

Currently, aquaculture is the fastest growing industry in food production sector, and aquafeed production for 1995-2020 has been estimated to be 465% increase and leached 70 million metric tonnes (Tacon et al. 2011). Fish requires much higher dietary protein than terrestrial animals (Wilson 2002) and FM has been used as main protein source of diet especially for carnivorous fish species. However, in recent years, the decline of fishery influenced the global fish meal (FM) productions. Consequently, it caused a reduction of FM availability and cost oscillations. Therefore, FM has been the limitation for the rapid expansion of aquaculture. In the meantime, plant protein (PP) sources have been receiving considerable attention over the past few decades as a partial or total FM replacer in aquafeed industry (Tacon et al. 2011). Among the ingredients being investigated as alternatives to fish meal, the products derived from soybeans are one of the most promising because of its stable supply, low price and relatively good protein/amino acid balance (Lim et al., 1998; Hardy, 1999; Storebakken et al., 2000; Swick, 2002; Gatfin et al., 2007).

However, the presence of antinutritional factors (ANFs), imbalance of amino acid and other disadvantages of PP in diet cause negative effects on growth performance, feed efficiency and health problems of fish. It has been found that only 20-50% of the FM protein could be replaced by conventionally processed soy bean meal (SBM) in the diets of red sea bream, *Pagrus major* (Biswas et al., 2007; Takagi et al, 2001); gilthead sea bream, *Sparus aurata* (Kissil et al, 2000); Japanese flounder, *Paralichthys olivaceus* (Deng et al., 2006); turbot, *Scophthalmus masimus* (Day & González, 2000); Korean rockfish, *Sebastes schlegeli* (Lim et al., 2004); and spotted rose snapper, *Lutjanus guttatus* (Silva et al, 2012). In contrast, other studies have reported from 75% replacement level of the FM without compromising the growth performance in Senegalese sole (*Solea senegalensis Kaup* 1858), juvenile cobia（*Rachycentron canadum*）, red sea bream（*Pagrus major*） and red drum (*Sciaenops ocellatus*) (Aragão et al., 2003; Salze et al., 2010; Kader et al., 2012; Ross et al., 2015;) with various combinations of SBM, soy protein, marine and animal by-products, AA. Many studies demonstrated that the alcohol soluble fraction in SBM and/or

soyasaponin caused morphological changes in the distal intestine of several fish species such as rainbow trout (Bureau et al., 1998; Heikkinen et al., 2006; Romarheim et al., 2008; Yamamoto et al., 2008), Atlantic salmon (Knudsen et al., 2007, 2008; Krongdahl et al., 2003; Baeverfjord and Krogdahl, 1996; Refstie et al., 2000, 2005), Atlantic cod (Olsen et al., 2007) as well as common carp (Uran et al., 2008). Soyasaponins were associated with the onset of morphological changes known as SBM induced enteritis in Atlantic salmon (Knudsen et al., 2007, 2008).

In other teleostean species, particularly in salmon and trout, high sensitivity to SBM induced entritis was well documented (van den Ingh et al., 1991; Rumsey et al., 1994; Baeverfjord and Krogdahl, 1996; Bakke-McKellep et al., 2000; Krogdahl et al., 2003). However, sensitivity to this pathological condition in other species including red sea bream was not well studied. In japan, red sea bream was second most cultured species in terms of production volume (Annual Report of Fisheries and Aquaculture Industry Statistics. 2016.) and there are many reports on availability of dietary plant protein sources (Ukawa et al., 1994; Yamamoto et al., 1996; Takagi et al., 1999; Takagi et al., 2000a, Hanini et al. 2012; Biswas et al. 2017; Matsukura et al., 2017). In some of these studies, effect of supplemental taurine was also examined (Takagi et al., 2006; Hanini et al., 2013). However, effect of taurine was examined mainly on bile acid conjugation and green liver syndrome in red sea bream and no study examined effect of taurine on intestinal morphology of red sea bream. Further, none of these studies examined does dependent effect of taurine.

Plant proteins are limited in a number of nutrients, including taurine, which might be necessary for the optimal performance and metabolism of farmed aquatic animals, particularly for carnivorous fish. Recently, with the study of FM replacement, the utilization of taurine in fish has also received extensive attention. Taurine has various roles such as neurotransmission, vision, reproduction, osmoregulation and immune response except above mentioned physiological roles. It has been demonstrated that dietary taurine deficiency can cause growth retardation and some pathological phenomena in fish, such as green liver syndrome and hemolytic anemia (Maita M, 1997; Watanabe T,

1998; Goto T, 2001; Takagi S, 2005). These pathological changes can be improved by exogenous addition of taurine (Takagi S, 2005, 2006a; 2006b). Also, taurine transporter is needed for cellular taurine intake and TNF-alpha, another kind of inflammatory cytokine, stimulated expression of taurine transporter（Mochizuki et al., 2005）. Inhibition of morphological abnormality of distal intestine of rainbow trout fed SBM based diet was reported by supplementation of 1% dietary cholyltaurine（Iwashita et al., 2008）. In addition, amelioration of intestinal inflammation and reduced expression of inflammatory cytokine by 0.2% taurine supplementation were reported in European seabass（Rimoldi et al., 2016). Also, combined supplementation of enzyme complex and 0.2% taurine improved growth performance of red sea bream fed low FM diet formulated with SBM and CGM (Hanini et al. 2013). These reports suggest that taurine supplementation help recovery from negative impact of SBM. However, these studies use single supplemental level of taurine and no study examining dose dependent effect of taurine.

Therefore, this study aimed at investigating the effect of graded level of taurine supplementation on PP based low or non FM diet on growth performance, intestinal morphology, inflammatory cytokine gene expression and nutrient digestibility of red sea bream was conducted.

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Chapter 2. Literature review

2.1. Aquaculture of red sea bream

Aquaculture is the world's fastest-growing animal agricultural industry. Aquaculture producers are seeking more efficient and sustainable ways to cultivate healthy species to satisfy growing customers' demand.

Red sea bream *Pagrus major* is a very economically cultured marine fish species in Asian countries, especially in Korea and Japan. It is considered a potential species for aquaculture throughout the world due to its high commercial value and human's traditional food habits. Red sea bream has a special place in Japan. Known as madai in Japanese, its unique red colour is considered lucky, and the fish is often served whole on New Year's Day, wedding ceremonies and other auspicious occasions. The aquaculture production of red sea bream was the second largest in Japan after yellowtail (Koshio, 2002). Red sea bream is a strictly carnivorous fish which is generally fed with feeds formulated with high FM content. This habit also places increasing demands on global supply of FM and the associated rise in its price.

For red sea bream, the unit price of cultured red sea bream has been rising in recent years in line with a decline in the production volume. For individual operators, fishery earnings in 2012 recorded a surplus of 10.9 million yen. Corporate operators also made a fishery profit of 2.4 million yen in 2012. Red sea bream has a cost structure where the feed and seed costs account for a large proportion of the total cost. The percentage of feed and seed costs in the total cost has been about or higher than 70% since 2006. (2015 Annual Report on Aquaculture in Japan)

2.2. Protein sources used in aquafeed industry

The availability of quality protein ingredients for aquafeeds is a critical concern of aquaculture producers and feed manufacturers.

Fish meal (FM) is the major protein in aqua-feeds due to its high protein content, excellent amino acid composition, high nutrient digestibility and high availability for farmed aquatic animals (Lovell, 2002; Gatlin et al. 2007). The aquaculture industry recently consumed 65% of global annual

fish meal production, respectively (Tacon & Metian, 2008; Hardy, 2010). The rapid development of aquaculture will result in a high demand and a shortage of supply for fish meal. Historically, 35% of global fishery is used to produce FM. However, in recent years, the decline of fishery influenced the global fish meal productions. The price of FM has been risen year by year. Furthermore, the contamination of certain fish meal with dioxins has reduced the quality of fish meal as a raw material to be included in fish feed. In order to reduce diet costs and increase the production profitability, to look for alternative protein sources to replace FM has been a certain trend. The most pressing issue is not just finding replacement ingredients for FM but identifying a consistent supply of high quality ingredients with a suitable available nutrient content. Lastly, an important aspect of potential ingredients is its effect on the final product quality, including but not limited to, changes in fish texture and flavor and whether the ingredient alters or confers additional human health benefits.

Various protein sources including terrestrial animal by-product meals, oilseed meals and by-products, aquatic plants, single-cell proteins, and legumes and cereal by-products have been widely evaluated for their suitability to partially or totally replace FM from aqua-feed (Wu et al., 1994, 1996; Fontainhas-Fernandes et al., 1999; Richter et al., 2003; Coyle et al., 2004; Abdel-Tawwab et al., 2008; Schaeffer et al., 2010).

Animal proteins such as poultry by-product, meat and bone meal have been used to replace fish meal in fish feed. Animal proteins are good protein sources with low price, which can be used to partially replace fish meal. However, due to the occurrence of BSE, consumers are questioning feeding practices based on the use of animal proteins as raw materials in animal feed. In some countries, animal proteins are banned in animal feed. Therefore, future development of animal feed goes towards a vegetable-based formulation.

Plant protein sources are increasingly used to satisfy the growing demands of the aqua-feed industry (Hardy, 2010). Plant-based protein sources that have been tested include corn gluten meal (CGM), soy bean meal (SBM), soy protein concentrate (SPC), wheat gluten meal, barley protein

concentrates, cottonseed meal, and canola (rapeseed) meal. Potential alternative ingredients have been evaluated with different degree of success in rainbow trout (Oliva-Teles et al., 1994; Gomes et al., 1995), salmon (Torstensen et al., 2008; Pratoomyot et al., 2010), tilapia (El-Saidy & Gaber, 2003, Richter et al., 2003; Coyle et al., 2004).

SBM is one of the most appropriate alternative protein sources used in the formulation of aquaculture feeds, as a substitution of FM. Several studies found that 20-50% of the FM protein could be replaced in the diets of red sea bream (*Pagrus major*) (Takagi et al., 1999, 2001); gilthead sea bream (*Sparus aurata* L.)(Kissil et al., 2000); turbot (*Scophthalmus masimus* L.) (Day and González, 2000). However, it can't be used in higher quantities as they contain anti-nutritional factors (ANFs) (Rumsey *et al*., 1994; Anderson & Wolf, 1995) as well as imbalanced amino acid profile (Wilson, 1989; Floreto *et al*., 2000).

The nutritive value of SBM can be improved using fractionation to produce SPC (Vielma et al., 2000; Collins et al., 2012). The highly refined SPC has a similar protein content and apparent dietary protein and amino acid digestibility to FM (Hardy, 2008). In SPC, the anti-nutritional components are eliminated during processing (Bowyer et al., 2013). Compared to FM, SPC has advantage of high protein and amino acids digestibility co-efficiency, readily available and consistent quality. Literature studies demonstrate that SPC is a good alternative to FM in shrimp and fish diets. In shrimp diet, 40% of fish meal can be replaced by soy protein concentrate without negative influence on growth performance. In fish, 40-100% of FM can be replaced by soy protein concentrate. SPC is a renewable plant protein product that can help reduce pressure on natural fisheries stocks and help provide sustainability to the growing demand for aquatic products.

CGM is considered as a good and palatable source of protein has been used in fish feeding in the USA since the 1970s (Raven et al., 1980). It is a protein-rich feed, containing about 60%-70% crude protein, and it is low in fiber, rich in vitamins B and E and is known to contain no antinutritional factors (Regost C, 1999). CGM is now generally included at 15-50% in trout diets

with other plant protein sources such as soybean products (SPC, SBM) (Aksnes et al., 2006; El-Haroun et al., 2007; Sarker et al., 2011; Prachom et al., 2013). However, increasing CGM in salmon diets resulted in lower digestibility, growth and feed intake. Feeding salmon on a mixture of SBM and CGM also had a negative effect on dressing percentage (Mundheim et al., 2004). These studies indicated that when FM was substituted by plant protein in high level, growth and feed utilization efficiencies will get impaired.

2.3. Use of plant proteins in red seabream diets

Nutrient sources of plant origin are found to contain a wide variety of antinutritional substances. They are seen to be toxic to animals, exhibiting deleterious effects, affecting growth and health. Most of the antinutrients, at levels incorporated as protein sources in fish feed, do not lead to mortality but produce adverse physiological effects, decreased growth and health conditions. Reduced food conversion efficiency, hypoglycemia (reduction in blood glucose level), pancreatic hypertrophy, liver damage and other pathological lesions are seen. Therefore, use of plant ingredients in fish feed without proper treatments may cause significant challenges in fish health.

Lectins cause a reduction in absorption of nutrients from the gut or alimentary canal. Aquatic species vary in their sensitivity to soy products. Most omnivorous freshwater fish species have a high tolerance to SBM and are not affected by soy antinutritional factors, particularly when high soy inclusion aquafeeds are manufactured using extrusion technology. Many marine fish species also appear to have a high tolerance for SBM, including Japanese sea bass, red drum, cobia, cod, yellow croaker, pompano and gilthead sea bream. Other marine fish species have a low tolerance for SBM, such as salmon, yellowtail and amberjack, many sea bass species, groupers and others. These species require the use of low-antigen SPC to negate the effects of ANFs found in SBM. However, even in species with a high tolerance for SBM, the maximum inclusion level of SBM in feeds is generally restricted to about 35% due to the high nutrient density of the feeds. There is not sufficient space in the feed to include higher levels of protein ingredients like SBM and still meet the nutritional

requirements of the aquatic species being cultured. Low-antigen SPC can be used to complement SBM in these diets to produce plant protein feeds with significantly lower reliance on FM. This extraction process removes the soluble carbohydrate and significantly lowers the levels of lectins, trypsin inhibitors, glycinin, B-conglycinin, saponins and oligosaccharides that are considered to be ANFs in regular SBM.

Imbalance in the nutrient composition is another drawback in plant ingredients. This limitation is appearing in amino acid profile and the fatty acid profile in plant ingredients. Amino acid profile of plant ingredients is not totally compensated EAA (Essential Amino Acid) requirement of fish (e.g. soybean; higher in lysine but deficient in methionine, cysteine; corn gluten: low in lysine). This leads to the combined use of one or more plant ingredients to correct balance of the AA profile of fish. One major example is a mixture of corn gluten (high in methionine but low in arginine and lysine) and soybean (high in arginine and lysine, low in methionine) meal to compensate the deficient AA requirement.

Although there are several undesirable characteristics, the value of plant ingredients in aquaculture practices is innumerable.

Table 1 Common anti-nutritional substances and effect on fish

ANFs	Effect on the fish
Protease inhibitor	Reduce apparent digestibility of protein and lipids(Atlantic salmon); Growth retardation in (Nile Tilapia, rainbow trout)
Lectins	Alter nutrient metabolism; Reduced nutrient digestibility and inflammation at distal intestine (Atlantic salmon)
Saponins	Damages to gill epithelium of fish; Cause soybean enteritis (Intestinal inflammation)(salmonids, at moderate to high inclusion level); Lower growth performance(rainbow trout and Nile tilapia)
Phytates	Reduce bioavailability of minerals; Damages in the pyloric caeca (chinook salmon); Hypertrophy and vacuolization of cytoplasm of intestinal epithelium cells (carps)
Oligosaccharides	Decreased nutrient utilization and digestibility (trout)
Glucosinolates	Growth retardation and effect on the thyroid function (rainbow trout)

2.4. Taurine supplement

Taurine is known to be the most abundant free amino acid in various tissues in fish or aquatic animals. It is a nutrient that plays an array of critical roles in its free form (Yamamoto et al., 1998). Taurine has various roles such as neurotransmission, vision, reproduction, osmoregulation and immune response. Except above mentioned physiological roles, in mammals, taurine is involved in a particularly wide variety of functions including constituent of bile, osmoregulation, cell membrane stabilization, anti-oxidation, and calcium signaling required in vertebrates for normal cardiac, skeletal muscle, nervous, and retinal function (Huxtable, 1992; Bouckenooghe et al., 2006).

In recent years, a number of studies have demonstrated the essentiality of dietary taurine for many commercially relevant species, especially marine teleosts.

On the other hand, plants contain less than 1% of the taurine levels. Consequently, the removal of taurine-rich dietary ingredients such as FM may induce a deficiency, of which symptoms include reduced growth and survival, increased susceptibility to diseases, and impaired larval development.

It has been demonstrated that dietary taurine deficiency can cause growth retardation and some pathological phenomena in fish, such as green liver syndrome and hemolytic anemia (Maita M, 1997; Watanabe T, 1998; Goto T, 2001; Takagi S, 2005). These pathological changes can be improved by exogenous addition of taurine (Takagi S, 2005, 2006a; 2006b). Also, taurine transporter is needed for cellular taurine intake and TNF-alpha, another kind of inflammatory cytokine, stimulated expression of taurine transporter（Mochizuki et al., 2005）.

Taurine-deficiency will result in reduced growth performance and immune response, such as of dietary taurine deficiency included reduced weight gain (Jirsa et al., 2014), increased mortality and incidence of green liver syndrome (Takagi et al., 2005). Taurine utilization in plant protein based diet has caused widespread concern. It was reported that dietary taurine supplementation was shown to improve growth rate and feed efficiency in Japanese flounder (Park et al., 2002; Kim et al., 2005), European sea bass (*Dicentrarchus labrax)* (Martinez et al., 2004), yellowtail (Matsunari et al., 2005), juvenile cobia (*Rachycentron canadum*) (Lunger et al., 2007) and more recently golden pompano (*Trachinotus ovatus*) (Wu et al., 2015).

Diets with proper nutrient balance are important in enhancing fish health and higher fish production.

2.5. Inflammation and cytokines

Intestinal health is vital for body health. Once normal intestinal function is damaged, it will cause many diseases, such as loss of appetite, low food intake, growth retardation and the decline of digestive and absorptive capacity of nutrients. With the rapid intensification of aquaculture, infectious disease occurrences that are attributed to bacterial, viral and fungal pathogens have increased. These diseases continue to be one of the most important problems in aquaculture and lead to considerable economic loss (Smith et al., 2003). Higher inclusions of plant ingredients in fish diet have a significant effect on fish health. These include immune response, stress and histological alterations.

Inflammation is one of the first responses of the immune system to infection. The symptoms of inflammation include redness and swelling, which are caused by increased blood flow into the tissue. Inflammation is caused by eicosanoids and cytokines, which are released by injured or infected cells. Common cytokines, which regulate inflammatory response, include interleukins (ILs) that are responsible for communication between white blood cells, tumor necrosis factors (TNFs), transforming growth factor (TGF), chemokines that promote chemotaxis, and interferons (IFNs) that have anti-viral effects. Cytokines, which are protein mediators produced by immune cells, are mainly responsible for host innate defense mechanisms. Inflammatory cytokines can be divided into two groups: those involved in acute inflammation and those responsible for chronic inflammation.

Since cytokines are important regulators of the immune system, investigation of cytokine functions may provide data that can be used as a basis for the development of vaccines and immunostimulants for aquaculture (Sahoo and Sakai, 2010).

IL-1 is a pro-inflammatory cytokine that activates a variety of immune and inflammatory cells, mainly secreted by monocytes, macrophages, neutrophils and endothelial cells. IL-1β also promotes vascular endothelial-leukocytic adhesion. The expression of molecules, chemotactic neutrophils and other inflammatory cells enter the intestinal lesions, causing a series of intestinal inflammation and tissue destruction

IL-8 is a potent neutrophil chemotaxis and activating factor. Mainly be secreted by monocytes, epithelial cells, epidermal cells, fibroblasts, and T lymphocytes under the stimulation of IL-1, TNF, and exogenous factor bacteria polysaccharides (LPS). Its main biological role is to chemotaxis and activate neutrophils, promote neutrophil lysosomal enzyme activity and phagocytosis, and also have certain chemotaxis effect on basophils and T cells. It is currently believed that the inflammatory responses induced by TNF, IL-1 and IL-6 are largely mediated by the induction of chemokines represented by IL-8. IL-8 levels are significantly high in lesion location. It is elevated and is positively correlated with the degree of general inflammation of the lesion. The mRNA detection can

be used as an indicator to judge the severity and efficacy of the disease.

TNF-a is mainly produced by monocytes, macrophages and T cells. TNF-α can promote the secretion of intestinal epithelial cells and the expression of IL-8 gene, and up-regulate the number of T cells, eosinophils and basophils. TNF-α can synergize with IFN-γ to alter the morphological structure and barrier properties of intestinal epithelial cells, leading to increased mucosal permeability. In addition, TNF-α can also induce colonic epithelial cell apoptosis.

TGF-b has many biological effects. In the immune response, in addition to participating in the inflammatory response, it also inhibits the proliferation and differentiation of immune cells and the production of certain cytokines.

Although in general the effects of cytokines are exerted locally at the site of their production (autocrine and paracrine), TNF-a and TNF-b, as well as IL-1 and IL-6, have major systemic (endocrine) effects when either produced acutely in large amounts, as in the case of bacterial sepsis, or chronically in lesser amounts, as in the case of chronic infections.

Inflammation induced by soybean is characterized by flattening of the intestinal mucosa, increased cell proliferation and apoptosis, lack of absorptive vacuoles, widening of lamina propria, leucocyte infiltration, reduced brush border enzyme activity, and activation of the immune system (Baeverfjord and Krogdahl, 1996; Bakke-McKellep et al., 2000, 2007; Krogdahl et al., 2003; van den Ingh et al., 1991).

Table 2. Common cytokines in fish and their effect on fish

Groups	**Pro-inflammatory cytokines:**	**Anti-inflammatory cytokines**
Common cytokine	Interleukin 1β(IL-1β)	Interleukin – 10(IL-10)
	Interleukin – 8(IL-8)	Interleukin – 4(IL-4)
	Tumor Necrosis Factor-α (TNF-α)	Interleukin – 6(IL-6)
	Interferon (IFN-γ)	Transforming Growth Factor (TGF-β)
Effects	Activate the body's innate and acquired immune system when pathogens invade the body, then destroy the invaders	Mainly eliminate the inflammation after the invaders is destroyed, to make the body recover to normal immune and physiological levels.

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Chapter 3. Effect of plant protein based low fishmeal diet on growth performance, histology and gene expression of inflammatory cytokines of juvenile red sea bream (*Pagrus major*)

Abstract

A 12-week feeding trial was conducted to investigate high level replacement of fishmeal (FM) with soybean meal (SM) and CGM (CGM) in diets for red sea bream on growth performance, histology and gene expression of inflammatory cytokines. Red sea bream (mean initial weight; 7.6 ± 2.2g) was fed one of four diets. (1)Fish meal based diet (50% anchovy meal) was a control diet, designated as FM; (2)Fish meal was replaced by SM and CGM at a level of 60%, designated LFM; (3)LFM diet supplemented with 0.5% taurine, designated as TLFM; (4)LFM diet supplemented with 0.5% taurine and 0.05% enzyme complex, designated as TELFM. Fish were fed to apparent satiation twice daily.

After 12 weeks, fish fed LFM showed significantly lower weight gain (%WG) and specific growth ratio (%SGR) than control group while TLFM and TELFM groups showed similar growth performance with control. Feed efficiency in terms of feed efficiency ratio (FCR) and protein efficiency ratio (PER)in LFM group was significantly lower than control and TELFM groups. Daily feed intake (DFI) showed no difference among diet groups. Typical pathological change (Neutrophil infiltration and blood congestion) mainly appeared in intestine submucosa of fish fed with LFM. The expression of inflammation-relative genes (IL-1β, IL-8, TNF-α and TGF-β1) were up-regulated in intestine of fish fed low FM diet. And in LFM group, fish have significantly higher expression level than the control. However, in liver, the cytokine genes expression levels showed no difference among diet groups. Results in this experiment suggested that fish fed LFM indicated poorer growth, feed utilization and severer inflammatory response while taurine and/or enzyme complex supplementation ameliorated soybean-induced adverse impacts.

3.1. Introduction

Fish meal is principal source of protein and lipid in aqua feed around the world. Recenly, production of the fish meal is significantly affected by sustainable issues of marine capture fisheries, variable climatic events and increasing prices of fish meal. Over the past few years, research on finding out cost-effective alternatives to replace the FM component of aquafeeds has achieved considerable progress. A vast array of proteins from both animal and plant sources have been investigated widely for their suitability for partial or total replacement of FM in aquafeeds （Wu et al., 1994; Richter et al., 2003; Schaeffer et al., 2010; Prachom et al., 2013; Plaipetch & Yakupitiyage, 2014; Lu et al., 2015）. Soybean meal (SBM) and corn gluten meal (CGM) are the most promising plant proteins due to their abundance and relatively low cost. However, the use of plant protein meal in red sea bream diets is often limited because of the presence of anti-nutritional factors, poor palatability and deficiency in some indispensable amino acids such as taurine, methionine, and lysine and other undesirable characteristics which may affect the growth of fish (Fowler 1980; Lee et al. 1991).

CGM contains about 60% crude protein and has a good dietary essential amino acid profile, except for deficiencies of lysine and arginine; and does not contain compounds that negatively affect digestion. However, it is usually priced higher than SBM, that is likely to limit its use. SBM and SPC have been used effectively to replace a portion of fish meal in the diets of various fishes. However, wider use of soybean-based diets has been hindered by negative effects associated with high soybean inclusion levels. High levels of soybean products in compounded diets have caused reduction of feed intake, decreased growth, poor nutrient utilization and digestibility, negative physiological effects, and histological abnormalities among some fishes (Krogdahl et al., 2010).

SBM-induced enteropathy (intestine inflammation) was well documented in salmon, trout and European sea bass (van der Ingh et al. 1991; Rumsey et al. 1994; Baeverfjord and Krogdahl 1996;

Bakke-McKllep et al. 2000; Krogdahl et al. 2003; Rimoldi et al., 2016) where the taurine deficiency might be one of the most important reasons. And only few papers reported the impact of SBM on the intestine of red seabream (Amano et al. 2012; Khorsavi et al. 2015; Matunari et al. 2015). Further, although one of them demonstrated the shortening of enterocyte and microvillus height (Khorsavi et al. 2015), none of these studies observed inflammatory changes in the intestine of red seabream.

Plant proteins are limited in taurine content. Taurine is a nutrient that plays an array of critical roles in its free form (Yamamoto et al., 1998). Taurine deficiency will result in reduced growth performance and immune response, signs of dietary taurine deficiency included reduced weight gain (Jirsa et al., 2014), increased mortality and incidence of green liver syndrome (Takagi et al., 2005). Improvement of enteropathy in the distal intestine of rainbow trout fed SBM based diet was reported by supplementation of 1% dietary cholyltaurine (Iwashita et al. 2008a, 2008b, 2009). In addition, it was reported that 0.2% taurine supplementation ameliorated intestinal inflammation and reduced expression of inflammatory cytokine in European seabass *Dicentrarchus labrax* (Rimoldi et al. 2016). Also, strong expression of taurine transporter was reported in distal intestine of Senegal sole (Pinto et al. 2012) where is more sensitive for SBM-induced enteropathy in rainbow trout (Bureau et al. 1998; Heikkinen et al. 2006; Romarheim et al. 2008; Yamamoto et al. 2007) and Atlantic salmon (Baeverfjord and Krogdahl 1996; Refstie et al. 2005; Krogdahl et al. 2003). Based on these studies, it was hypothesized that taurine may be able to improve SBM-induced enteropathy in fish. Previously, effect of supplemental taurine was examined in red seabream (Takagi et al. 2006; Hanini et al. 2013). However, the effect of taurine was examined mainly on liver function and pathogenesis of green liver syndrome, but few studies examined the effect of taurine on the intestinal morphology of red seabream.

Our objectives here were to evaluate the effects of high level of FM replacement with PP in the diet supplemented with taurine and enzyme complex of juveniles red seabream, on growth performance, feed utilization efficiency, histology and gene expression of inflammatory cytokines.

3.2. Materials and methods

3.2.1. Experimental diets

Four iso-nitrogenous (47% protein) experimental diets were employed: FM-based control diet (FM); and three plant-based diets were formulated to fulfill the known nutritional requirement of juvenile red sea bream. Ingredient composition of diets is given in **Table 3**, respectively. In the control diet, fish meal was the main protein source. In the other three diets, 60% fish meal component of the diet were replaced with SBM, soy protein concentrate (SPC) and CGM. And among low FM diets, one diet was supplemented with 0.5% taurine, one was supplemented with 0.5% taurine and 0.05% enzyme complex. The names of the four diets were designated according to the FM content and supplementation, namely FM, LFM, TLFM and TELFM. The calculated lysine and methionine contents of the LFM diets (calculation was based on amino acid composition of ingredient) were insufficient to meet the essential amino acid requirements of red seabream, and therefore methionine and lysine-HCl were used to compensate for this deficiency. Bonito extract was added to LFM diets as attractive.

Table 3. Ingredient composition (%) of experimental diets fed juvenile red sea bream, *Pagrus major*

Ingredients	FM	LFM	TLFM	TELFM
Fish meal	50.0	20.0	20.0	20.0
Defatted soybean meal	-	12.0	12.0	12.0
Soy protein concentrate	-	24.0	24.0	24.0
Corn gluten meal	11.0	12.0	12.0	12.0
Wheat flour	11.0	5.5	5.5	5.5
Pre-gelatinzed starch	5.0	5.0	5.0	5.0
Fish oil	6.0	6.0	6.0	6.0
Soybean oil	5.0	5.0	5.0	5.0
Ca(H2PO4)2	-	1.0	1.0	1.0
Enzyme complex	-	-	-	0.05
Taurine	-	-	0.5	0.5
Bonito extract	-	1.0	1.0	1.0
Methionine	-	0.3	0.3	0.3
Lys-HCl	-	0.5	0.5	0.5
Cellulose	7.4	3.1	2.6	2.55
Others[a]	4.6	4.6	4.6	4.6

[a] Others: Vitamin premix [b] (3%); Mineral premix [c] (1%); Vitamin E (0.1%); Choline chloride (0.5%).

[b] Vitamin premix composition (unit.kg^{-1}): vitamin A, 2,420,000 IU; vitamin D3, 2,420,000 IU; vitamin K3, 6,050 mg; thiamine, 3,025 mg; riboflavin, 3,630 mg; pyridoxine, 2,420 mg; cyanocobalamin, 6.0 mg; L-ascorbic acid, 368,902 mg; nicotinic acid, 24,200 mg; D-pantothenic acid, 6,050 mg; inositol, 121,000 mg; d-biotin, 363 mg; folic acid, 908 mg; para-aminobenzoic acid, 3,025 mg.

[c] Mineral premix composition (%): sodium chloride, 5.0; magnesium sulfate, 74.5; iron (III) citrate n-hydrate, 12.5; trace element mix, 5.0; cellulose, 3.0. [The trace element mixture contains (%): zinc sulfate heptahydrate, 35.3; manganese sulfate, 16.2; copper (II) sulfate pentahydrate, 3.1; aluminum chloride hexahydrate, 1.0; cobalt chloride, 0.3; potassium iodate, 0.1; cellulose, 44.0].

The proximate composition of the experimental diets is given in Table 4, respectively. Crude protein content was about 46 to 48 % in all test diets and crude lipid content was about 13 to 14 % in all test diets.

Table 4. Proximate composition of the test diets (%) fed juvenile red sea bream, *Pagrus major*

parameters	FM	LFM	TLFM	TELFM
Crude protein（% D.W base）	47.4	46.7	47.2	46.8
Crude lipid（% D.W base）	13.1	13.6	14.0	13.8
Ash（% D.W base）	8.5	6.4	6.9	6.5
Moisture	5.1	4.8	5.9	4.5

Values represent averages of duplicate samples from each test diet (*n* = 2).

* Dry weight base.

The total amino acid composition of experimental diets is given in **Table 5**, respectively.

Table 5. Total amino acid content of the test diets (g/100 g, d.b.) fed juvenile red sea bream, *Pagrus major*

	FM	NFM+1.0T	NFM+1.5T	NFM+2.0T
EAAs[a]				
Arginine	2.35	1.91	2.06	1.96
Histidine	1.09	0.87	0.93	0.90
Isoleucine	1.42	1.30	1.41	1.21
Leucine	3.81	4.01	3.90	4.17
Lysine	3.14	2.93	2.74	3.02
Methionine	1.08	0.80	0.93	0.83
Phenylalanine	2.02	2.11	1.94	2.19
Threonine	2.03	1.59	1.67	1.65
Valine	1.44	1.47	1.44	1.50
Conditional EAA				
Taurine	0.39	0.16	0.58	0.56

[a] EAAs, essential amino acids.

Values represent averages of duplicate samples from each test diet (*n* = 3).

Before pelleting, the ingredients were ground to pass through a sieve (0.5 mm mesh) and then thoroughly mixed by using a horizontal mixer (ACM-50 L1.AT, Aikohsha mfg., Tokyo, Japan) for 45 minters. The mixture was then moistened by adding distilled water (30~40%) and mixed for another

15 min prior to pelletizing by a meat chopper (LCM22; Hitachi Kouki, Tokyo, Japan). Pellets were dried in a vacuum freeze dryer (RLE-II 206; Kyowa Vacuum Tec Co, Saitama, Japan) for 16 hs. After preparation, diets were stored at 5 °C until use.

3.2.2. Experimental fish, experimental conditions and feeding

The feeding trial was carried out at the Laboratory of Fish Nutrition, Tokyo University of Marine Science and Technology, Tokyo, Japan. The juvenile red seabreams were obtained from a local hatchery (Marua Suisan Co., Ehime, Japan) and transported to the laboratory by delivery service. The fish were acclimatized in eight 60 L rectangular glass tanks to for 2 weeks. During this period, a commercial diet (50% crude protein; Higashimaru, Japan) was supplied to the fish. After the acclimatization period, 160 juveniles (average body weight, 7.6±2.2g) were bulk-weighed and randomly allocated into previously prepared 8 tanks. The experimental condition is given in **Table 6.** A group of 20 fish were stocked in each tank with duplicate per dietary treatment. All fish were fed the respective experimental diets to the satiation level by hand twice a day six days per week for 12 weeks. Any uneaten feed left was removed one h after feeding by shiphoning and dried using a freeze dryer. The feeding trial was conducted in re-circulated artificial seawater (Sea Life, Japan) system where each tank was equipped with an inlet, outlet, and continuous aeration. Approximately 50% of the seawater in the recirculating system was replaced every 2 weeks. A flow rate of rearing water was maintained at 700-800 ml min^{-1} throughout the experimental period. During the feeding trial, the re-circulating system was equipped with electric heaters (1000W) and water temperature was maintained at 21.0 ± 1.8 ˚C and other water quality parameters (means ± S.D.) were pH 7.1 ± 0.5 and salinity 33.1 ± 0.5. These ranges are considered within optimal values for juvenile red seabream. The tanks were maintained under natural light/dark regime.

Table 6. Experimental condition for feeding trial of juvenile red sea bream, *Pagrus major*

Initial body weight	7.6 ± 2.2
Tank volume (L)	60
Number of fish (ind. / tank)	20
Water temperature (^{o}C)	21.0 ± 1.8
pH	7.0
Photoperiod (h)	12L:12D
Water flow rate (L min^{-1})	0.7-0.8
Feeding	Apparent satiation (twice a day)
Culture period	12 weeks

3.2.3. Carcass sampling

At the beginning of feeding trial, ten fish from initial stock tank were sacrificed by using an overdose of 2-phenoxyethanol (Wako Pure Chemical Industries, Osaka, Japan) and stored at -30 ˚C for chemical analysis.

During the 12-week experiment, the fish in each tank were individually measured with an electrical balance (AUW220D, Shimadzu Co.), respectively, at every four weeks.

At the end of the experiment, fish in each tank were bulk weighted to obtain growth parameters. The rest of the fish was collected, stored at -30 ˚C and transferred to Laboratory of Fish Nutrition, Tokyo University of Marine Science and Technology. Frozen samples were minced by a centrifugal mill (Retsch ZM 1, Haan, Germany) fitted with a 0.25 mm screen and kept at-30 ˚C until analysis.

Weight gain (WG, g), specific growth rate (SGR, %), daily feed intake (DFI, %), food conversion ratio (FCR), survival rate (SR, %) and protein efficiency ratio (PER) were calculated by using the following equations to compare fish growth, nutrient utilization efficiency, and body indices among treatments.

WG (%) = (final weight−initial weight) ×100/initial weight

SGR (%, day^{-1}) = 100 × {(Ln (final weight) −Ln (initial weight))/duration (84 days)}

DFI (%, day^{-1}) = total feed intake ×100／{(initial number of fish× mean of initial body weight + final number of fish× mean final body weight+ dead fish weight) × duration (84 days×2)}

FCR= feed intake (g)/weight gain (g)

PER= weight gain (g)/dry protein intake (g)

SR (%) =100× (final no. of fish/initial no. of fish)

3.2.4. Chemical analysis

Feed ingredients, diets and carcass samples were analyzed in accordance with standard procedures for chemical analysis (AOAC 2005). All the samples were finely ground and analyzed in triplicate.

Dry matter content was calculated from the weight loss after drying of the sample by dry oven (NDO-450, EYERA Co., Tokyo, Japan) at 105 ˚C until it reached a constant weight.

Ash content was determined after the incineration of samples in in electric furnace (FO200, Yamato Co., Tokyo, Japan.) at 650˚C for 8 h.

Crude protein was measured by the Kjeldahl method. The samples were wrapped in weighing paper and put into the digestion tube; in order to digest, one Kjeltab and 12ml concentrated H_2SO_4 solution were added into each tube. Digestion was performed by quick digester at 420 ^oC 1h, and then 80 ml distilled water was added into each sample. It was measured by Kjeltec Auto (Kjeltec 2400, FOSS Co., Tokyo, Japan); an empirical factor of 6.25 was used to convert amount of nitrogen into protein.

Crude lipid was extracted according to the method of Folch et al. (1957). The samples stored in homogenizer container were added 60 ml chloroform / methanol solution (2 / 1, v / v) then it was homogenized by homogenizer for 5 minutes at 13,000 rpm. Secondly, homogenized sample was

transferred to butcher funnel for suction filtration with 60 ml chloroform / methanol solution. The filtrate was transferred to the separate funnel, which filled 24 ml of the 0.03 mol L^{-1} magnesium chloride, and it was shaken well for about 1 minute until no more gas is released and stand overnight. The lower layer in separate funnel was filtered and it was transferred to the previously weighed round bottom flask. In order to obtain the weight of lipid, solvent was evaporated until dryness using the rotary evaporator (SB-650, SB-651, TOKYO RIKAKIKAI Co., Ltd.) until constant weight was obtained.

Total amino acid content was determined by automatic amino acid analyzer (JLC500, JEOL Co.), which analyzed amino acid automatically; procedure is described below.

Samples (0.002 ~ 0.004 mg) were put into digestion tube, and then added 1 ml methanesulfonic acid solution by using a micropipette. Vacuum pump was used for 10 minutes in order to remove air in digestion tube. Heater was set at 110 ^{o}C and put digestion tube on heater for 24 h, and secondly, 1 ml 3.5 N NaOH solution was added into digestion tube to neutralize this solution. It was moved from digestion tube to 10 ml volumetric flask by needle; and diluted by distilled water until the volume up to 10 ml. The solution was transferred from volumetric flask to stocking tube and injected into automatic amino acid analyzer (JLC500, JEOL Ltd., Tokyo, Japan).

3.2.5. Histology analysis

Liver and intestine sampling

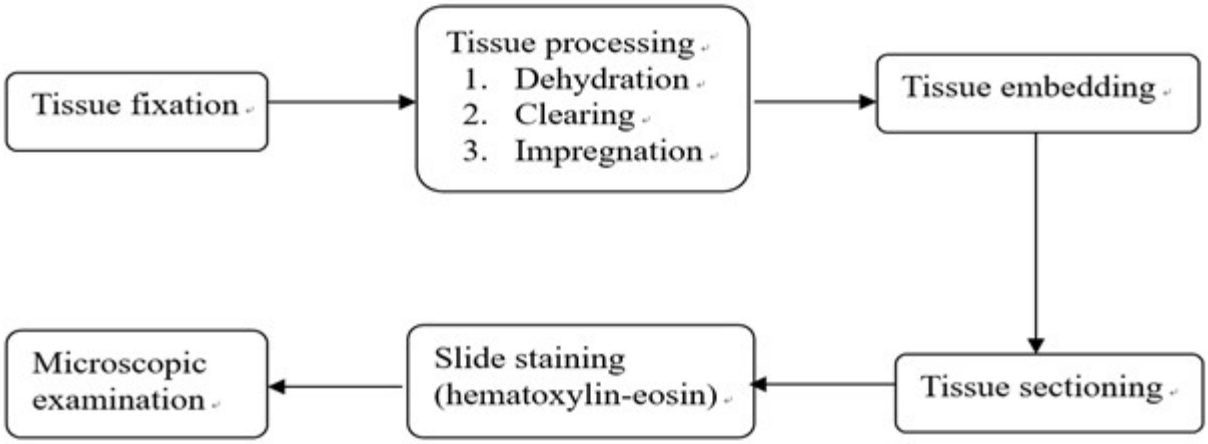
At the end of feeding trial, 3 fish per tank were selected randomly. Liver and intestine from these fish were collected for histology analysis. The occurrence of possible histopathological damages was studied in liver, anterior and posterior intestine. Tissue portions taken from a given part of the organ were fixed in 10% buffered formalin.

Experimental approach

The basic approach is set out schematically in **figure 1.** After euthanasia with overdoes of

anesthetics, body cavity was opened with a scissor and a knife, whole intestine was dissected. Latter half next to the anus was designated as distal intestine. Tissue samples (approximately 5 mm × 5 mm) were taken from the distal intestine. Intestinal samples were processed and fixed in 4% formaldehyde for histological examination. The formalin fixed tissues were dehydrated through graded ethanol solution and then embedded in paraffin wax. Sections of approximately 5 μm thickness were cut and stained with hematoxylin and eosin (HE) (Menke et al. 2011). Section was observed under a light microscopy (Nikon Eclipse E600, Nikon Instruments, Tokyo, Japan). Pathological change was graded as no(−), slight (+), moderate (++), and marked (+++).

Figure 1. Histology procedure



3.2.6. Gene expression analysis

Liver and intestine sampling

After 12-week feeding trial, 3 fish from each tank were randomly selected. Liver and intestine were sampled for total RNA extraction and cytokine gene expression analysis. The fish were dissected under sterile conditions. To reduce the individual variation of gene expression, the sample from each tank was pooled and homogenized using a glass homogenizer and stored at -70 °C.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from 50 to 100 mg of liver and intestine tissues by the standard Trizol extraction method (Invitrogen, Carlsbad, CA, USA) and recovered in 100 µl of molecular biology grade water. Total RNA samples were pre-treated with RNase-free DNase (Promega, Madison, WI, USA) according to the manufacturer's instructions to remove possible genomic DNA contaminations. First strand cDNA was synthesized in 20 µL RT reactions with 1 µg total RNA template, MultiScribe™Reverse transcriptase, 10X RT buffer, 25X dNTP mix, 10X RT random primers (Applied Biosystems, USA).

Quantitative real time PCR

The expression of relative expression of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-8 (IL-8); transforming growth factor-β (TGF-β) genes of liver and intestine tissues of red sea bream fed three diets under were analyzed by quantitative real time PCR (qRT-PCR). The expression of target gene was normalized using β-actin as a housekeeping gene. PCR primers for β-actin, TNF-α, IL-1β, IL-8 and TGF-β were designed according the Red sea bream (Table 5).

A qRT-PCR amplification was carried out using SYBR green PCR master mix (Applied Biosystems, USA) in duplicate and an ABI7300 quantitative PCR system (Applied Biosystems, USA) following the manufacturer's instructions. The conditions of quantitative PCR as follows: an initial denaturation step of 1 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 1 min, and final step dissociation stage followed by 1 cycle 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s, 60 °C for 15 s as described by Zhao et al. (2015).The β-actin was used as an internal control for sample normalization of the target primers. The specificity of the real time PCR reactions was confirmed by the agarose gel electrophoresis for all samples.

The comparative cycle threshold method (CT method) was used for qRT-PCR data analysis, where CT values refer to the number of cycles at which monitored fluorescence emissions in the qRT-PCR reactions exceed a manually set threshold. The relative gene expression model was used to evaluate the fold changes in mRNA expression between fish in different treatments using the ΔΔCT method

(Livak and Schmittgen, 2001). Relative gene expression ratios (R) between treated and control groups were calculated using the formula; R = 2^-ΔCT with ΔCT = CT (target gene)−CT (β- actin) and with ΔΔCT =ΔCT (treatment) - ΔCT (control). Fish reared with FM was used as the respective control. Thus, dietary induced changes are presented as n-fold differences relative to the corresponding control set to 1.

Table 7. Primers sequence used for qPCR

Gene	Primer	GenBank Accession No.	Primer sequence (5'------- 3')	Annealing temp. (C)	PCR product (bp)
β-actin	β-actin-F	AB036756.1	TTCCTCGGTATGGAGTCCTG	60	115
	β-actin-R		TGGTACCTCCAGACAGCACA		
Interleukin1β	IL-1β-F	AY257219.1	GCGAGCAGAGGCACTTAGTC	60	109
	IL-1β-R		AGGTAGGTCGCCATGTTCAG		
Interleukin 8	IL-8-F	KF693767.1	CCATCCCTGATGGTGTTGAT	60	114
	IL-8-R		ACCTCTTGGCCTGTCCTCTT		
Tumor Necrosis Factor-α	TNF-α-F	AY314010.1	CGGACACTGCTGAGAAAACA	60	107
	TNF-α-R		CGAACCCTCGTCTTCATCAT		
Transforming growth factor β	TGF-β-F	FJ767724.1	ATCCCTCAAAACGTCAGCAG	60	104
	TGF-β-R		AAGCTCCTCACACAGCAGGT		

3.2.6. Statistical analysis

The effects of different diets on red seabream whole body proximate composition, growth, survival, feed utilization and relative gene expression were calculated and as subjected to one-way analysis of variance (ANOVA) in SPSS version 20.0 (SPSS Inc., Chicago, IL, USA). When ANOVA identified differences among groups, the differences in mean values were compared using Tukey's HSD test at the 5% level of significance ($P < 0.05$). All data are expressed as mean ± standard deviation.

3.3. Results

3.3.1. Growth performances and feed utilization efficiencies

The experimental diets affected growth performance and feed utilization efficiencies of fish (Table 6). At the end of the 12-week feeding trial, juvenile weight gain ranged from 46 to 56g. The lowest growth parameters were observed in the group fed LFM. Final body weight (FW), weight gain (WG), specific growth rate (SGR) and feed conversion rate (FCR) were not significantly improved in TELFM (P>0.05). Feed intake (FI) was depressed when fish were fed LFM and TELFM, however, there was no significant difference among the three dietary treatments. Compared to fish fed FM and TELFM, fish fed LFM showed significantly higer FCR than other two treatments. There was no significant difference between FM and TElFM. That revealed dietary taurine and enzyme complex can improve feed utilization efficiency.

Table 8. Growth performance and feed utilization efficiencies of whole fish body of Red sea bream fed experimental diets for 12 weeks.

	FM	LFM	TLFM	TELFM
IW (g)	7.60±0.12	7.64	7.65	7.66
FW (g)	55.5	46.0	51.0	52.6
WG(%)	630.7a	503.3b	567.4ab	586.9ab
SGR	2.37a	2.14b	2.26ab	2.29ab
DFI(%)	0.48	0.49	0.48	0.48
FCR%	1.10a	1.21b	1.14ab	1.12a
PER	1.93a	1.76b	1.86ab	1.90a
SR%	85.0	77.5	82.5	85.0

Mean values in same row with different superscripts are statistically different (p <0.05)

[1] IBW: initial body weight; FBW: final body weight; WG: weight gain; SGR (%): specific growth rate (%); FCR: food conversion ratio.

3.3.2. Whole body proximate composition

The nutritional quality of fish was determined based on proximate composition and amino acid composition. Body composition of red sea bream fed experimental diets is shown in Table 7.

The data for whole body proximate analysis reflects that the crude protein value in group fed LFM was significantly lower than other two groups ($P< 0.05$). The FM group showed highest protein value. However, there was no significant difference on percentage crude lipid, moisture and ash among different dietary groups. The value of ash content was lowest in the group fed LFM compared to the other groups while crude lipid and moisture showed higher value than other groups.

Table 9. Proximate compositions of whole fish body of Red sea bream (% wet basis, n=3)

parameters	FM	LFM	TLFM	TELFM
Crude protein（% D.W base）	47.3	46.7	47.2	46.8
Crude lipid（% D.W base）	13.1	13.6	14.0	13.7
Ash（% D.W base）	8.52	6.44	6.91	6.46
Moisture	5.05	4.83	5.87	4.52

Values are mean± S.D. Means with the same letter in a same row are not significantly different (P>0.05)

3.3.3. Histology study

From visual observation, no occurrence of green liver syndrome was found in all diet groups.

The liver structures are shown in **Figure 2.** The structures were very similar in FM, TLFM and TELFM fish (**Fig. 2. A, B**). The average diamater of hepatocyte is 23μm and the nuclear is in the middle of the cellular. However, it showed some morphology changes in fish fed LFM (**Fig. 2. C, D**). **Fig. 2C.** showed some atrophic hepatocyte(a) of LFM group. Compared to other two groups, the size of cellula apparently shrinked and the average diamater of hepatocyte is only15μm. **Fig. 2.D.** showed hydropic degeneration(b), hepatocyte apparently enlarged, and the average diameter is 30μm. In **Fig. 2.D**, letter **c** indicated nuclear dislocation. Compared with other two groups, the nuclear shift to the cytomembrane. According to statistic, 30% of hepatocytes in LFM group were observed this phenomena.

Figure 2. Hematoxylin and eosin stained sections of liver of Red sea bream fed with experimental diets: FM (A), TELFM (B) or LFM (C, D). (a) indicates atrophic hepatocyte; (b) indicated hydropic degeneration; (c) indicates nuclear dislocation. (Scale bar = 50 µm)

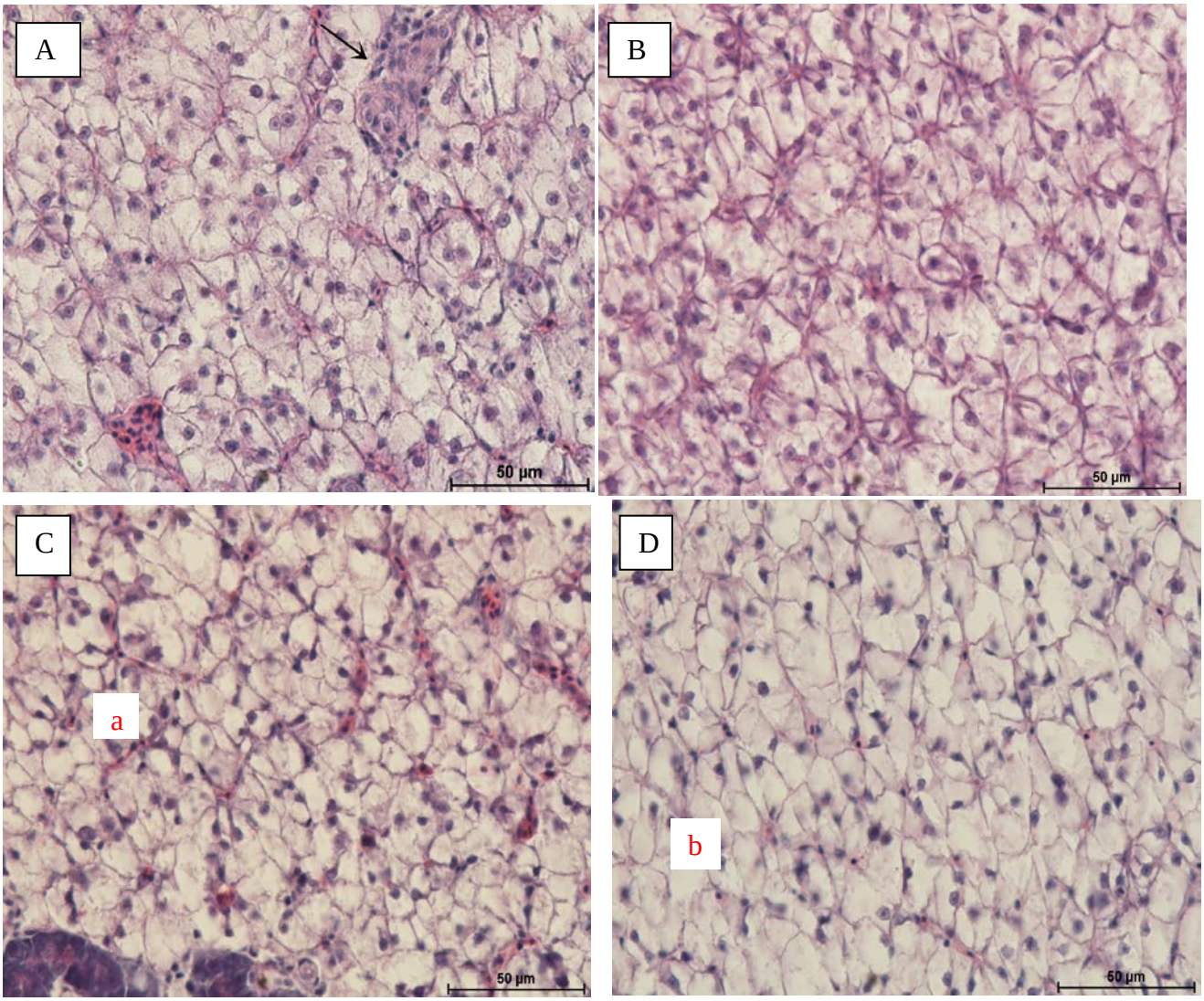


Figure 3.Histological characteristics of intestinal mucosa from red seabream fed a fishmeal based control diet and three LFM diets. (A) FM (control); (B, C, D) LFM. The black arrowheads (a, b) represent the infiltrated neutrophils. (c). Inflammatory response. Scale bar = 50 μm

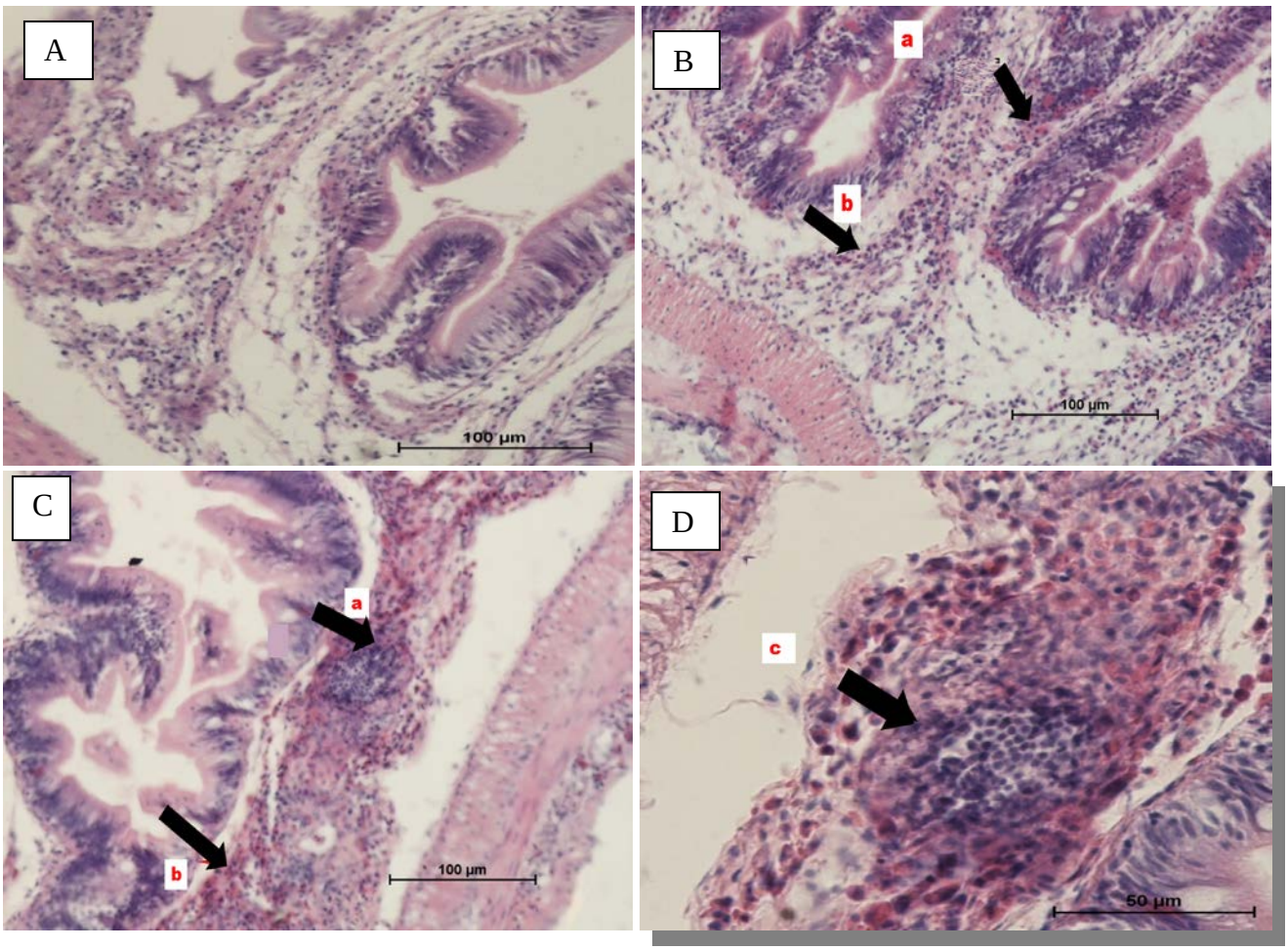


Table 10. Pathological change in Red sea bream after 12 weeks feeding trial.

Parameter	FM	LFM	TLFM	TELFM
a. Neutrophil infiltration	**(-) 5/6**	**(+) 4/6**	**(-) 5/6**	**(-) 5/6**
	(+) 1/6	**(++) 2/6**	**(+) 1/6**	**(+) 1/6**
b. Blood congestion	**(-)**	**(-) 3/6**	**(-) 5/6**	**(-) 5/6**
		(+) 3/6	**(+) 1/6**	**(+) 1/6**
c. Inflammatory response		**(-) 4/6**		
		(+) 2/6		

The intestine structures are shown in **Figure 3.** The structures were very similar in FM and TELFM fish (**Fig. 3.A, B**), also in anterior and posterior intestine. However, some pathological changes in fish fed LFM (**Fig. 3.C, D, E**) were observed Pathological change was graded as negative (−), slight (+, C), moderate (++, D, E), and marked (no). Letter a indicated neutrophil infiltration of LFM group. Letter b indicated blood congestion and letter c indicated inflammatory response.

Table 10. shows the pathological changes in Red sea bream after 12 weeks feeding trial. Typical pathological alterations were observed in fish fed LFM. Fish fed TLFM and TELFM also showed some pathological changes, but the changes were very slight. Inflammatory response was only observed in fish fed LFM, statistically 33% fish in LFM group had the response. The result suggested that dietary effects on physical health of red sea bream.

3.3.4. Relative gene expression

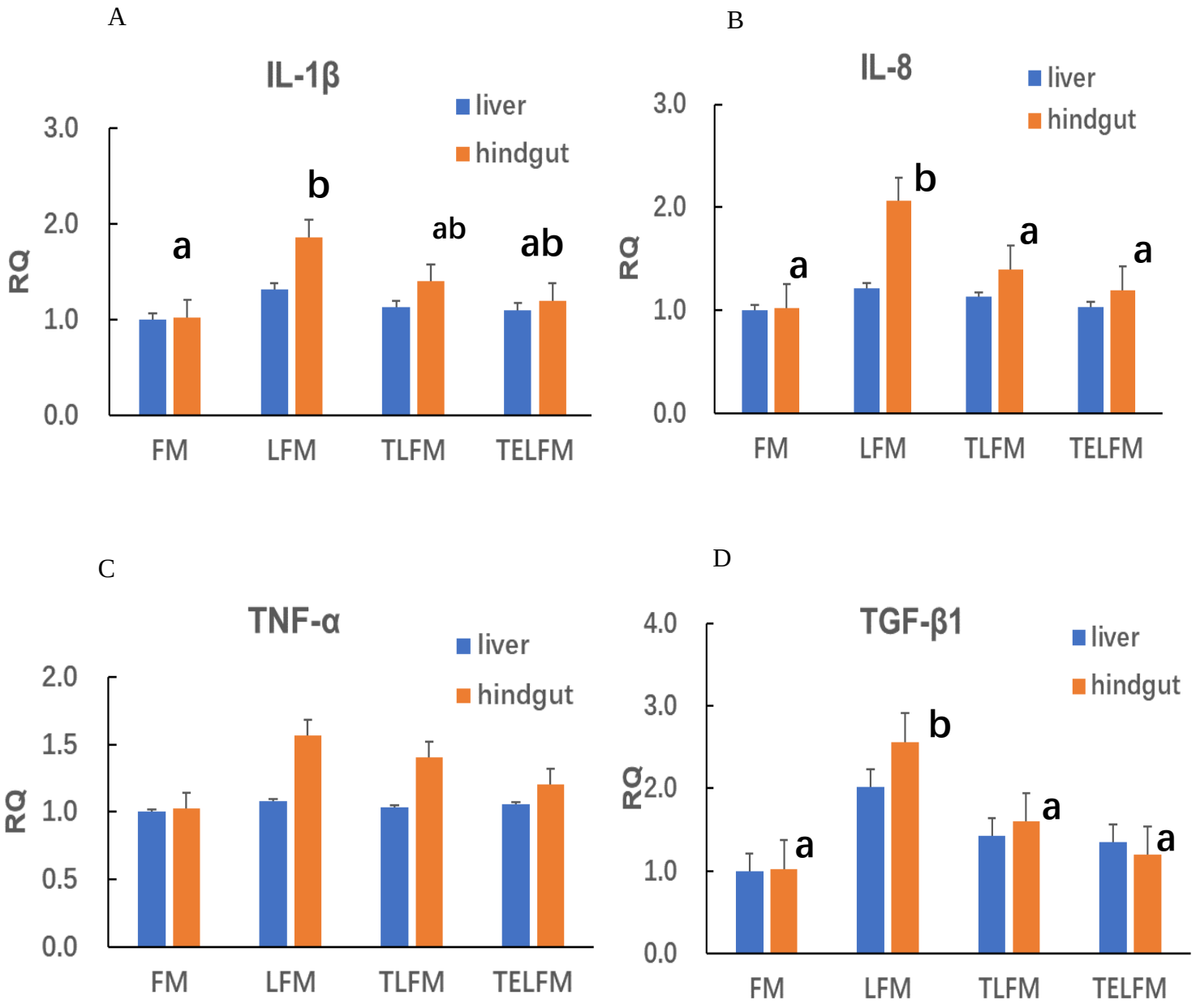
Relative expression of IL-1b, IL-8 and TNF-a and TGF-b were shown in **Fig. 4**. The gene expression of fish fed low FM diets were up-regulated both in liver and hindgut compared to fish fed FM and the highest values were found in LFM group. However, cytokine gene expression levels in liver did not differ significantly among diet treatments. However, hindgut showed something

different: cytokine gene expression was significantly influenced by the experimental diets.

In hindgut, IL-1β expression level in LFM group was significantly higher than FM groups while taurine and enzyme complex supplemented low FM diet groups shows no difference comparing to control group. Gene expression level of TNF-a showed no difference among the dietary treatments even though the value was highest in LFM group.

Expression of IL-8 and TGF-β1 showed very similar trend in intestine of red sea bream even though IL-8 belongs to pre-inflammatory cytokine and TGF-β1 belongs to anti-inflammatory cytokine. LFM group showed significantly higher expression level than control group and significantly higher than fish fed TLFM and TELFM diets. This result indicated that taurine and enzyme complex can effectively down-regulate the expression of IL-8 and TGF-β1 affected by PP ingredients.

Figure 4. mRNA expression of 4 cytokine genes associated with gut inflammation in red seabream: IL-1β (A); IL-8 (B); TNF-α (C); TGF-β1 (D). Relative mRNA levels were determined by the comparative Ct method using β-actin gene as the control. Relative mRNA expression was evaluated by real-time quantitative PCR. Values are means ± S.D. (n = 4). Bars of the same gene bearing with different letters are significantly different by Tukey HSD test ($p < 0.05$).



3.4. Discussion

In recent years, an amount of research has been conducted on the replacement of FM by PP in different level. Growth performance and feed efficiency are the necessary indicators to evaluate the suitability of this replacement. Some studies also focus on immunity of fish fed the low fish meal diet. In the present study, the effects of FM replacement were studied not only on growth performance, feed efficiency, but also on the inflammation response of fish body.

In present study, retardation of growth parameters was observed in fish fed LFM. The finding was in correspond with the previous studies that suggested partial replacement of FM with plant protein caused growth depression in red sea bream (Takagi et al. 1999, 2000a, 2000b). Higher growth rate always correlates with the feed intake. Previous studies suggested that higher inclusion levels of soybean proteins resulted in lower feed intake (FI) because of imbalanced amino acids and decreased palatability of diets (Imsland et al., 2001, Rubio et al., 2005, Wang et al., 1997). However, daily feed intake in this study showed no difference among diet treatments, which quite differed from previous research. One reason maybe the supplementation to low FM diets of 1% bonito extract (a kind of phagostimulant) helped improving the palatability of feed. Feed utilization in LFM groups was significantly lower than FM groups. The detrimental effects probably due to the presence of anti-nutritional factors in SBM in diet (Vaintraub and Bulmaga 1991; Liener 1994). Dietary enzyme supplementation may improve the nutrition digestibility in several fish species (Jackson et al. 1996, Debnath et al. 2005, Giri et al. 2003, Drew et al. 2005, Refstie et al. 1999; Odetallah et al. 2005). In present study, significantly higher feed utilization in fish fed TELFM than LFM group was in line with the previous studies.

The whole body proximate compositions of our experimental fish were not affected by the dietary ingredients. Whole body composition of fish fed PP based diets was similar to that in the control. This result suggests that partial replacement of FM in the red seabream diet with PP had no

negative impact on the final nutritional quality of fish products.

In the present studies, only some morphology changes were observed in liver of fish fed LFM. However, no occurrence of green liver syndrome was found in low FM groups. Differing from this finding, Goto et al, 2001 demonstrated the incidence of green liver of red seabream fed low fish meal diets in six months (180 days). In this study, feeding trial was only lasted for 84 days, so it is thought that the no incidence of green liver or decline in growth were observed perhaps because of the time limitation. The histologic study of intestine submucosa revealed some pathological changes in fish fed LFM. The pathological changes were not clear to demonstrate inflammation. In previous trials taken on rainbow trout (Buttle et al., 2001; Krogdahl et al., 1994; Nordrum et al., 2000; Romarheim et al., 2008; Rumsey et al., 1994; Sealey et al., 2009), clear signs expected inflammatory potential of distal intestinal inflammation were observed in fish fed SBM based diet. The infiltration of granular neutrophils in the intestine submucosa found in the current study is similar to that induced by soybean in Atlantic salmon (Baeverfjord and Krogdahl, 1996), gilthead sea bream (A. Sitjà et al., 2005). In Atlantic salmon, SBM produced a decrease in mucosal enzymes, which were coincidental with an impaired feed conversion (Krogdahl et al., 2003). In present case, FCR showed the similar effect in fish fed with LFM. However, the factor in plant protein caused tissue histology alteration was unclear.

Inflammation is characterized by a massive neutrophil infiltration and generation of cytokines. Cytokines are the key mediators in response to microbial invasion and tissue injury and can stimulate immune responses. Previous studies reported that increased cytokines production may also result in increased phagocytic activity (Low et al., 2003). In the present study, relative expression of imflammatory cytokine genes was significantly affected by the diet. However, gene expression level was no more than 2 which cannot indicate the inflammation caused by plant protein in LFM group. Combining with the histology analysis, only some inflammatory reponses appeared in LFM group when living in the nomal rearing condition.

Relative expression of fish fed LFM showed up-regulation effect. Similar up-regulation of IL-1β and TNF-α gene expression was observed in turbot treated with nucleotides (Low et al., 2003) and in common carp fed with spirulina (Watanuki et al., 2006) or with human IFN-α (Watanuki et al., 2009). In this study, IL-10 gene expression was up-regulated, that agreed with the preivious study taken on sea bass treated with LPS (Buonocore et al., 2007) and common carp fed with human IFN-α (Watanuki et al., 2009). In contrast to current study, spirulina-treatment of common carp (Watanuki et al., 2006) showed down-regulated the expression of the IL-10 gene, also dietary baker's yeast extract in common carp (G. Biswas et al., 2012). In other cytokine gene, some species were reported to have the similar up-regulated expression effect.

Soya saponins and soya lectins are suspected to be the main inducers of inflammation (Iwashita et al. 2008a, 2009; Chen et al. 2011). In the present study, red seabream fed LFM diet showed pathological changes in the hindgut, and the main performance was the appearance of amount of infiltration of inflammatory cells identified as neutrophils in submucosal. These results were consistent with previous findings in carp (Urán et al. 2008a), rainbow trout (Romarheim et al. 2008), giant grouper (García-Ortega et al. 2016), and orange-spotted grouper (Wang et al. 2017), which indicated intestinal inflammation when fish fed SBM based diet. However, studies taken on red seabream previously (Amano et al. 2012; Khorsavi et al. 2015; Matunari et al. 2015), failed to observe SBM induced enteropathy in red seabream. The reason why inconsistent results were obtained was unclear. However, they fed the diets only for 6 weeks. One may suppose that feeding SBM based diet 6 weeks is too short to induce pathological changes of intestine of red seabream. In the other hand, they successfully observed pathological changes of liver in red seabream. And these pathological change in hepatocyte was the same with the present study. This suggests that liver seems to be more sensitive than intestine in red seabream. In addition, the pathological changes, especially the invasion of neutrophil, was gradually ameliorated in low FM diets with taurine supplementation. These findings suggest that taurine could partly prevent pathological changes in the

intestine of red seabream fed PP based diets. This hypothesis was agreed with Rimoldi et al. (2016) who revealed that taurine exerted a mitigating effect on the inflamed distal intestine of European sea bass fed SBM. Similarly, Iwashita et al. (2009) also indicated that morphological changes in the distal intestine of rainbow trout fed SBM-based diets can be prevented by supplementation of cholyltaurine.

In the present study, expression of proinflammatory cytokines such as IL-1β, IL-8 and TNF-α was significantly up-regulated by PP in low FM diets. Gene expression analysis of inflammatory cytokines along with progression of SBM-induced enteropathy in Atlantic salmon and common carp revealed that elevated expression of proinflammatory cytokine genes such as IL-1 and IL-8 in early phase of the enteropathy (Lilleeng et al. 2009; Urán et al. 2008a, b). Although recovery of intestine has not been reported in Atlantic salmon, adaptation and recovery of intestinal tissue was suggested in common carp fed SBM based diet. Specifically, after 4 weeks onwards, recovery of intestine was observed and increased expression of TGF-β was initiated to be observed after 3 weeks of feeding SBM diet in common carp (Urán et al. 2008b). Although it is unknown whether adaptation to SBM and recovery also takes place in red seabream, the present study successfully observed increased expression of TGF-β in red seabream fed low FM diets. Lower expression level in fish fed low FM diet with taurine supplementation indicated that inclusion of taurine could alleviate the inflammatory response in intestine. On the other hand, taurine supplementation failed to improve intestinal injury as well as inflammatory response in Atlantic salmon (Kortner et al. 2016). The reason why taurine was not effective in their study was unclear, but one possibility is the taurine level used was below effective concentration.

3.5. Conclusion

Partial replacement of FM with plant proteins had detrimental effects on the growth performance, feed utilization, liver and intestine morphology and intestinal cytokine gene expression.

In low FM diets, supplementation with taurine in feed resulting in enhanced growth, and soybean-induced enteropathy can also be ameliorated.

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Chapter 4. Non fishmeal diet supplemented with taurine: effects on growth performance, feed utilization, morphology and cytokine gene expression in intestine of juvenile red seabream

Abstract

Red sea bream (5. 4 ± 0.03 g) were fed one of the four iso-nitrogenous (46% crude protein) and iso-lipidic (12% crude lipid) diets formulated with 50% fishmeal diet (FM, control) or soy and corn protein based non-FM diets supplemented with increasing level of 1.0-2.0% taurine (NFM+T1.0, NFM+T1.5, and NFM+T2.0) for 10 weeks. Fish fed NFM diets performed equally well final weight (FW) and daily feed intake (DFI) compared with the control group. Growth parameters in terms of weight gain (% WG) and specific growth rate (% SGR） in FM group were significantly higher than those in NFM groups. Among non-FM groups, WG and SGR showed no significant difference with increasing levels of taurine supplementation. Fish fed NFM+1.0T and NFM+1.5T performed significantly lower feed utilization efficiencies in terms of feed conversation ratio (FCR) and protein efficiency ratio (PER) than control. Apparent digestibility coefficient (ADC) of protein and lipid were significantly higher in the FM group than the NFM+1.0T group but not NFM+1.5T and NFM+2.0T groups. Typical pathological change for soybean enteropathy such as neutrophil infiltration was observed in the intestinal submucosa of fish fed NFM-based diets. Inflammatory cytokine genes were significantly up-regulated in the intestine of NFM groups than the FM group. However, the gene expression levels significantly decreased with increasing taurine supplementation levels in the NFM groups. These results demonstrated that the complete replacement of FM by plant protein (PP) showed no compromising growth effect. However, PP caused pathological changes and high expression of cytokine genes in intestine, but these changes can be ameliorated by taurine in red seabream.

4.1. Introduction

In response to the rising demand for, and cost of, FM, the use of dietary alternatives is becoming a common practice in aqua-feed formulations. To successfully achieve the goal of replacement of FM in aquafeed, the premise should be paid attention to: 1 do not affect the growth of aquaculture animals: mainly from the balance of nutrients; 2 no adverse effect on the survival of aquaculture animals: improve the immunity of aquaculture animals by using some functional feed additives; 3 do not affect the palatability of aquafeeds: through appropriate technical measures or add some attractive to eliminate the negative effect caused by ANFs in alternatives to improve the palatability of feed; 4 do not affect the quality of aquaculture animals: alternatives in feed cannot shadow nutritional quality and safety.

Fish meal inclusion levels for both omnivorous and carnivorous fishes have been reducing significantly at present (Hardy, 2010) on account of fish meal supply becoming significantly low together with its huge demand and higher prices in the market (Edwards et al., 2004; De-Silva and Hasan, 2007; Hung et al., 2007). FM replacement levels can be accelerated to 50–60% in juvenile and 70–90% in yearling red sea bream by combining several protein sources (Aoki et al., 1998; Kader et al., 2010; Takagi et al., 2000b). FM completely replaced by mainly soy products has achieve success in rainbow trout (Kaushik et al., 1995) and in cobia (Salze et al., 2010), whereas in marine finfish such as red sea bream and yellowtail, SBM-based diets with low or no FM content have been shown to depress the growth and feed efficiency, and cause physiological abnormalities. However, Kader et al. (2012a) indicated that dietary SBM supplemented with fish soluble, krill meal and squid meal is capable to a total replacement of FM for red sea beam.

The intestines of aquatic animals are not only the digestive and absorbing organs, but also the largest immune organs, metabolic organs and endocrine organs (Gosain, 2005). The intestinal tract of aquatic animals is susceptible to damage due to its quite simple structure. When intestinal tract got

damaged, the digestion and absorption of nutrients always be affected and then affected growth performance. At the same time, intestine damage always causes the pathogens invade the body. It can cause intestinal diseases in aquatic animals. Therefore, the design of aquafeed formulations should take the intestinal health of aquaculture animals into account. FM replaced by plant proteins, which contains anti-nutritional factors, is a major factor in inducing enteritis in aquatic animals is an important factor in inducing enteritis in aquatic animals. Based on the maintenance of intestinal health in aquatic animals, to supplement some functional feed additives to improve intestinal health during feed formulation is necessary.

Intestinal inflammation induced by soybean is characterized by flattening of the intestinal mucosa, increased cell proliferation and apoptosis, lack of absorptive vacuoles, widening of lamina propria, leucocyte infiltration, reduced brush border enzyme activity, and activation of the immune system (Baeverfjord and Krogdahl, 1996; Bakke-McKellep et al., 2000, 2007; Krogdahl et al., 2003; van den Ingh et al., 1991). It was demonstrated that the alcohol soluble fraction of SBM and 47 soyasaponin caused morphological changes in the distal intestine of several fish species 48 such as rainbow trout Oncorhynchus mykiss (Bureau et al. 1998; Heikkinen et al. 2006; Romarheim et al. 2008; Yamamoto et al. 2007), Atlantic salmon Salmo salar (Baeverfjord and Krogdahl 1996; Refstie et al. 2005; Krogdahl et al. 2003), Atlantic cod Gadus morhua (Olsen et al. 2007) as well as common carp Cyprinus carpio (Urán et al. 2008a). However, sensitivity to SBM was not well studied in other species including red seabream. In japan, red seabream was second most cultured species in terms of production volume (Ministry of Agriculture, Forestry and Fisheries 2016) and there are many studies on availability of dietary PP sources for this species (Ukawa et al. 1994; Yamamoto et al. 1996; Takagi et al. 1999, 2000a; Hanini et al. 2013; Biswas et al. 2017; Matsukura et al. 2017). PPs are limited in several important nutrients such as taurine, which is thought to be conditionally essential for farmed aquatic animals, particularly for carnivorous fish (El-Sayed et al. 2014; Salze and Davis 2015). Taurine has various roles such as neurotransmission, vision, reproduction, osmoregulation and

immune response (Huxtable 1992).

As described previously, there are several reports documenting positive effect of taurine on feed performance of low FM diet (Hanini et al. 2013; Linn et al. 2014; Matsukura et al. 2017; Hu et al. 2018; Zhang et al. 2018). Improvement of enteropathy by supplementation of taurine or its derivatives to SBM based diet has been reported in several fish species, including rainbow trout fed (Bureau et al. 1998; Heikkinen et al. 2006; Romarheim et al. 2008; Yamamoto et al. 2008; Iwashita et al. 2008a, 2008b, 2009), European seabass (Rimoldi et al. 2016), sole (Pinto et al. 2012) and Atlantic salmon (Baeverfjord and Krogdahl 1996; Refstie et al. 2005; Krogdahl et al. 2003). Based on these studies, it was hypothesized that taurine maybe able to improve SBM-induced enteropathy in red seabream. However, in studies mentioned above, they used single supplemental level of taurine and no study examining different dose of taurine except for studies in rice field eel Monopterus albus and black carp Mylopharyngodon piceus (Hu et al. 2018; Zhang et al. 2018). For red seabream, as long as we know, only few papers examined impact of SBM on intestine of red seabream (Amano et al. 2012; Khorsavi et al. 2015; Matunari et al. 2015) and one of them demonstrated shortening of enterocyte and microvillus height (Khorsavi et al. 2015), none of these studies observed inflammatory changes of intestine of red seabream.

Our objectives here, therefore, were to evaluate the effect of graded level of taurine supplementation on plant protein based no FM diet on growth performance, intestinal morphology and inflammatory cytokine gene expression of red seabream.

4.2. Materials and Methods

4.2.1. Experimental diets

Four iso-nitrogenous (46% crude protein) and isolipidic (14% total lipid) experimental diets (FM-based control diet and three PP based diets) were formulated to fulfill the known protein, lipid and indispensable amino acid requirement of juvenile red seabream (National Research Council 2011). The formula and chemical composition of the experimental diets are shown in Table 11. The

control diet was a 50% FM based diet (FM). Non FM (NFM) diets were supplemented with graded level of taurine at 1.0, 1.5, and 2.0%, designated as NFM+T1.0, NFM+T1.5 and NFM+T2.0, respectively (Table 11.). Bonito extract was also supplemented in NFM diets as feed stimulant (Table 11.). Before pelleting, the ingredients were ground to pass through a sieve (0.5 mm mesh) and then thoroughly mixed by using a horizontal mixer (ACM-50 L1.AT, Aikohsha mfg., Tokyo, Japan) for 45 minters. The mixture was then moistened by adding distilled water (30~40%) and mixed for another 15 min prior to pelletizing by a meat chopper (LCM22; HITACHI KOUKI., Tokyo, Japan). Pellets were dried in a vacuum freeze dryer (RLE-II 206; Kyowa Vacuum Tec Co, Saitama, Japan) for 16 hs. After preparation, diets were stored at 5 °C until use.

Table 11. Ingredient composition of experimental diets fed red seabream

Ingredients	**FM**	**NFM+ 1T**	**NFM+ 1.5T**	**NFM+ 2T**
Fish meal	50	0	0	0
Defatted soybean meal	-	12	12	12
Soy protein concentrate	-	28	28	28
Corn gluten meal	11	26	26	26
Wheat flour	11	5.5	5.5	5.5
Pre-gelatinized starch	5	2.5	2	1.5
Fish oil	6	6	6	6
Soybean oil	5	5	5	5
Taurine	-	1	1.5	2
Methionine	-	0.3	0.3	0.3
Lys-HCl	-	0.8	0.8	0.8
$Ca(H_2PO4)_2$	-	3	3	3
Bonito extract	-	1.5	1.5	1.5
Cellulose	7.4	3.3	3.3	3.3
Others*	4.6	4.6	4.6	4.6

Table 12. Proximate composition (% dry matter basis, n = 3)

Parameters (%)	FM	NFM+ 1T	NFM+ 1.5T	NFM+ 2T
Crude protein	46.0	46.8	46.5	46.6
Crude lipid	12.9	12.5	12.0	12.4
Ash	8.8	6.0	5.9	6.0
Moisture	7.0	4.1	5.3	5.4

Dietary amino acid composition of the diets is given in Table 12. All the NFM diets were supplemented with crystalline lysine and methionine to compensate for the essential amino acid deficiencies of the experimental diets (Table 13.).,

Table 13. Total amino acid content of experimental diets for red sea bream, *Pagrus major*. (g/100g dry matter, n = 3)

	FM	**NFM+ 1.0T**	**NFM+ 1.5T**	**NFM+ 2.0T**
Arginine	3.1	2.7	2.6	2.6
Histidine	1.4	1.0	1.0	1.0
Isoleucine	1.5	1.4	1.3	1.5
Leucine	3.9	4.3	4.3	4.5
Lysine	3.4	3.0	2.9	3.0
Methionine	1.3	0.9	0.8	0.9
Phenylalanine	2.4	2.4	2.1	2.0
Threonine	2.3	1.7	1.8	1.8
Valine	1.8	1.6	1.5	1.6
Taurine	0.4a	0.9b	1.3c	1.6d

4.2.2. Experimental fish and growth trial

The feeding trial was carried out at the Laboratory of Fish Nutrition, Tokyo University of Marine Science and Technology, Tokyo, Japan. The juvenile red seabreams were obtained from a

local hatchery (Marua Suisan Co., Ehime, Japan) and transported to the laboratory by delivery service. The fish were acclimatized in eight 60 L rectangular 121 polycarbonate tanks to for 2 weeks. During this period, a commercial diet (50% crude 122 protein; Higashimaru, Japan) was supplied to the fish. After the acclimatization period, 123 160 juveniles (average body weight, 5.4 ± 0.06 g) were bulk-weighed and randomly 124 allocated into previously prepared eight tanks at a stocking density of 20 fish per tank 125 with duplicate per dietary treatment. All fish were fed the respective experimental diets 126 to the satiation level by hand twice a day six days per week for 10 weeks. Any uneaten 127 feed left was removed 1 h after feeding by shiphoning and dried using a freeze dryer. 128 The feeding trial was conducted in re-circulated artificial seawater (Sea Life, Nihon Kaisui 129 Co., Tokyo, Japan) system where each tank was equipped with an inlet, outlet, and 130 continuous aeration. Approximately 50% of the seawater in the recirculating system was 131 replaced every 2 weeks. A flow rate of rearing water was maintained at 700-800 ml/min 132 throughout the experimental period. During the feeding trial, the monitored water quality 133 parameters (means ± S.D.) were water temperature 24.1 ± 1.7 ℃; pH 7.1 ± 0.5 and 134 salinity 33.1 ± 0.5. The tanks were maintained under natural light/dark regime.

4.2.3. Sample collection

A pooled sample of 10 fish at the beginning was stored at −30 ℃ for whole body proximate chemical composition analysis. During the feeding trial, all fish were weighed in bulk at 4 weeks interval to determine growth and to check health condition. At the end of the experiment, fish were fasted for 24 h and anaesthetized with 0.2 ppm 2-phenoxyethanol before being handled. The total number and individual body weight of fish from each tank were measured. A pooled sample of five fish from each duplicate tank were randomly collected and stored at −30 ℃ for final chemical analysis. Frozen samples were minced in a centrifugal mill (Model ZM 1; Retsch, Haan, Germany), freeze-dried in a vacuum freeze-dryer, and kept at −30 ℃ until analysis. Weight gain (WG, g), specific growth rate (SGR, %), daily feed intake (DFI, %), food conversion ratio (FCR), survival rate

(SR, %) and protein efficiency ratio (PER) were calculated by using the following equations to compare fish growth, nutrient utilization efficiency, and body indices among treatments.

WG (%) = (final weight−initial weight) ×100/initial weight

SGR (%, day^{-1}) = 100× {(Ln (final weight) −Ln (initial weight))/duration (70 days)}

SR (%) =100× (final no. of fish/initial no. of fish)

DFI (%, $days^{-1}$) = total feed intake×100⁄ {(initial number of fish× mean of initial body weight + final number of fish× mean final body weight+ dead fish weight) × duration (70 days×2)}

FCR= feed intake (g)/weight gain (g)

PER= weight gain (g)/dry protein intake (g)

4.2.4. Chemical analysis

The diets and whole fish body were analyzed for moisture, crude protein, total lipid and ash, in triplicate, using standard methods (Association of Official Aanalytical Chemists 2005). All the samples were finely-ground and analyzed in triplicate.

Dry matter content was calculated from the weight loss after drying the sample by dry oven (NDO-450, EYERA Co.) at 110 ˚C until it reached a constant weight. Ash content was determined after the incineration of samples in electric furnace (FO200, Yamato Co.) at 650˚C for 8 h. The Kjeldhal method was used for crude protein content (N content × 6.25). It was measured by Kjeltec Auto (Kjeltec 2400, FOSS Japan Co., Tokyo, Japan). Crude lipid content was extracted by using a mixture of chloroform and methanol (2: 1) and determined by the gravimetric method (Folch et al. 1957).

Total amino acid composition was analyzed according to Boonyoung et al. (2013). Samples were digested at 110 °C for 22 h with 4 M methanosulphonic acid (Sigma-Aldrich, St. Louis, MO, USA); the digested solution was neutralized with 3.5 N NaOH, passed through a 0.45-μm membrane filter and injected into the automatic amino acid analyzer (JLC500, JEOL Ltd., Tokyo, Japan) fitted

with a multi-plate column packed with ion exchange resin (4 mm × 120 mm).

4.2.5. Histological examination

Four fish per treatment were sampled for histological examination of the intestinal tract. After euthanized with 0.5 ppm of anesthetics, body cavity was opened with a scissor and a knife, whole intestine was dissected. Latter half next to the anus was designated as distal intestine. Tissue samples (approximately 5 mm × 5 mm) were taken from the distal intestine. Intestinal samples were processed and fixed in 4% formaldehyde for histological examination. The formalin fixed tissues were dehydrated through graded ethanol solution and then embedded in paraffin wax. Sections of approximately 5 μm thickness were cut and stained with hematoxylin and eosin (HE) (Menke et al. 2011). Section was observed under a light microscopy (Nikon Eclipse E600, Nikon Instruments, Tokyo, Japan) .

We classified degree of neutrophil infiltration of distal intestine into three categories; normal, moderate, and severe. No distribution of neutrophil in mucosa or submucosal layer, it was classified as normal; When focal distribution of neutrophil in the mucosa or submucosa, it was classified as moderate; When extensive distribution of mucosa or submucosa. it was classified as severe. We also classified lamina propria (LP) into normal and abnormal by morphology. When there was no distribution of connective tissue in LP, it was classified as normal. When LP was widened with hyperplastic connective tissue, it was classified as abnormal.

4.2.6. RNA isolation and gene expression analysis

Intestine samples of five fish per treatment were used for total RNA extraction and subsequent gene expression analysis.

The expression of Interleukin (IL)-1β, IL-8, tumor necrosis factor (TNF)-α and transforming growth factor (TGF)-β1 in intestine of red seabream were analyzed by quantitative real time PCR (qRT-PCR). IL-1β, IL-8 and TNF-α are the pro-inflammatory cytokines, whereas TGF-β1 is the anti-

inflammation cytokine. PCR primers for β-actin, IL-1β, IL-8, TNF-α and TGF-β1 were designed against sequences registered in gene bank (**Table 14.**). A qRT-PCR amplification was carried out in triplicate using SYBR green PCR master mix (Applied Biosystems, USA) and an ABI7300 quantitative PCR system (Applied Biosystems, USA) following the manufacturer's instructions. The conditions of quantitative PCR as follows: an initial denaturation step of 1 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 1 min, and final step dissociation stage followed by 1 cycle 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s, 60 °C for 15 s as described by Zhao et al. (2015). The β-actin was used as an internal control for sample normalization of the target primers. The relative gene expression model was used to evaluate the fold changes in mRNA expression among the treatments using the $\Delta\Delta C_T$ method (Livak and Schmittgen 2001). Fish fed FM diet was used as the respective control. Thus, dietary induced changes are presented as n-fold differences relative to the corresponding control set to 1.

Table 14. Primers sequence used for qPCR

Gene	**Primer**	**GenBank Accession No.**	**Primer sequence (5‘------- 3')**	**Annealing temp. (C)**	**PCR product (bp)**
β-actin	β-actin-F	AB036756.1	TTCCTCGGTATGGAGTCCTG	60	115
	β-actin-R		TGGTACCTCCAGACAGCACA		
Interleukin1β	IL-1β-F	AY257219.1	GCGAGCAGAGGCACTTAGTC	60	109
	IL-1β-R		AGGTAGGTCGCCATGTTCAG		
Interleukin 8	IL-8-F	KF693767.1	CCATCCCTGATGGTGTTGAT	60	114
	IL-8-R		ACCTCTTGGCCTGTCCTCTT		
Tumor Necrosis Factor-α	TNF-α-F	AY314010.1	CGGACACTGCTGAGAAAACA	60	107
	TNF-α-R		CGAACCCTCGTCTTCATCAT		
Transforming growth factor β	TGF-β-F	FJ767724.1	ATCCCTCAAAACGTCAGCAG	60	104
	TGF-β-R		AAGCTCCTCACACAGCAGGT		

4.2.7. Digestibility trial

For estimation of apparent digestibility coefficient (ADC) of the experimental diets, chromic oxide (Cr_2O_3) (Sigma-Aldrich, St. Louis, USA) was included in the diets as an inert indicator at a concentration of 0.5%. The other ingredients were the same with those in diets used the growth trial except the cellulose content (0.5%). The culture condition was the same with growth trial. New batches of red seabream with mean body weight of 40 g were stocked into 4 tanks at a density of 15 fish per tank and each group of fish was fed one of the test diets. Before the trial, fish were acclimatized to the feed for 1 week and feces were collected over the next 14 days. Feces were collected according to Tokyo University of Fisheries (TUF) column system (Satoh et al. 1992). All feces were frozen at − 20 ℃ for analysis. Chromium oxide content of diet and feces samples was analyzed by the method described by Divakaran et al. (2002).

4.2.8. Statistical analysis

The effects of different diets on red seabream whole body proximate composition, growth, survival, feed utilization, ADC for protein, lipid and dry matter and relative gene expression were calculated and subjected to one-way analysis of variance (ANOVA) in SPSS version 20.0 (SPSS Inc., Chicago, IL, USA). When ANOVA identified differences among groups, the differences in mean values were compared using Tukey's HSD test at the 5% level of significance ($P < 0.05$). All data are expressed as mean ± standard deviation.

Calculation was made by the following formula;

$ADC_{dm}\% = 100 \times (1 - Cr_{feed}/Cr_{feces})$

$ADC_{Protein}\ (\%) = \{1 - [(\%Cr_2O_{3\ feed}) \times (\%N_{feces})/ (\%Cr_2O_{3\ feces}) \times (\%N_{feed})]\} \times 100.$

ADClipid (%) = {1- [(%Cr_2O_3 feed) ×(%lipidfeces)/ (%Cr_2O_3 feces) × (%lipidfeed)]} ×100

4.3. Result

4.3.1. Growth performances, feed utilization efficiencies and protein digestibility

Growth performance, feed utilization, protein digestibility and survival of fish are presented in **Table 15.** Fish fed NFM diets performed equally well FW compared with fish fed the control diet. Significantly higher %WG and SGR were observed in FM group than NFM groups. However, among all NFM groups, no significant difference in WG and SGR was observed. No significant difference was observed in DFI and SR among all groups. Significantly better feed efficiency (1/FCR) and PER were observed in FM group than control group. Meanwhile, significantly better PER were observed in NFM+2.0T group than NFM+1.0T group. Significantly higher PER was observed in FM group than NFM+2.0T group. ADC of protein seemed like be affected by experiment diets. FM showed the best ADCp than non FM groups.

Table 15. Growth parameters, feed utilization and protein digestibility of red seabream fed experimental diets for 10 weeks.

Parameters	Diet group			
	FM	NFM+ 1.0T	NFM+ 1.5T	NFM+ 2.0T
IW[a]	5.4±0.12	5.4±0.12	5.4±0.13	5.4±0.12
FW[b]	33.6±1.21	27.8±1.25	29.2±1.72	30.6±1.20
WG[c]	520.1±20.86[a]	421.7±5.30[b]	440.3±8.24[b]	465.4±5.49[b]
SGR[d]	2.61±0.05[a]	2.36±0.01[b]	2.41±0.03[b]	2.47±0.01[b]
DFI[e]	0.53±0.01	0.55±0.0	0.54±0.02	0.53±0.01
FCR[f]	1.04±0.01[a]	1.27±0.03[c]	1.19±0.05[bc]	1.13±0.01[ab]
PER[g]	2.08±0.02[a]	1.69±0.04[b]	1.82±0.08[bc]	1.91±0.01[c]
ADCp[h] (%)	91.9	84.7	86.5	87.6
SR[i]	85.0±0.0	70.0±7.07	72.5±3.54	77.5±3.54

[a] IW: initial weight (g); [b] FW: final weight (g); [c] WG: percent weight gain (%); [d] SGR: specific growth rate (% day^{-1});

[e] DFI: daily feed intake (% day^{-1})'; [f] FCR: feed conversation ratio; [g] PER: protein efficiency ratio; [h] ADCp: apparent digestibility coefficiency of protein; [i] SR: survival rate (%)

4.3.2. Proximate composition of whole body

The whole body proximate composition of fish at the start and end of the feeding trial is shown in **Table 16.** Final fish in all groups tended to have higher protein and lipid contents but lower moisture and ash content than initial fish (**Table 16.**). However, no trend in protein, lipid, ash, or moisture content of whole fish body was apparent among treatment groups, indicating no observed effect of PP inclusion levels on these components of body composition.

Table 16. Proximate composition of whole body of red seabream fed experimental diets for 10 weeks.

	Initial	FM	NFM+ 1.0T	NFM+ 1.5T	NFM+ 2.0T
Protein (% wet basis)	10.09±0.32	16.74±0.25	15.53±0.41	15.63±0.34	15.72±0.90
Lipid (% wet basis)	1.38±0.09	4.27±0.15	4.07±0.20	4.01±0.15	4.40±0.18
Ash (% wet basis)	3.94±0.15	3.39±0.39	3.26±0.36	3.09±0.17	3.45±0.29
Moisture (%)	87.62±0.12	74.52±0.59	75.70±0.11	75.85±0.26	73.86±0.43

Values (Mean ± S.D.) (n = 3) with the different letters in the same line are significantly different at $P < 0.05$ by the Tukey HSD test

4.3.3. Intestinal morphology

Fig. 5. showed the pathological changes in fish fed experimental diets after 10 weeks. Apparent inflammatory changes were observed in the several individuals fed the NFM+1.0T, while slight abnormalities were observed in NFM+1.5T group. No pathological changes were observed in NFM+2.0T compared with FM groups (**Fig. 5.**). The typical signs of the pathological changes: LP appeared widened with increased cellularity; an increased presence of neutrophils in the LP and submucosa. There are also some subtle differences were noted in some individuals in NFM group that a loss of the normal supranuclear vacuolization (SNV) of the enterocytes and a decrease of goblet cell (GC).

In the present study, the most obvious inflammatory response was the neutrophil infiltration in the intestinal LP and submucosa. Focal distribution of neutrophil mainly appeared in LP and/or

submucosa in fish fed NFM diets while several individuals in NFM+1.0T showed extensive distribution of neutrophil infiltration in LP and/or submucosa in distal intestine compared with FM group. In addition, the pathological changes were getting milder as the level of taurine gradually increased in NFM groups (Table 17.)

Table 17. Morphological changes in terms of neutrophil infiltration of the distal intestine of red seabream fed the experimental diets for 10 weeks

	FM	NFM+1.0T	NFM+1.5T	NFM+2.0T
Neutrophil				
Normal	4	1	3	3
Moderate[1]	-	2	1	1
Severe[2]	-	1	-	-
Lamina propria				
Normal	4	1	1	2
Abnormal[3]	-	3	3	2

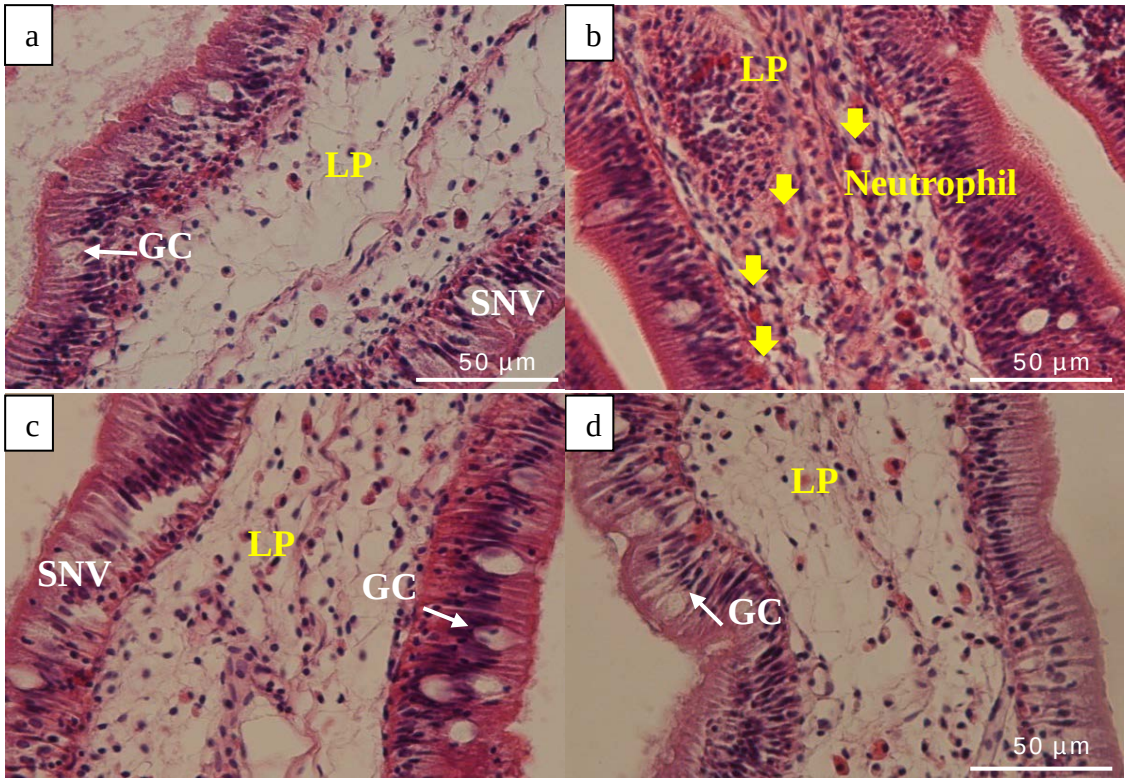
Fish number n = 4.

[1] Moderate: focal distribution of neutrophil in the mucosa or submucosa.

[2] Severe: extensive distribution of neutrophil in mucosa or submucosa.

[3] Abnormal: the LP was widened with hyperplastic connective tissue.

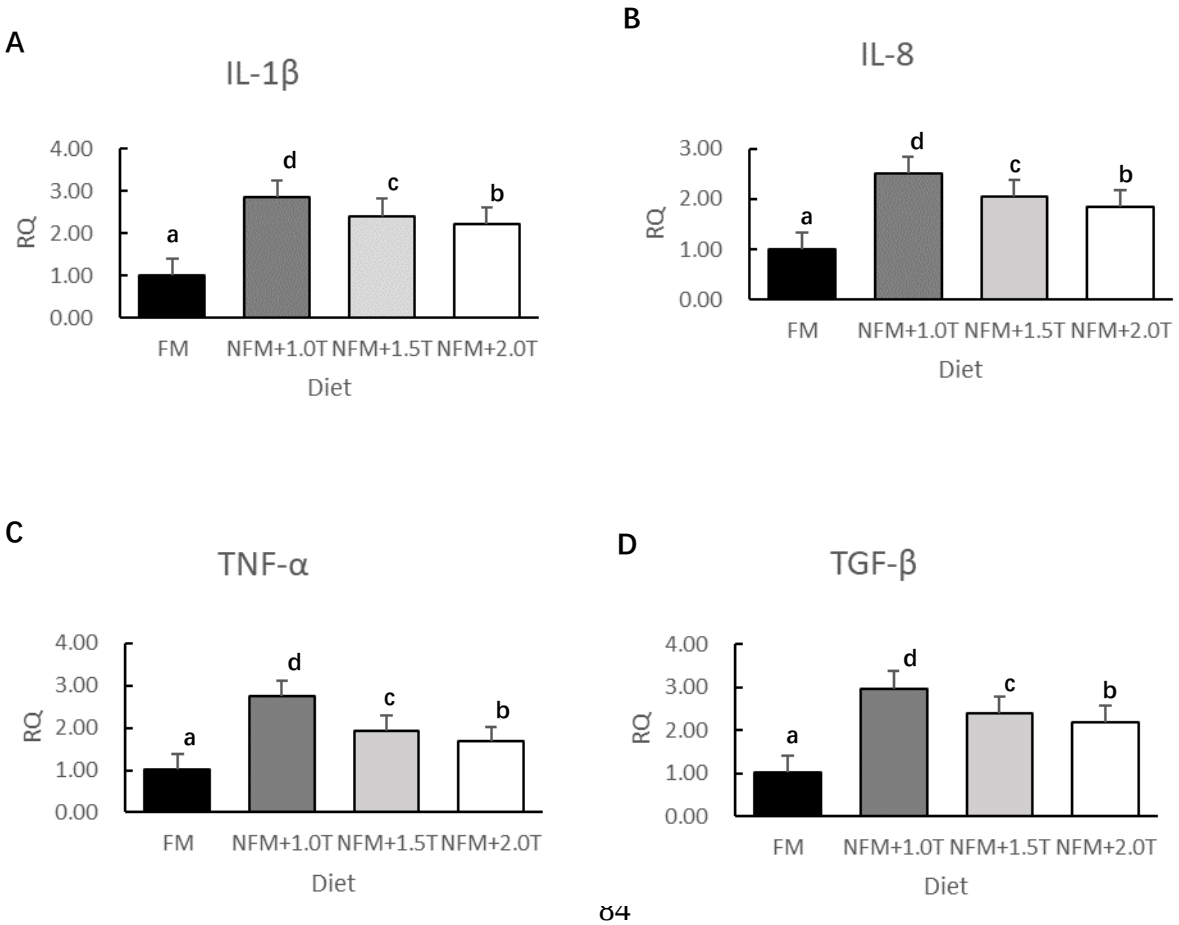
Figure 5. Histological characteristics of distal intestinal mucosa from red seabream fed a fishmeal based control diet and three NFM diets. (a) FM (control); (b) NFM+1.0T; (c) NFM+1.5T; (c) NFM+2.0T. SNV, supranuclear vacuoles; GC, goblet cells; LP, lamina propria. The yellow arrowheads in (b) represent the infiltrated neutrophils. Scale bar = 50 μm



4.3.4. Relative gene expression of IL-1β, IL-8, TNF-α and TGF-β1

Relative expression of hepatic IL-1β, IL-8, TNF-α and TGF-β1 was significantly influenced by the dietary treatments. Significant increase in expression levels of the inflammatory cytokines was observed (Fig.6. A-D, $P < 0.05$). In NFM groups, with increasing taurine supplemental levels, the transcription levels of the cytokine genes became significantly lower in the NFM groups, although the expression levels of these groups were still significantly higher than the control group (Fig.6. A-D, $P < 0.05$)

Figure 6. mRNA expression of 4 cytokine genes associated with gut inflammation in red seabream: IL-1β (A); IL-8 (B); TNF-α (C); TGF-β1 (D). Relative mRNA levels were determined by the comparative Ct method using β-actin gene as the control. Relative mRNA expression was evaluated by real-time quantitative PCR. Values are means ± S.D. (n = 4). Bars of the same gene bearing with different letters are significantly different by Tukey HSD test ($p < 0.05$).



4.4. Discussion

The present study was designed to generate data on practical diets in which fish meal protein was replaced with plant protein supplemented with taurine.

The results from the present study showed that growth parameters in term of WG and SGR were decreased in NFM groups. It was well documented that ANFs in PP sources caused growth retardation of fish fed PP based diet (Rumsey et al. 1994; Baeverfjord and Krogdahl 1996; Bakke-McKllep et al. 2000; Krogdahl et al. 2003). Besides, one of the superiorities of CGM as the substitution of FM is its low inclusion of ANFs and several studies reported that certain level of CGM in diet has no adverse effects on fish growth due to the lack of ANFs in its composition (Alexis et al. 1985 and Hardy 2000). Therefore, the inferior growth performance in NFM groups most likely due to the presence of ANFs in SBM (Vaintraub and Bulmaga 1991; Liener 1994). The inferior growth performance in NFM groups was in line with several previous studies that have shown considerable growth depression in partial replacement of FM with PP in red seabream (Takagi et al. 1999, 2000a, 2000b). Feed utilization efficiency showed similar tendency with the growth performance among dietary groups. The result indicated that inclusion of PP depressed the diet utilization of red seabream. Additionally, content of some essential amino acid in the NFM diet were lower than the FM diet, which may account for the significantly lower WG and SGR of fish fed the NFM diets than those of the FM fed fish. This could be a possible reason of lower feed utilization in NFM groups. For fish fed NFM diets, growth performance and feed utilization gradually improved with increasing level of taurine supplementation. This is in agreement with the previous findings that the different supplementation level of taurine (from 0.5% to 2%) improve feed utilization and growth performance in Japanese flounder and juvenile red sea bream (Park et al. 2002; Kim et al. 2005; Matsunari et al. 2008). 1840 Therefore, taurine supplementation is necessary in diets that are either partially or totally substituted with plant protein (Lunger, McLean, Gaylord, Kuhn, & Craig, 2007; Goto, Tiba, Sakurada, & Takagi, 2001; Kim et al., 2005; Takagi et al., 2006).DFI was similar in all

diet groups, indicating that there were no palatability problems in the present study as fish in all groups readily accepted the experimental diets. In this study, similar whole body protein content was observed among all groups, as reported in previous studies testing high PP dietary levels (Cabral et al. 2011; Kaushik et al. 2004; Regost et al. 1999; Silva et al. 2009; Valente et al. 2011).

In this study, relatively low survival rate was recorded. This probably because 330 several fish seemed to be affected by pathological symptoms such as skin hemorrhage 331 in tail and body swelling due to unknown bacterial disease. However, no obvious effect 332 was seen in growth parameters. Also, improving trend was observed in SR of NFM 333 groups with increasing taurine supplementation.

Several studies have reported that PPs reduced the digestive enzyme activity of fish (Glencross et al. 2007; Hu et al. 2018; Krogdahl et al. 2003; Zhang et al. 2018). However, ADC of protein and lipid showed that fish fed NFM diets had comparable ADCs with fish fed FM diet except for NFM+1.0T group. In this study, although there was no significant difference in ACD of protein and lipid, supplementation of taurine tended to improve protein and lipid digestion and absorption. Similarly, the addition of taurine to feed significantly increased the activity of amylase and trypsin and promoted the utilization of starch and protein in juvenile cobia (Salze et al. 2012). On the other hand, Hanini et al. (2013) reported that addition of taurine to a low FM diet did not affected the ADC of protein. In this case, taurine content in the feed was 0.31% in the taurine-supplemented low FM diet and was lower than that in our study (0.9-1.5%). It is assumed that 0.31% dietary taurine seems to be unable to improve protein digestibility of red seabream. Another explanation is that possible upregulation of trypsin precursor by taurine, which was previously reported in tongue sole *Cynoglossus semilaevis* (Zheng et al. 2016). It is assumed that elevated expression of trypsin precursor could improve protein digestibility in red seabream in our study.

Soya saponins and soya lectins are suspected to be the main inducers of inflammation (Iwashita et al. 2008a, 2009; Chen et al. 2011). In the present study, red seabream fed NFM+1.0T diets, showed pathological changes in the distal intestine, such as shortening of the mucosa fold; a loss of the normal SNV of the enterocytes, a slight widening of the LP and small amount of infiltration of inflammatory cells identified as neutrophils in LP and submucosal of the distal intestine occurred. These results are consistent with previous findings in carp (Urán et al. 2008a), rainbow trout (Romarheim et al. 2008), giant grouper (García-Ortega et al. 2016), and orange-spotted grouper (Wang et al. 2017). In addition, the pathological changes, especially the invasion of neutrophil, was gradually ameliorated in NFM groups as increasing taurine supplementation. These findings suggest that taurine could partly prevent pathological changes in the distal intestine of red seabream fed PP based diets. This hypothesis was agreement with Rimoldi et al. (2016) who revealed that taurine exerted a mitigating effect on the inflamed distal intestine of European sea bass fed high level SBM. Similarly, Iwashita et al. (2009) also indicated that morphological changes in the distal intestine of rainbow trout fed SBM-based diets can be prevented by supplementation of cholyltaurine.

Matsunari et al. (2015) and Amano et al. (2012) failed to observe SBM induced enteropathy in red seabream even though they fed SBM based low or NFM diets. However, in apparent contrast, we here observed feeding SBM based NFM diet caused pathological changes in distal intestine. The reason why inconsistent results were obtained was unclear. However, they fed the diets only for 6 weeks. One may suppose that feeding SBM based diet 6 weeks is too short to induce pathological changes of intestine of red seabream. However, they successfully observed pathological changes of liver in red seabream although they failed to observe negative impact of SBM on intestinal structure. This suggests that liver seems to be more sensitive than intestine in red seabream. Khosarvi et al. (2015) also failed to observe defective changes of intestinal structure in red seabream even though they observed smaller diameter of intestinal tract, shorter microvilli and enterocyte height in red seabream fed soy protein concentrate-based diet. The reason why they failed to observe pathological

changes in intestine is unclear. However, we should point out that they only examined morphology of proximal intestine but not distal intestine where is thought to be most sensitive for SBM-induced enteropathy in salmon (Krogdahl et al. 2003). So, one possible explanation is that proximal intestine is not as sensitive as distal intestine regarding occurrence of SBM induced pathogenesis in red seabream.

In the present study, expression of proinflammatory cytokines such as IL-1β, IL-8 and TNF-α was significantly enhanced by replacing FM with PP. Gene expression analysis of inflammatory cytokines along with progression of SBM-induced enteropathy in Atlantic salmon and common carp revealed that elevated expression of proinflammatory cytokine genes such as IL-1 and IL-8 in early phase of the enteropathy (Lilleeng et al. 2009; Urán et al. 2008a, 2008b). Although recovery of intestine has not been reported in Atlantic salmon, adaptation and recovery of intestinal tissue was suggested in common carp fed SBM based diet. Specifically, after 4 weeks onwards, recovery of intestine was observed and increased expression of TGF-β was initiated to be observed after 3 weeks of feeding SBM diet in common carp (Urán et al. 2008b). Although it is unknown whether adaptation to SBM and recovery also takes place in red seabream, the present study successfully observed increased expression of TGF-β in red seabream fed NFM diets.

In the present experiment, we observed improving morphological defects and amelioration of inflammation of intestine by supplemental taurine whose dose was surplus of its requirement for red seabream (5 mg taurine/g diet) (Matsunari et al. 2008). It is known that nutrient requirement differs depending on which kind of physiological phenomena researchers focused. For instance, cataract in Atlantic salmon is caused by histidine deficiency. To prevent development of cataract, surplus histidine beyond its requirement that is estimated by growth trial (8 g/kg, National Research Council 2011) is needed (Waagbbø et al. 2010). High expression of taurine transporter was reported in distal intestine as well as brain, eye, and heart in Senegale sole (Pinto et al. 2012). In our study, high expression of TNFα was observed in NFM group. It was reported that TNFα stimulated taurine

transporter (Mochizuki et al. 2005). In this case, elevation of taurine uptake in enterocytes by increased taurine transporter could reduce taurine availability and eventually results in higher demand of dietary taurine. This hypothesis is consistent with our finding that amelioration of inflammatory response was observed in fish fed diets supplemented with surplus level of taurine. On the other hand, taurine supplementation failed to improve intestinal injury as well as inflammatory response in Atlantic salmon (Kortner et al. 2016). The reason why taurine was not effective in their study was unclear, but one possibility is the taurine level used was below effective concentration.

As a conclusion, based on the findings in the present study, we successfully demonstrated that PP based diet induced inflammatory response of distal intestine of red sea bream. We also demonstrated that high inclusion of dietary taurine tended to improve fish growth performance, feed utilization and intestinal cytokine gene expression and ameliorates pathological changes of intestine of red sea bream.

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Chapter 5. Effect of graded levels of taurine supplementation to non fishmeal diet on growth performance, feed utilization and nutrient digestibility of juvenile red seabream

Abstract

A 12-week feeding trial was conducted to investigate the effects of complete replacement of dietary FM with plant proteins (PPs) and graded levels of taurine supplementation on growth performance, feed efficiency and nutrient digestibility. Six isonitrogenous (46% crude protein) and isolipidic (13% crude lipid) practical diets were formulated. 50% FM diet was designated as FM (control); soy and corn protein based non FM diets supplemented with increasing level of taurine (0, 0.5%, 1.0%, 1.5%, 2.0%) were designated as NFM, NFM+T0.5, NFM+T1.0, NFM+T1.5 and NFM+T2.0, respectively. 270 fish averaged with initial weight of 5.78 ±0.8g were divided into 18 tanks and fed one of the four diets to apparent satiation with triplicated treatments.

Growth performance in terms of %WG in fish fed NFM or NFM+0.5T diets was significantly lower than that in control group. Significantly lower SGR was observed in NFM group than in control ($P < 0.05$). There was no difference in DFI among diet groups. Feed utilization in terms of feed efficiency (1/FCR) and PER in fish fed NFM diets was significantly lower than that in control group. Significantly higher SGR was recorded in non-FM group with increasing taurine supplementation ($P < 0.05$). Growth parameters and feed utilization tended to be improved in non FM groups with as elevation of taurine supplementation. %WG in fish fed NFM+1.5T or NFM+2.0T was significantly higher than that in fish fed NFM or NFM+0.5T diets. There was no significant difference in survival among all groups.

Apparent digestibility coefficient (ADC) of protein and lipid were significantly higher in FM group than NFM+1.0T group but not NFM+1.5T and NFM+2.0T groups. No significant difference was observed in ADCs among all NFM groups.

Keywords: Soybean meal; FM replacement; apparent digestibility coefficiency

5.1. Introduction

Costs of feed containing fish meal as a primary protein source can be expected to rise as fish meal prices increase in response to static supply and growing demand. Alternatives to fish meal are needed to reduce production costs in many aquaculture enterprises. Soybean meal (SBM) and other plant protein (PP) sources have high protein nutritional value, reasonable amino acid composition, and low sugar content compared with cereals. And now PPs have become hotspot of research on alternative protein sources.

However, PP often contains high levels of carbohydrate and fiber. Most fish cannot digest them effectively. Appropriate amount of fiber can increase the peristalsis of feed in the gastrointestinal tract of aquatic animals and improve the utilization of nutrients. However, if it exceeds a certain range, it will lead to malnutrition and hinder the growth of aquatic animals. Carbohydrates in PP also can alter populations of micro-flora in the gastrointestinal tract of fishes, sometimes with negative effects. In addition, improperly processed SBM has anti-trypsin, blood lectin and other anti-nutritional factors, thereby limiting the widely use of SBM.

In general, herbivorous fish can make good use of plant protein sources. Omnivorous fish also have high utilization of plant protein sources, and FM in feed can be partially or even completely replaced by plant protein sources. However, carnivorous fish mainly feed on animal protein sources. Generally, the digestive tract is short, and the digestive enzyme system is mainly suitable for animal protein. Therefore, the utilization of plant protein by carnivorous aquatic animals is relatively poor.

Low acceptance of diets containing a high proportion of plant ingredient has been reported by several papers. Previous reports have appealed that plant proteins fed to fish affect the nutrient digestibility (Fontainhas-Fernandes *et al*., 1999; Chong *et al*., 2002; Gaylord *et al*., 2004; Santigosa *et al*., 2008; Richard *et al*., 2011; Santigosa *et al*., 2011a; Santigosa *et al*., 2011b; Li *et al*., 2013). The reason of the poorer digestibility can be summarized as: 1) PPs contain ANFs in which hinder

the digestibility of nutrients; 2) excess levels of fiber; 3) changes in the intestinal micro flora with regard to feeding plant proteins. Chong *et al*. (2002) suggested that the anti-protease inhibitors for protein digestion was identified when fed with higher levels of SBM, wheat meal and winged bean. It was reported that digestive and absorptive enzymes activity were lower in grass carp when fed with high-level of plant proteins; but enzymes activity got reversed when lysine and methionine supplemented to diet (Jiang *et al*., 2016). Santigosa *et al*. (2008) also noticed that digestive activity of sea bream got reduced when fed with plant protein sources; but growth rates were similar to that of FM diets as compensation mechanisms were discovered in this fish i.e. increase in the relative intestinal length and up-regulation of trypsin activity. Santigosa *et al*. (2011a, 2011b) reported that sea bream and rainbow trout fed with plant protein sources delayed the intestinal nutrient absorption.

It is well-known that taurine can promote digestion by promoting the secretion of digestive enzymes and improve digestive enzyme activity. The addition of taurine to feed can significantly increase the activity of amylase and trypsin in juvenile cobia (Salze et al., 2012), and promote the utilization of starch and protein of cobia. In addition, several studies have reported that plant proteins reduced the digestive enzyme activity of fish (Gaylord et al., 2008; Glencross et al., 2007; Hu et al., 2018; Zhang et al. 2018). However, it has also been reported that red seabream feed diet containing SBM may increase the lipase activity (Murashita et al. 2016). Therefore, it is necessary to elucidate whether or not the enzyme activity is reduced by the PP firstly, and then to confirm the the effect of taurine.

Nutrient availability in feedstuffs has species-specific. The evidences are available that PP based feeding had no adverse effect on the digestibility of nutrients in fish. Hansen *et al*. (2006) reported that Atlantic cod may be fed with PP based feeds up to 44 % without any adverse impact to nutrient digestibility. Bonaldo *et al*. (2011) showed that turbot fed with higher plant protein (mixture of SBM, wheat gluten meal and CGM) in the diet did not cause the digestibility of ingredients and gut histology.

Evaluation of ADC of feedstuffs utilized in fish diets is one of the most important steps in formulating properly balanced diets to satisfy the nutrient requirements of fish. Knowledge of the digestibility of these various ingredients is a basic requirement for formulating diets. Few studies have been taken to evaluate the apparent digestibility coefficients of non FM- based diet for red seabream species. Therefore, the objective here were to 1) evaluate the effects of completely FM replacement with plant protein in the diet of juvenile red seabream on growth performance, feed utilization efficiency; 2) compare the nutrient digestibility of diets with different protein sources supplemented with different level of taurine, and 3) determine the most suitable dose of taurine supplementation to non FM diet for this fish species.

5.2. Materials and methods

5.2.1. Experimental diets

Six isonitrogenous (46% crude protein) and isolipidic (13% crude lipid) experimental diets (one FM-based control diet and five PP based diets) were formulated to fulfill the known protein, lipid and indispensable amino acid requirement of juvenile red seabream (National Research Council 2011). The formula of the experimental diets is shown in Table 18.

Table 18. Ingredient composition (%) of experimental diets fed juvenile red sea bream, *Pagrus major*

Ingredients	FM	NFM	NFM+0.5T	NFM+1T	NFM+1.5T	NFM+2T
Fish meal	50	0	0	0	0	0
Defatted soybean meal	-	12	12	12	12	12
Soy protein concentrate	-	28	28	28	28	28
CGM	11	26	26	26	26	26
Wheat flour	11	5.5	5.5	5.5	5.5	5.5
Pre-gelatinzed starch	5	4	3.5	3	2.5	2
Fish oil	6	6	6	6	6	6
Soybean oil	5	5	5	5	5	5
Taurine	-	-	0.5	1	1.5	2
Cr_2O_3	0.5	0.5	0.5	0.5	0.5	0.5
Methionine	-	0.3	0.3	0.3	0.3	0.3
Lys-HCl	-	0.8	0.8	0.8	0.8	0.8
$Ca(H_2PO4)_2$	-	3	3	3	3	3
Bonito extract	-	1.5	1.5	1.5	1.5	1.5
Cellulose	6.9	2.8	2.8	2.8	2.8	2.8
Others*	4.6	4.6	4.6	4.6	4.6	4.6

[a] Others: Vitamin premix [b] (3%); Mineral premix [c] (1%); Vitamin E (0.1%); Choline chloride (0.5%).

[b] Vitamin premix composition (unit.kg^{-1}): vitamin A, 2,420,000 IU; vitamin D3, 2,420,000 IU; vitamin K3, 6,050 mg; thiamine, 3,025 mg; riboflavin, 3,630 mg; pyridoxine, 2,420 mg; cyanocobalamin, 6.0 mg; L-ascorbic acid, 368,902 mg; nicotinic acid, 24,200 mg; D-pantothenic acid, 6,050 mg; inositol, 121,000 mg; d-biotin, 363 mg; folic acid, 908 mg; para-aminobenzoic acid, 3,025 mg.

[c] Mineral premix composition (%): sodium chloride, 5.0; magnesium sulfate, 74.5; iron (III) citrate n-hydrate, 12.5; trace element mix, 5.0; cellulose, 3.0. [The trace element mixture contains (%): zinc sulfate heptahydrate, 35.3; manganese sulfate, 16.2; copper (II) sulfate pentahydrate, 3.1; aluminum chloride hexahydrate, 1.0; cobalt chloride, 0.3; potassium iodate, 0.1; cellulose, 44.0]

The control diet was a 50% FM based diet (FM). Non FM (NFM) diets were supplemented with graded level of taurine at 0, 0.5, 1.0, 1.5, and 2.0%, designated as NFM, NFM+T0.5, NFM+T1.0, NFM+T1.5 and NFM+T2.0, respectively (Table 1). All the NFM diets were supplemented with lysine and methionine to compensate for the essential amino acid deficiencies of the experimental diets. Bonito extract was also supplemented in NFM diets as feed stimulant. Before pelleting, the ingredients were ground to pass through a sieve (0.5mm mesh) and then thoroughly mixed by using a horizontal mixer (ACM-50 L1.AT, Aikohsha mfg., Tokyo, Japan) for 45 minters. The mixture was then moistened by adding distilled water (30~40%) and mixed for another 15 min prior to pelletizing

by a meat chopper (LCM22; HITACHI KOUKI., Tokyo, Japan). Pellets were dried in a vacuum freeze dryer (RLE-II 206; Kyowa Vacuum Tec Co, Saitama, Japan) for 16 hs. After preparation, diets were stored at 5 °C until use.

The proximate composition of the experimental diets is given in Table 19, respectively. Crude protein content was about 46 to 47 % in all test diets and crude lipid content was about 13 % in all test diets.

Table 19. Proximate composition of the test diets (%) fed juvenile red sea bream, *Pagrus major*

Parameters (%)	**FM**	**NFM**	**NFM+ 0.5T**	**NFM+ 1T**	**NFM+ 1.5T**	**NFM+ 2T**
Crude protein	46.31	46.38	46.04	46.90	46.85	46.31
Crude lipid	13.36	13.47	13.42	13.59	13.68	13.36
Ash	8.30	6.89	7.03	6.41	6.16	8.30
Moisture	6.74	7.86	7.57	7.32	7.13	6.74

Values represent averages of duplicate samples from each test diet (n = 2).

* Dry weight base.

The total amino acid composition of experimental diets is given in Table 20, respectively.

Table 20. Essential and Conditionally essential amino acid content of the test diets (g/100 g, d.b.) fed juvenile red sea bream, *Pagrus major*

	FM	**NFM**	**NFM +0.5T**	**NLFM +1T**	**NFM +1.5T**	**NFM +2T**
Arginine	2.94	2.36	2.52	2.34	2.33	2.61
Histidine	1.26	1.06	1.16	0.95	0.92	0.97
Isoleucine	1.14	1.32	1.45	1.20	1.19	1.50
Leucine	2.15	4.80	3.22	4.14	4.19	4.40
Lysine	3.05	2.49	2.83	2.79	2.81	2.93
Methionine	1.03	1.01	0.88	0.73	0.73	0.74
Phenylalanine	2.15	2.34	2.39	2.14	2.16	2.25
Threonine	1.89	1.68	1.79	1.59	1.59	1.71
Valine	1.33	1.48	1.60	1.35	1.31	1.62
Taurine	0.40	0	0.43	0.85	1.32	1.67

Values represent averages of duplicate samples from each test diet (n = 3).

5.3.2. Experimental fish and growth trial

The feeding trial was carried out at the Laboratory of Fish Nutrition, Tokyo University of Marine Science and Technology, Tokyo, Japan. The juvenile red seabreams were obtained from a local hatchery (Marua Suisan Co., Ehime, Japan) and transported to the laboratory by delivery service. The fish were acclimatized in eight 60 L rectangular glass tanks to for 2 weeks. During this period, a commercial diet (50% crude protein; Higashimaru, Japan) was supplied to the fish. The experimental condition is given in Table 21. After the acclimatization period, 270 juveniles (average body weight, 5.78 ± 0.8g) were bulk-weighed and randomly allocated into previously prepared 18 tanks at a stocking density of 15 fish per tank with duplicate per dietary treatment. All fish were fed the respective experimental diets to the satiation level by hand twice a day six days per week for 10 weeks. Any uneaten feed left was removed one h after feeding by shiphoning and dried using a freeze dryer. The feeding trial was conducted in re-circulated artificial seawater (Sea Life, Japan) system where each tank was equipped with an inlet, outlet, and continuous aeration. Approximately 50% of the seawater in the recirculating system was replaced every 2 weeks. A flow rate of rearing water was maintained at 700-800 ml / min throughout the experimental period. During the feeding trial, the monitored water quality parameters (means ± S.D.) were water temperature 22.7 ± 2.2°C; pH 7.1 ± 0.5 and salinity 33.1 ± 0.5. These ranges are considered within optimal values for juvenile red seabream. The tanks were maintained under natural light/dark regime.

Table 21. Experimental condition for feeding trial of juvenile red sea bream, *Pagrus major*

Initial body weight□	5.78 ±0.8
Tank volume (L)	60
Number of fish (ind. / tank)	15
Water temperature (^{o}C)	22.7 ± 2.2
PH	7.1±0.5.
Photoperiod (h)	12L:12D
Water flow rate (L min^{-1})	0.7-0.8
Feeding	Apparent satiation (twice a day)
Culture period	12 weeks

5.2.3. Sample collection

A pooled sample of 10 fish at the beginning was stored at −30 °C for whole body proximate chemical composition analysis. During the feeding trial, all fish were weighed in bulk at 4 weeks interval to determine growth and to check health condition. At the end of the experiment, fish were fasted for 24 h and anaesthetized with 0.2 ppm 2-phenoxyethanol before being handled. The total number and individual body weight of fish from each tank were measured. A pooled sample of five fish from each duplicate tank were randomly collected and stored at −30 °C for final chemical analysis. Frozen samples were minced in a centrifugal mill (Model ZM 1; Retsch, Haan, Germany), freeze-dried in a vacuum freeze-dryer, and kept at −30 °C until analysis. Weight gain (WG, g), specific growth rate (SGR, %), feed intake (FI, g), food conversion ratio (FCR), survival rate (SR, %) and protein efficiency ratio (PER) were calculated by using the following equations to compare fish growth, nutrient utilization efficiency, and body indices among treatments.

WG (%)=(final weight−initial weight)×100/initial weight

SGR (%, day^{-1})= 100×{(Ln (final weight)−Ln(initial weight))/duration (84 days)}

DFI (%, day^{-1}) = total feed intake×100⁄{(initial number of fish× mean of initial body weight + final

number of fish× mean final body weight+ dead fish weight) × duration (84 days×2)}

FCR= feed intake (g)/weight gain (g)

PER= weight gain (g)/dry protein intake (g)

SR (%) =100× (final no. of fish/initial no. of fish)

5.2.4. Chemical analysis

The diets and whole fish body were analyzed for moisture, crude protein, total lipid and ash, in triplicate, using standard methods (Association of Official Aanalytical Chemists 2005). All the samples were finely-ground and analyzed in triplicate.

Dry matter content was calculated from the weight loss after drying the sample by dry oven (NDO-450, EYERA Co., Tokyo, Japan) at 110 ˚C until it reached a constant weight. Ash content was determined after the incineration of samples in electric furnace (FO200, Yamato Co., Tokyo, Japan) at 650˚C for 8 h. The Kjeldhal method was used for crude protein content (N content × 6.25). It was measured by Kjeltec Auto (Kjeltec 2400, FOSS Japan Co., Tokyo, Japan). Crude lipid content was extracted by using a mixture of chloroform and methanol (2: 1) and determined by the gravimetric method (Folch et al. 1957).

Total amino acid composition was analyzed according to Boonyoung et al. (2013). Samples were digested at 110 °C for 22 h with 4 M methanosulphonic acid (Sigma-Aldrich, St. Louis, MO, USA); the digested solution was neutralized with 3.5 N NaOH, passed through a 0.45-µm membrane filter and injected into the automatic amino acid analyzer (JLC500, JEOL Ltd., Tokyo, Japan) fitted with a multi-plate column packed with ion exchange resin (4 mm × 120 mm).

5.2.5. Digestibility trial

For estimation of apparent digestibility coefficient (ADC) of the experimental diets, chromic oxide (Cr_2O_3) (Sigma-Aldrich, St. Louis, USA) was included in the diets as an inert indicator at a

concentration of 0.5%. The other ingredients were the same with those in diets used the growth trial except the cellulose content (0.5%). The culture condition was the same with growth trial. New batches of red seabream with mean body weight of 50 g were stocked into 12 tanks at a density of 15 fish per tank and each group of fish was fed one of the test diets in triplicate. Before the trial, fish were acclimatized to the feed for 3 days and feces were collected over the next 14 days. Feces were collected according to TUF column system (Satoh et al. 1992). All feces were frozen at − 20 °C for analysis. Chromium oxide content of diet and feces samples was analyzed by the method described by Divakaran et al. (2002).

Calculation was made by the following formula:

$ADC_{dm}\% = 100 \times (1 - Cr_{feed}/Cr_{feces})$

$ADC_{Protein}$ (%) = {1- [(%Cr_2O_3 feed) ×(%N_{feces})/ (%Cr_2O_3 feces) × (%N_{feed})]} ×100.

ADClipid (%) = {1- [(%Cr_2O_3 feed) ×(%lipidfeces)/ (%Cr_2O_3 feces) × (%lipidfeed)]} ×100

5.2.6. Statistical analysis

The effects of different diets on red seabream growth, survival, feed utilization and ADCs were calculated and subjected to one-way analysis of variance (ANOVA) in SPSS version 20.0 (SPSS Inc., Chicago, IL, USA). When ANOVA identified differences among groups, the differences in mean values were compared using Tukey's HSD test at the 5% level of significance ($P < 0.05$). All data are expressed as mean ± standard deviation

5.3. Results

5.3.1. Growth performances and feed utilization

Growth performance and feed utilization are presented in **Table 22.** Fish fed NFM diets performed equally well final weight compared with the control group. % WG in non FM groups with less inclusion of taurine (0, 0.5%) were significantly lower than the control. SGR in fish fed NFM diet was also significantly lower than the control group. No significant difference were observed in DFI and SR among all groups. Fish fed NFM and NFM+0.5T performed significantly lower feed

utilization efficiencies in terms of feed efficiency (1/FCR) and PER than the other groups. Among non FM groups, growth parameters and feed utilization efficiency were improved with graded levels of taurine supplementation to diets. Significantly higher %WG was observed in non FM groups with higher inclusion of taurine (1.5, 2%) than NFM and NFM+0.5T groups. Significantly higher 1/FCR and PER was observed in NFM+2T than NFM group($P < 0.05$).

Table 22. Growth performance and feed utilization of Red sea bream fed experimental diets for 12 weeks

Parameters	FM	NFM	NFM+ 0.5T	NFM+ 1.0T	NFM+ 1.5T	NFM+ 2.0T
IW (g)	5.71	5.74	5.73	5.74	5.68	5.69
FW (g)	44.3	25.8	30.4	36.6	38.7	39.5
WG (%)	675.9a	344.2c	487.9b	538.2ab	604.9a	642.8a
SGR (%/day)	2.85a	2.09b	2.32ab	2.57ab	2.66ab	2.69ab
DFI (%/day)	0.49	0.47	0.48	0.49	0.50	0.49
FCR	1.08a	1.38b	1.25bc	1.19ac	1.17ab	1.14ac
PER	2.00a	1.56c	1.74bc	1.80b	1.83b	1.87b
SR (%)	88.9	66.7	73.3	75.5	80.0	82.2

Values (Mean ± SD.) (n = 3) with the different letters in the same line are significantly different at $P < 0.05$.

5.3.2. Liver observation

Six fish from each diet group were sampled and dissected, but occurrence of green liver syndrome was only found in fish fed NFM or NFM+0.5T diet. In fish sampled, all fish from NFM group were observed green liver syndrome while four fish from NFM+0.5T showed the occurrence of green liver.

5.3.3. Digestibility

The effects of dietary taurine on the apparent digestibility coefficients (ADCs) of the feed are listed in **Table 23.** Fish fed FM had significantly higher ADC for dry matter than NFM groups. However, ADCs of protein and

lipid in FM group were significantly higher than fish fed non FM diet with taurine supplementation level lees than 1.0% (NFM, NFM+0.5T, NFM+1.0T). Among the non FM groups, the ADC values were tended to elevate with increasing taurine supplementation. ADC of dry matter and ADC of lipid in NFM group was significantly lower than other non FM groups. ADC of protein in NFM group was significantly lower than other NFM+2T.

Table 23. Apparent digestibility coefficients of protein, lipid and dry matter of red seabream fed experimental diet.

Apparent digestibility coefficient (%)	FM	NFM	NFM +0.5T	NFM +1.0T	NFM +1.5T	NFM +2.0T
Dry matter	76.23a	62.18c	66.24bc	68.97b	69.48b	70.13b
Crude protein	86.87a	77.23c	79.93bc	82.23bc	82.68ab	82.95ab
Crude lipid	83.76a	73.88c	78.03bc	78.62b	80.81ab	80.93ab

Values (Mean ± SD.) (n = 3) with the different letters in the same line are significantly different at $P < 0.05$.

5.4. Discussion

Rising fish meal prices are driving efforts worldwide to identify economical alternatives to fish meal in marine fish diets. Results from the present study showed that growth and feed utilization were dramatically decreased in NFM groups. The inferior growth in NFM groups, probably due to the presence of anti-nutritional factors in SBM (Vaintraub and Bulmaga 1991; Liener 1994). The inferior growth performance in NFM groups was in line with several previous studies that have shown considerable growth depression in partial replacement of FM with PP in red seabream (Takagi et al. 1999, 2000a, 2000b).Feed utilization efficiencies in fish fed diet with lesser inclusion of taurine supplementation were lower than those in non FM groups with higher taurine supplementation levels and control The similar growth performance and feed utilization between FM and NFM+2T, suggested that taurine supplemented to NFM diet can efficiently promote growth and feed utilization of red seabream.

. In the present study, taurine deficiency in 0 or 0.5% taurine supplemented non-fish meal diet groups, might be the reason of occurrence of the green liver syndrome because the requirements of taurine of this species cannot be satisfied. In the present study, all fish at the end of rearing were not examined. So we cannot clearly indicate no occurrence in all fish

The current study was conducted over a ten-week period for fish to acclimatize to feed and another two-week to allow adequate collection of fecal samples. The duration of the experiment also could have contributed to the low ADCs reported in the current study. Negative effects of plant proteins are likely magnified as exposure time is increased. ADCs only represent a snapshot of digestibility when conducted over a short period. Animals and their gut micro-flora communities can adapt to changes in diet composition to increase digestive efficiency, however chronic exposure to high levels of plant products could decrease digestive efficiency of species susceptible to antinutritional factors in plants.

Published data on nutrient availability in feedstuffs is not only species-specific, but also diet-specific. Plant products incorporated into feeds as protein sources also contain carbohydrates and fiber. Many fish have limited ability to digest carbohydrates and no ability to digest fiber. ADCs in this study were lower than those previously reported for red seabream and some other carnivorous fishes (Chong et al., 2002; Fontainhas-Fernandes et al., 1999; Gaylord et al., 2004; Li et al., 2013; Richard et al., 2011; Santigosa et al., 2008; Santigosa et al., 2011a; Santigosa et al., 2011b). Krogdahl et al,（2003）also suggested that inclusion of SBM to Atlantic salmon diets reduced the lipid digestibility. Therefore, the significant ADCs among dietary groups in the present study can be explained by this argument. In addition, digestibility and gastric evacuation rate(GER) of fish vary from feed protein, and it has been suggested that SBM is faster in GER than fish meal, feather meal, and CGM, and it is difficult for it to be digested by intestinal enzymes (Touhata et al. al., 2017). Therefore, digestibility always be reduced when FM is replaced by SBM. Takigi et al, 2000 suggested that supplementation of soybean trypsin inhibitor can compensate the decrease in trypsin activity of red sea bream and increase the absorption of nutrients by promoting the retention time of feed in digestive tract and the secretion of digestive enzymes. However, the increase of retention time of plant ingredient in digestive tract would increase the time of digestive tract exposed to soybean saponin and lectin, and as a result, the incidence of lesion in the intestine might increase. Soyasaponins was associated with the onset of morphological changes known as SBM induced enteritis in Atlantic salmon (Knudsen et al., 2007, 2008). Once normal intestinal structure is damaged, it always causes the decline of digestive and absorptive capacity of nutrients. These studies revealed that various antinutritional factors in PP sources may play causative roles of pathological changes and help dissect negative impact of each compounds on gastrointestinal tract of fish.

Taurine is well documented that it may interfere with fish lipid metabolism (El-Sayed, 2014; Salze and Davis, 2015). And it is well-known that taurine can promote digestion by promoting the secretion of digestive enzymes and improve digestive enzyme activity (Gaylord et al., 2008;

Glencross et al., 2007; Hu et al., 2018; Zhang et al. 2018). Salze et al., (2012) also demonstrated that addition of taurine to feed can significantly increase the activity of amylase and trypsin in juvenile cobia and promote the utilization of starch and protein of cobia. In red sea bream, lower nutrient digestibility and digestive hormone and enzyme gene expression were reported by feeding SBM based diet（Murashita et al. 2018). Therefore, we hypothesised that the low ADCs maybe account by the low enzyme activity. In present study, ADC of protein gradually improved with increasing taurine levels, this finding is also in line with previous study. However, it has also been reported that red seabream feed diet containing SBM may increase the lipase activity (Murashita et al. 2016). Because of lacking data for digestibility enzyme, we failed to confirm the enzyme activity is reduced or not. Consequently, we cannot tell the low ADCs is caused by digestive enzyme activity. In addition, when taurine level reached to certain dose, the difference became insignificant. This result may be caused by the excessive content of taurine in diet. Therefore, it is necessary to elucidate whether or not the enzyme activity is reduced by the PP firstly, and then to confirm the mechanism.

There are numerous factors that can affect digestibility measurements, including diet composition, feed intake, fish size, fecal collection method and diet processing, among others. It can be difficult to determine the reasons for variations in nutrient digestibility measurements among laboratories, or even within a laboratory during a period, although lack of methods standardization is a factor.

5.5. Conclusions

FM completely replaced by PP will cause growth retardation and feed utilization decrease. Fish fed PP based diet performed lower digestibility for nutrients. Insufficient dose of taurine in PP based diet can cause occurrence of green liver syndrome. These adverse effects were tended to elevate with increasing taurine supplementation.

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Chapter 6. General Conclusion

Plant proteins are the most important and cost-effective alternative for fish meal in the aquafeed to reduce diet costs and increase the production profitability. A number of studies have been taken to evaluated partial or totally replacements for FM and have got different degree of success. Despite the drawbacks, PP has shown to be a promising protein ingredient for several fish species without impairing growth performance. In future, high or complete supplementation levels of plant proteins are likely to be held in the fish feed. Development of effective all-plant diets for red seabream could increase the profitability of red seabream aquaculture, facilitate increased commercial red seabream production, and assist the creation of sustainable aquaculture methods for related marine fish species.

Taking all parameters studied in the present study into account, we can conclude that: FM completely replaced by plant proteins was feasible under the conditions of this experiment. The results of this study provide evidence that PP based diet with sufficient nutrient supplements showed no impairment of growth performance and feed efficiency of red sea bream. The present study provides evidence that plant proteins as FM alternatives caused intestinal inflammation response in red seabream. Pathological change in fish intestine and enteropathy related gene expression can be ameliorated when supplemented with taurine.

However, the study was designed to evaluate the effect of experimental diet on growth, nutrient digestibility, intestinal morphology and cytokines gene expression in juvenile red seabream. The rearing period in each experiment in this study is lasted for around 12 weeks, which is too short for fish to perform growth retardation or enteritis. Therefore, long term feeding trial is needed for evaluating the experimental diet is suitable for intensive fish farming or not. In addition, the mechanism of taurine modulating of intestinal inflammation in red seabream remains largely unknown. Accordingly, systematic studies to investigate the relation among plant protein, taurine, digest enzyme and intestinal immune function and further attempts to explore the molecular mechanisms in fish are quite necessary.