Studies on utilization of ω3 rich oilseed Camelina (*Camelina Sativa*) in diets for red sea bream (*Pagrus major*)

マダイ飼料におけるカメリナ(*Camelina Sativa)*の利用性に関する研究

KUMBUKANI MZENGEREZA

2022

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KUMBUKANI MZENGERZA

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Abstract

The cost of aquaculture operations continue rise due to high cost of aqua-feed resulting from price fluctuations and scarcity of traditionally conventional and highly nutritious fish meal and fish oil. The current high aquaculture feed cost, it is apparent that further research is necessary to investigate the use of low cost and available feed ingredients for formulation of practical diets for red sea bream. Camelina oil (CO) was incorporated in diet of red sea bream as the first step to evaluate its nutritional efficacy as a lipid source on growth performances, lipid profiles, blood health parameters and proximate contents. Five experimental diets were formulated to contain varying levels of Camelina oil at 0%, 10%, 30%, 50% and 90% designated (D1, D2, D3, D4 and D5, respectively) Growth performance diminished with addition of CO with subsequent decreasing of EPA and DHA and 30% CO registered similar growth of fish oil (FO) diets. Feed intake (FI) lowered with CO addition showing poor acceptability leading to subsequent low growth. Blood function was not altered probably signifying apparent maintenance of health in all diets. Lipids were more pronounced in total and neutral lipids in storage form of TGA in tissues as CO gradually replaced FO. In an effort to optimise the use of CO a separate second experiment was undertaken with supplementation of Soybean lecithin to the previous feed formulation and utilized very smaller animals (red sea bream averaging 1.9g at stocking). Upon completion of the experiment, it was observed that CO can completely replace fish oil as long as EPA and DHA are incorporated at recommended 0.5-1% of diets. SGR, FI, FCR, CF, HSI and survival rates were unaffected in all diets. Similarly, whole-body composition was similar in feed groups except for the crude lipid content, which had highest in the control group. Blood chemistry levels were not influenced in response to test diets: haematocrit, Glu, T-pro, T-Chol, BUN, TG, ALT and

AST. Values of CAT and SOD activities were not altered in plasma and muscles between CO and FO diets and lower in liver at FO substitution with 100% CO, whilst highest values were observed in the control. The experiment confirmed that red seabream may have capacity to maintain LC-PUFAs and CO substitution could reduce lipid peroxidation as well as maintain digestibility. Another 60-day experiment was conducted to investigate effect of fish meal (FM): camelina meal (CM) on growth response and relative gene expression of growth-related gene expression, feed utilization capacity, stress tolerance and immune response of *Pagrus major*. Four formulated and designated as camelina meal, CM0, Soyabean meal, SBM20.5, CM20.5, and CM33 based on protein contents. Upon completion of this experiment, marginal addition of camelina meal in place of fish meal dimished growth performance. The lowest growth response, feed utilization, enzyme activity, and digestibility were recorded in fish high CM diet (330g/kg). Hematocrit levels were depressed at highest camelina inclusion while T-Pro, T-cho, TGA, BUN, T-bil, AST, and ALT were not altered. Non-specific immune variables were relatively lower in higher CM than in lower CM inclusion for diets. Stress indicators: SOD, CAT and TBars showed that increasing CM in diets of red sea bream lowers resistance to oxidative stress. Significantly higher hepatic *IGF-1* and *IGF-2* mRNA expression was found in fish-fed diet groups containing low CM than fish fed high CM. The experiment showed that the addition of CM up 205 g/kg to diet maintains growth performance, feed utilization potency, digestive enzymes activity, immune response and stress tolerance of red sea bream.

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Declaration

I declare that work presented in this thesis is entirely my own with all exceptions being clearly indicated or/and properly cited in the context

Dedication

Above everything this thesis is a manifestation of the existence of Almighty God through Jesus Christ my saviour. I take refuge in Jeremiah 29:11.

Thank you my entire family and friends for your love and support. Many thanks to my mother, Emily Pitalumbiri Mzengereza (nee Gondwe), My sisters: Tamala, Liz, Khumbo and Thoko, my brother Tchinga as well as my friend Sam Manda and all that come from far and wide too numerous to mention for your moral, spiritual and emotional support.

List of publications and presentations

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Viliame Waqalevu , Akinobu Honda , Serge Dossou , Tran Nguyen Duy Khoa , Hideaki Matsuia , **Kumbukani Mzengereza** , Hanlin Liub , Manabu Ishikawa , Kazuhiro Shiozaki , Tomonari Kotani ,(2019). Effect of oil enrichment on *Brachionus plicatilis* rotifer and first feeding red sea bream (*Pagrus major*) and Japanese flounder (*Paralichthys olivaceus*), *Aquaculture,*510,73–83. https://doi.org/10.1016/j.aquaculture.2019.05.039.

Zaineldin A.I., Hegazi S., Koshio S., Ishikawa M., Dawood M.A.O., Dossou S., Yukun Z., **Mzengereza K**. (2020). Singular effects of *Bacillus subtilis* C-3102 or *Saccharomyces cerevisiae* type 1 on the growth, gut morphology, immunity, and stress resistance of red sea bream (*Pagrus major*), Annals of Animal Science, DOI: 10.2478/aoas-2020-0075

Dossou, S.; Dawood, M.A.O.; Zaineldin, A.I.; Abouelsaad, I.A.; **Mzengereza, K.**; Shadrack, R.S.; Zhang, Y.; El-Sharnouby, M.; Ahmed, H.A.; El Basuini, M.F.

Dynamical hybrid system for optimizing and controlling efficacy of plant-based protein in aquafeeds. *Complexity* 2021, *2021*, 9957723.

Serge Dossou, Shunsuke Koshio, Manabu Ishikawa, Saichiro Yokoyama, Mohammed F. El Basuini, Amr I. Zaineldin, **Kumbukani Mzengereza**, Amina Moss, Mahmoud A. O. Dawood,(2018), Effects of replacing fishmeal with fermented and non- fermented rapeseed meal on the growth, immune and antioxidant responses of red sea bream (*Pagrus major*). *Aquaculture Nutrition* ;1–10. ; https://doi.org/10.1111/anu.12876.

Mohammed F. El Basuini, Islam I. Teiba, Mohamed A.A. Zaki, Ahmed N. Alabssawy, Abdelaziz M. El-Hais, Ahmed A. Gabr, Mahmoud A.O. Dawood, Amr I. Zaineldin, **Kumbukani Mzengereza**, Ronick S. Shadrack, Serge Dossou.(2020), Assessing the effectiveness of CoQ10 dietary supplementation on growth performance, digestive enzymes, blood health, immune response, and oxidative related genes expression of Nile tilapia (*Oreochromis niloticus*),*Fish and Shellfish Immunology*、*98 420–428,* https://doi.org/10.1016/j.fsi.2020.01.052.

Conference in contribution

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List of Acronyms and Abbreviations

ω3	Omega- 3
AA	Amino acid
ADC	Apparent digestibility
ALA	Alpha-linoleic acid
ALT	Alanine aminotransferase
ANFs	anti-nutritional factors
ANOVA	Analysis of Variance
ARA	Arachidonic acid
AST	Aspartic aminotransferase
BAP	Biological antioxidant potential
BUN	Blood urea nitrogen
CAT	Catalase
CF	Condition Factor
DHA	Docosahexaenoic acid
DM	Dry matter
DO	Dissolved oxygen
d-ROM	Reactive oxygen metabolites
ECL	Equivalent chain length
EPA	Eicosapentaenoic acid
FA	Fatty acid
FAMES	Fatty acid methyl esters
FAO	Food and Agriculture Organization of the United Nations
FBW	Final body weight
FCR	Feed conversion ratio
FER	Feed efficiency ratio
FFA	Free fatty acids
FI	Feed intake
FM	Fish meal

FO	Fish oil
GE	Gross energy
Glu	Glucose
GM	Genetically modified
GOT	Glutamyl oxaloacetic transaminase
GPT	Glutamic pyruvate transaminase
H&E	Hematoxylin and eosin
HCT	Hematocrit
HPLC	High –performance liquid chromatography
HSI	Hepatosomatic Index
HUFAs	Highly unsaturated fatty acids
IGF	Insulin-like Growth Factor
LA	Linoleic acid
LC	Long chain
LNA	Linoleic acid
LSD	Least square difference
LT_{50}%	Tolerance against low salinity stress
MMT	Million Metric Tons
MUFA	Monounsaturated fatty acids
NBT	Nitroblue tetrazolium
NL	Neutral lipid
NRC	National Research Council
OD	Optical density
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PE	phosphatidylethanolamine
Pl	Phosphatidylinositol
PPT	Part per thousand

PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acids
S.E .	Standard Error
SA	Saturated fatty acids
SBM	Soybean meal
SGR	Specific growth rate
SOD	Superoxide Dismutase Enzyme
sp.	Species
SR	Survival rate
TAA	Total amino acid
TBARs	Thiobarbituric Acid Reactive substances
T-Bil	Total bilirubin
TCA	Trichloroacetic acid
T-Cho	Total cholesterol
TG	Triglyceride
TL	Total lipids
T-Pro	Total protein
VO	Vegetable oil
VSI	Visceral somatic index

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CHAPTER 1. General Introduction

1.0. Global aquaculture perspective

Trends show that aquaculture production has been the fastest growing food production sector in the past three decades (FAO, 2018). 1950-1960s witnessed a world capture fisheries production boom for wild and marine's species (Pickova and Morkore, 2007). In the 1970s, the world experienced an equilibrium in exploitation and production of marine resources which led to the dwindling global fish stocks. Food and Agricultural Organisation (FAO, 2009) concedes that 75% of the fish stocks are either depleted, overexploited or fished at biological limit (FAO, 2009). Worm et al. (2006) forecasts that by the end 2048 all wild seafood species will have collapsed. World population increase places high demand for fish. The current maximum annual wild–capture fisheries production is 100 mmt (Watanabe, 2002; FAO, 2009), a production level that would culminate into global seafood shortage of 50-80 mmt by 2030 if left unchecked or no intervention is made. Farmed fish accounts for nearly half of all fish consumed globally, and this trend is expected to increase in the coming years (Turchini et al., 2009). Aquaculture is an important adventure in bridging the fish scarcity gap through provision of seafood's for the global population. Overdependence on fish meal (FM) and Fish oil (FO) as the main ingredients in aqua feed for carnivorous species will in the long run pose a threat to the sustainability and further growth of intensive aquaculture. As a result, it is important to strike a balance in providing nutritious, safe and high quality food whilst sustaining the environment. There is considerable interest in red sea bream (*Pagrus major*) aquaculture especially in South east Asia, particularly Japan. Japan is one of the

top aquaculture producers at regional and national level. Red sea bream farming has been established in the past five decades and is commercially relevant; notwistanding that interest in farming of alternative marine species such as yellowtail and Japanese flounder has grown, further research in aspects related to the farming of these species can help develop the aquaculture industry in this part of the world.

1.2. Fish nutrition in aquaculture

Animals including fish require all essential nutrients and energy to meet the physiological needs for good health and normal growth. Practical diets for fish must be fortified to supply the essential nutrients including: amino acids, fatty acids, minerals, vitamins, and energy-yielding macronutrients (protein, lipid and carbohydrate). (NRC, 2011) is a compendium of guidelines which profiles the minimum requirements of nutrient that is adequate to sustain growth of farmed fish species and avoid nutrient deficiency. Factors that affect nutritional requirement include species, life stage and the nutrient itself. Amino acids are the building blocks for synthesis and maintenance of available tissue protein; whilst surplus protein are biologically converted to energy (Wilson ,2002). In general, the protein requirements of fish diminish with increasing size and age and carnivorous fish have higher protein demand than omnivorous and species herbivorous fish (Lall and Tibbetts, 2009). Dietary lipids are responsible for supplying essential fatty acids and energy to fish species. The requirement for essential fatty acids can only be met by supplying long chain (LC) polyunsaturated fatty acids (PUFA). Marine animals do not have ability to biosynthesize essential fatty acids in bodies especially the limiting (LC-PUFA) eicosapentaenoic acid (EPA, 20:5ω3), docosahexaenoic acid (DHA, 22:6ω3) and arachidonic acid (ARA, 20:4ω6). Therefore, EPA and DHA must be fortified through

practical diets. On the other hand, freshwater fish are able to synthesize LC-PUAFA α-linoleic acid (ALA, 18:3ω3) and linoleic acid (LNA) which act as precursor for synthesis of both eicosapentaenoic acid (EPA, 20:5ω3), docosahexaenoic acid (DHA, 22:6ω3) (Sargent et al., 2002). ALA and LNA Dietary lipids are also important structural components of membranes, and act as precursors of steroid hormones and prostaglandins in fish. Dietary carbohydrates are an alternative source of energy for fish. However, utilization of carbohydrates for energy largely depends on species and their natural diets; for instance, carnivorous species have limited capacity to digest carbohydrates compared to omnivorous and herbivorous species (Lall and Tibbetts, 2009). Minerals are essential components for normal function and maintenance cellular structure, cellular respiration, oxygen transport, immunity and health status (Lall, 2002). Fish have peculiar physiological mechanisms to absorb and retain minerals from both their diet and from the aquatic environment (Oliva-Teles, 2012). Vitamins are organic compounds that act as substrates in some metabolic reactions (De Silva and Anderson, 1995). Feed formulation in aquaculture is tailored towards striking a balanced diet to satisfy targeted nutritional requirements for varying species, life stages and other purposes, such as medicated feeds.

1.3. Replacement of fishmeal and fish oil in aquaculture feeds

Protein sources is a major driving force towards the sustainability of livestock and aqua-feed. Animal feed ingredients especially fish meal is the traditional source of protein in practical aqua-feed. Fishmeal has a high nutrient content, high palatability and can easily be digested (Taufek et al., 2016). Conversely, the major bottleneck derailing aquaculture

production among several factors is fish feed (Iheanacho et al., 2018). Ogueji et al. (2020) corroborates that access to feeds in aquaculture is difficult due to lack of availability, low quality and astronomical costs. In fact, aqua-feed account for 80% of operating costs in fish farming (Taufek et al., 2016). The perpetual escalation of fish meal demand has resulted in skyrocketing of prices thus pushing aquaculture production cost extremely high (Jabir et al., 2012). The University of Stirling and International Fishmeal and Fish Oil Organisation—IFFO (2016) reported that fourteen million tonnes of edible fish taken from capture fisheries and two million seven hundred tonnes from fish by-products are approximately used to produce four million six hundred tonnes of fishmeal. About nine hundred thousand tonnes of fish oil are also produced currently alongside (Oguenji et al.,2020). In 10 years to come, the production of fishmeal is likely to increase by 25%–30% (FAO, 2016). To sustain the aquaculture venture, aqua feed manufactures should strive to produce nutritionally sound feeds that enhance growth and maintain health status of fish species at low cost (Iheanacho et al., 2018). Research on substitutes to fish meal is premised on the foregoing. Fish oil is the major lipid source in aqua-feeds (Cardinalleti et al., 2019) since it has abundant levels of highly unsaturated fatty acids (HUFAs), especially eicosapentaenoic (EPA) and docosahexaenoic (DHA). EPA and DHA are important for enhancing fish growth, regulating fish metabolism and improving immunity and stress tolerance (IFFO,2016). However, with global dwindling of fish oil production, identifying alternative lipid sources to substitute fish oil has become an important area worthy researching on in fish nutrition (Mzengereza et al., 2021).

1.4. Camelina plant as alternative ingredient in aquaculture feeds

The oilseed *Camelina sativa* (L. Crantz) is ancient leguminous plant first discovered by the Germans dating back around 600BC (Budin et al., 1995). It was cultivated around Europe especially central part before production took unprecented sudden nosedive during the Middle Ages (5th to 15th centuries) (Budin et al., 1995; Putnam et al., 1993). However, camelina continued to exist as weed with flax, coined as “false flax”. Camelina also known as “Gold of Pleasure”, is cruciferous (Brassicaceae family), along with other crops including mustards, rapes, canola, radish, turnip, broccoli, cabbage, collards, cauliflower, rutabaga, Brussels sprouts, kohlrabi and other weeds (Budin et al., 1995). Camelina has myriad agronomic advantages since it needs less inputs and therefore low cost of production. Camelina thrives under semi-arid areas, can be produced in soils with low salinity and fertility, a peculiar attribute for oil seed crops (Putnam, 1993). Arguably camelina has better nutritive edge than several oil crops including soy, sunflower and canola whose nutrient requirements are high (Budin et al., 1995). Putnam et al., (1993) reported that camelina survives low temperatures in late winter and spring (freeze and frost conditions), has low seedrate requirement and withstand insect and weed attacks. Recently, Canadian government has revitalised camelina production, notably in Atlantic Canada and the Prairies for biofuel and animal feed purposes.

Documented literature show that camelina seeds have oil contents ranging from 28% to 40% (Budin et al., 1995) of which 40 % is lipid fraction, a record high comparatively with other oilseed crops (Zubr, 1997; Ni Eidhin et al., 2003). Camelina oil contains higher proportions of omega-3 PUFA than numerous vegetable oil that have been previously used in aquaculture feeds, therefore camelina may have biochemically and nutritionally commercially superiority over plant oils which have commonly been used in recent years, including sunflower, soybean sesame, canola, corn, linseed, and or palm oil.

Fatty acid profile of camelina reported by (Ni Eidhin et al., 2003) reveal that camelina oil has the high proportions of LNA (19%), 18:1ω9 (18%) and 20:1ω9 (16%), higher content of PUFA and MUFA and low SFA levels. Theoretically, high PUFA levels increases risk of lipid peroxidation, however, camelina comprise of the most potent vitamin E isomer, ϒ-tocopherol which provide an endemic protection against lipid oxidation albeit high PUFA proportion in camelina (Ni Eidhin et al., 2003). Therefore, camelina is a potential alternative to fish oil as a lipid source (ω3 PUFA) in aquaculture feeds due to its economical, nutritional and biochemical characteristics (Ni Eidhin et al., 2003). Camelina meal or camelina pressed cake is a by-product remaining after the oil has been extracted from camelina seed oil crop using mechanical or solvent methods. The meal or cake contain 10% oil (Hixson et al., 2016). Camelina meal contains between 38-43% protein (Hixson et al., 2013, Mzengereza et al.,2021; Zubr,1997), around 11% crude lipid ,6% ash (Hixson, et al 2013,2014a; Mikersch, 1952). Data reported by Hixson et al. (2016) Mzengereza et al. (2021) show that amino acid profile of camelina meal compares favourably with other alternative plant proteins of note, that have been lately used to substitute fish meal, these include soybean meal, rapeseed meal and corn meal. The amino acid profile shows that there are 18 amino acids, 9 of which are essential and non-essential amino acids respectively in camelina meal. There are many anti -nutritional metabolites in camelina meal, the doeminant ones being glucosinolates (tannis and sinnapines) trypsin inhibitors, and phytates (Matthaus and Zubr, 2000). Tannins form aggregations that impede digestive capacity of proteins, enzymes, and essential amino Matthaus (1997). Camelina also contain trypsin inhibitor that may arrests or limit bioconversion of protein and render camelina meal ineffective as a protein source.

1.5. Purpose of study

Experts concede that low supply of fish meal and fish oil confounded with high global population has contributed to the rise in production cost in aquaculture. This situation calls for hot research to find affordable and accessible ingredients as replacement. To date, several initiatives aimed at finding substitutes have been undertaken such as use of vegetable or plant meals and oils from oilseed crops, use of algal oil or powder, insect meal, use of fermentation methods in low nutritious ingredients, supplementation with additives and function foods. Notably, the use of vegetable or plant oils and meals has been done extensively in carnivorous marine species producing mixed results. Recently, the Canadian government re –introduced camelina oilseed crop which was previously abandoned into its agricultural mainstream for both biofuels and livestock feed industries. World over, research on camelina oil and meals has been and still being conducted. To our knowledge, there has been tremendous responses in the of camelina products in aquaculture diets. In fact, *Camelina sativa* is a promising aqua feed ingredients. This study aimed at complementing the global research agenda and efforts in the utilization of affordable and high quality vegetable products as alternatives ingredients for marine ingredients in aqua- feed for carnivorous marine species. This research intends to build on existing knowledge of *Camelina sativa* products nutritional attributes and its suitability for use in aqua-feeds to develop a model practical diet for endemic species to Japan waters, red sea bream (*Pagrus major*). The experiments herein are intended to investigate the effects of supplementing camelina products in diets of red sea bream on growth performance while also maintaining overall health red sea bream. It is envisaged

that formulations adapted in this study can contribute to the optimization of practical feed development and its subsequent use in aquaculture. Results of the present study are expected to improve the understanding of nutritional requirements in using camelina meal and establish opportunities of further research in other marine species. It is hoped that results of the project will contribute significantly to the existing body of knowledge in enhancement of feeds for red sea bream.

1.6. Objectives

The present study aimed at addressed the following:

1. To evaluate the nutritional biochemistry on camelina oil and its implications as an alternative lipid source to fish oil in diets for red sea bream, (*Pagrus major*).
2. To determine the effect of incorporating soy lecithin in the substitution of FO by CO on growth performance, feed utilization efficiency, biochemical parameters and tissue morphology and health of red sea bream *(Pagrus major*).
3. To evaluate the response in Growth performance and related gene expression indices, feed utilization capacity, stress resistance level and immunity of red sea bream (*Pagrus major*) fed diets formulated by slowly replacing fish meal with camelina meal.

CHAPTER 2: Nutritional evaluation of *Camelina sativa* oil as substitute to fish oil for red sea bream, (*Pagrus major*).

Abstract

A 56-day feeding trial was conducted to investigate the effects of substituting fish oil with omega 3 rich oil seed Camelina (*Camelina sativa)* into the diets of juvenile red sea bream (*Pagrus major)*. Five experimental diets were formulated to contain varying levels of Camelina oil at 0%, 10%, 30%, 50% and 90% designated (Control, CO10%, CO30%, CO50% and CO90%, respectively) and fed to juvenile red sea bream (initial mean weight 15.2± 0.02 g). The results showed that weight gain, feed intake and specific growth rate declined significantly in CO50% and CO90%groups when compared with Control, CO10% and CO30%group ($p < 0.05$). Significant differences were not detected in proximate content, survival rate, visceral and hepatosomatic indices, among all fish fed diets containing several levels of Camelina oil ($p > 0.05$). Blood plasma showed slight variations ($p <0.05$) in glucose, total protein and Cholesterol while haematocrit, triglycerides and glutamyl oxaloacetic transaminase and glutamic pyruvate transaminase were not significantly altered ($p > 0.05$). Dietary lipid compositions reflected the proportions of a blend of CO and FO in and their distinctive fatty acid compositions However, tissue neutral and polar lipids of the fish reflect, selected essential fatty acids such as eicosapentaenoic acid were preferentially absorbed, resulting in marginal differences between lipid levels in diet treatments in comparison to what was found in tissue lipid. Our findings suggest that refined camelina oil is a suitable dietary lipid source for juvenile red sea bream under our test conditions.

2.0. Introduction

The seafood production trajectory has been plummeting in the last decade, aquaculture production has on the other hand been exponentially growing to offset the declining aquatic stocks by supplying half of the global food fish for human consumption. (FAO, 2012). Traditionally, fish oil is the main source of lipid in marine aqua-feed industry because to is easy to digest and has high long chain (LC) omega- 3 (ω3) polyunsaturated fatty acids (PUFA). Long chain PUFA, EPA and DHA in particular, are important for health related functions, and can help to prevent cardiovascular and inflammatory diseases and neurological disorders (Siriwardhana et al. 2012). DHA + EPA of 250 mg per day is recommended he World Health Organization (2008). Fish oil production is being threatened by its over-reliance on the ever dwindling wild fisheries stocks. Investigation into alternatives to fish oil has taken centre stage especially on vegetable oils. Different terrestrial oilseeds are commercially used in fish feeds (Turchini et al., 2009). Previously researched vegetable oils have limited contents of LC ω3 PUFA. Lipid sources that are easily digested and contain high LC ω3 PUFA, low levels of ω6 PUFA are best alternatives for fish oils in aqua-feeds to provide a high ω3/ ω6 ratio necessary for fish and human health (Takeuchi et al.1992). Recent evidence in applied aqua-feed trials show that *Camelina sativa* oilseed plant belonging to "Brasicaceae" family exhibit nutritional potential to substitute fish oil (Waraich et al., 2013). Camelina oil, is rich in α-linoleic acid (18:3n-3) and linoleic acid (18:2n-6), an n-3/n-6 ratio close to 2, which is uncommon in vegetable oils. It also has a high amount of antioxidants and can replace

nearly up to 100% fish oil in the diet without causing deletions effects on fish performance (Morais et al., 2012; Hixson et al., 2014b).

To date, there is no information on the use of Camelina oils in Japanese marine aquatic farmed animals. The present study seeks to examine the effects of substituting fish oil with Camelina oil as lipid source in the diets of farmed red seam bream (*Pagrus major*), an economically important marine cultured species in Japan. Fatty acids (FA) compositions, the lipid content and lipid class of the major lipid storage tissues in red sea bream fish were analysed.

2.1. Materials and methods

Table 1: Composition of experimental diets (100g kg−1)

Ingredient g/kg DM	Test diets				
	control	CO10%	CO30%	CO50%	CO90%
Brown Fish meal	450	450	450	450	450
Activated gluten	80	80	80	80	80
Soybean meal	225	225	225	225	225
Dextrin	50	50	50	50	50
Fish oil	90	80	60	40	0
Camelina oil	0	10	30	50	90
EPA	5	5	5	5	5
DHA	5	5	5	5	5
Mineral mix	40	40	40	40	40
Vitamin Mix	40	40	40	40	40
Stay C	3.5	3.5	3.5	3.5	3.5
alphacellullose	11.5	11.5	11.5	11.5	11.5
Proximate composition					
Crude protein	48.26	49.54	50.43	50.77	50.01
Total Lipid	14.01	13.14	11.11	14.0	14.15
Moisture	7.3	6.9	7.9	7.6	7.1
Ash	11.9	13.86	12.49	12.08	12.15
Energy(MJ/kJ)	22.1	21.9	21.5	20.8	21.5

[a] Defatted brown fish meal.

[c] Riken Vitamin, Tokyo, Japan.

[d] Kanto Chemical Co., Inc. Tokyo.

[e] Biopure oil, Box 1943, Fort Qu Appele, SK SOG ISO.

[f] Riken Vitamin, Tokyo, Japan.

[g] Riken Vitamin, Tokyo, Japan.

[i] Glico Nutrition Company Ltd. Osaka, Japan. Commercial name "A-glu SS.

[j] Minerals' mixture (mg/kg diet): MgSO4 (5.07), Na2HPO4 (3.23), K2HPO4 (8.87), Fe Citrate (1.1), Ca Lactate (12.09), Al (OH)3 (0.01), ZnSO4 (0.13), MnSO4 (0.03), Ca (IO3) 2 (0.01) and CoSO4 (0.04). h Amino-mix: RM0 (l-lysine 0, Methionine_0, Betaine_0.3), RM50 (l-lysine 0.34, Methionine_0.22, Betaine_0.3), FRM50 (l-lysine 0.49, Methionine_0.14, Betaine_0.3.

Vitamin mixture (mg/kg diet): ß- carotene (0.10), vitamin D3 (0.01), menadione NaHSO3·3H2O (K3) (0.05), dl-α-tocopherol acetate (E) (0.38), thiamine-nitrate (B1) (0.06), riboflavin (B2) (0.19), pyridoxine-HCl (B6) (0.05), cyanocobalamin (B12) (0.0001), bi-otin (0.01), inositol (3.85), niacin (nicotinic acid) (0.77), Ca Pantothenate (0.27), folic acid (0.01), choline chloride (7.87), ρ-amino benzoic acid (0.38) and cellulose (1.92).

Stay-C: L-Ascorbyl-2-Monophosphate-Na/Ca (DSM Nutrition Japan K. K.).

[m] Nippon Paper Chemicals, Tokyo, Japan.

[k] Calculated using combustion values for protein, lipid and carbohydrate of 23.6, 39.5 and 17.2 kJ/g, respectively.

respectively.

2.1.1. Husbandry

Juvenile red sea bream were collected from a fingering production hatchery, in Miyazaki prefecture, Japan, and transported alive into the Kamoike Marine Production Laboratory, Faculty of Fisheries, Kagoshima University, Japan. Collected fish were conditioned to new indoor rearing environment for a fortnight while being nourished on a commercial pelleted fish feed procured from Higashimaru, Kagoshima, Japan. A total of 225 fish (initial body weight 15.4 ± 0.2 g were divided into fifteen 100 Litre tanks at room temperature filtered seawater at uniform stocking density (15 fish tank $^{-}$1) in triplicate tanks per treatment. The water flow to the tanks was 1.6 L min^{-1}, artificial aeration and natural light/dark regime was applied in the trial. Recorded water temperatures ranged from 26.6 ± 0.6 °C during the feeding period, and fish were fed test diets (09:00 and 16:00

h) to satiation The pH and salinity of the tank water during the trial were 8.2 ± 0.2 and 32.1 ± 0.4 ‾1, respectively. Waste were siphoned every morning to maintain water quality. Uneaten feed were collected few hours before next feeding, air- dried and weighed to calculate preceding feed intake (FI) index. At the end of the feed trial, length and weight of individual fish were recorded. Six fish from each tank were killed by hypothermia method which entails immersion in ice cold precipates of sea water prepared and maintained at 3 ppt and ^{0}C consistent with recommendations by Kolumus et al (2008). Blood was collected using 1 ml syringes previously dipped in heparin (1800 IU mL $^{-1}$) to avoid blood coagulation, needle (25 G 9 1"). Blood was drawn by puncture of the caudal vein of individual fish. The blood sample was collected from 6 fish per tank and pooled into 2 separate 1.5ml capacity tubes. Liver was dissected out from three fish in each replicate tank, weighted individually to get heapatosomatic index and finally pooled together and stored.

Weight Gain (WG %) $= \frac{W_{56d} - W_{0d}}{W_{0d}} \times 100$

Specific Growth Rate (SGR % /day) $= \frac{\text{Ln } W_{56d} - \text{Ln } W_{0d}}{T} \times 100$

Feed Intake (FI, g /fish/ 56 d) = $\frac{\text{Dry diet given - Dry uneaten diet recovered}}{\text{No. of fish}}$

Feed Conversion Ratio (FCR) $= \frac{\text{FI (g)}}{\text{WG (g)}}$

Survival rate (SR %) $= \frac{N_{56d}}{N_{0d}} \times 100$

Condition factor (CF) $= \frac{W}{L^3} \times 100$

Where, W_{56d}= final body weight at 56 days; W_{0d}= initial body weight; T= the experimental period in days (d); N_{0d}= initial number of fish; N_{56d}= final number of fish; W= fish total weight (g); L= fish total length (cm).

$$\text{HSI} = \frac{\text{Liver weight, g}}{\text{Fish body weight, g}} \times 100$$

$$\text{VSI} = \frac{\text{Viscera weight, g}}{\text{Fish body weight, g}} \times 100$$

2.1.2. Experimental design

Prior to starting the experiment, a sample of 5 fish from each tank was collected and stored at −20 °C for determination of initial whole body proximate composition. At the end of the trial experimental fish were starved for 24 h. The survival and individual body weight and length of fish from each tank were measured. Fish from the replicate tanks were pooled and used for various analytical measurements. Fish from each treatment were randomly collected and stored at −20 °C for final whole body proximate analysis. In addition, fish for liver samples collection were killed by hypothermia method by immersion into a slurry of ice cold sea water prepared and maintained at 3 ppt and ^{0}C in accordance with Kolumus et al (2008). Filleting was conducted following the filleting Japanese method. Livers and muscles were pooled and kept at −80 °C for further analysis.

2.1.3. Proximate analysis

Protein, lipid, moisture and ash contents of the feed ingredients, diets and fish whole bodies were analysed according to the standard methods (AOAC, 1998). Fish whole body

were freeze dried (Eyela freeze dryer FD-1, Tokyo Rikakikai Co. Ltd., Japan). Moisture determination was performed in mechanical convection oven at 135 °C (Dk400, Yamato Scientific CO., Tokyo, Japan). Weight difference represented moisture amount. Crude protein was performed following the guidelines in Kjeldahl method measured in terms of nitrogen levels and subsequent protein evaluation in (Kjeltec System 1002 tecator, Sweden). Ash was determined by combustion in muffle furnace at 550 °C for 6 hours. Ether extraction for crude lipid evaluation was assayed by soxhelt extraction method.

2.1.4. Blood parameters collection

Heparinized disposable syringes were used to collect blood for measuring hematocrit level. To measure the health condition of the fish, plasma glucose (Glu), total cholesterol (T-cho), blood urea nitrogen (BUN), total bilirubin (T-bil), glutamyl oxaloacetic transaminase (GOT), glutamic pyruvate transaminase (GPT), total protein (T-pro), Triglyceride (TG) were measured. Plasma chemical paraments were measured spectrophotometric ally with an automated analyzer (SPOTCHEM EZ model SP-4430, Arkray, Inc. Kyoto, Japan.

2.1.5. Haematocrit and blood biochemical analysis

Six fish per tank were randomly selected, and their blood was collected by puncture of the caudal vein using heparinized (1,600 IU/ml, Nacalai Tesque, Kyoto, Japan) disposable syringes with a 26-gauge needle and pooled. Non-heparinized disposable

syringes were used to collect blood for serum analysis. Partial heparinized whole blood was used to analyse the haematocrit, whilst plasma and serum were obtained by centrifugation at 3,000 g for 15 min under 4°C and then stored at −80°C pending analysis. Haematocrit was determined using the micro haematocrit technique (Ren et al. 2005), serum chemical parameters were measured spectrophotometric ally with an automated analyser (SPOTCHEM TM EZ model SP-4430, Arkray, Inc. Kyoto, Japan).

2.1.6. Fatty acid analysis

Total lipid was extracted according to Bligh and Dyer (1959). Fatty acid (FA) compositions of camelina oils, diets, fillets and liver of fish were determined following the protocol proposed by Tamaru et al (1992). Methylation of total lipids was conducted by adding Boron Trifluoride Methanol, (14% in methanol) and Dichloromethane and heated for 1 hour. Hexane and saturated NaCl were added to separate impurities from the upper fatty acid methylate. Derived methyl ester were used for determination of fatty acid in total lipid fraction using gas A Shimadzu AOC-20I GC 2010 equipped with a flame ionization detector using an Omega wax 320 column (Supelco, Inc., Japan) at 260 ^{0}C, carrier gas He at 1 *ml*/min; column temperature at 200^{0}C; injector temperature at 250 ^{0}C and helium (He) served as the carrier gas. Fatty acids composition was quantified by addition of internal standard, 23 Methyl tricosanoate ester (Nu-Chek Prep. Inc) at 1.000 mg/*ml* hexane to samples before methyl esterification.

2.1.7. Lipid class analysis

To assess lipid class levels, total lipid was separated into neutral and polar lipid using sep-pac gas chromatography method using Sep-pak silica cartridge (Waters Corporation, Midford, Massachusetts, USA) (Juaneda and Rocquelin, 1985). Lipid class analysis was performed through thin layer chromatography with flame ionization detector (TFC/FID) using Iatroscan MK-6s (Iatron Laboratories, Tokyo, Japan) machine.

2.2. Statistical analysis

Firstly, data was subjected to normality test, homogeneity of variances verification and robust test for equality of means by Shapiro-wilk test, Levene's test and brown forsythe respectively. The statistical analyses were performed using an analysis of variance (package super-ANOVA 1.11, Abacus19 Concepts, Berkeley, California, (USA). Data were expressed as standard error of means ±S. E (n=3). Data were subjected to a one-way ANOVA while significant differences of mean values were determined at 5% level of probability using Least square difference (LSD).

2.3. Results

2.3.1. Camelina oil lipid profile

The summary of fatty acids (%) composition in camelina oil presented in Fig 1 show That is comprised of 18:3ω3 (42.53±2.6), 18:2ω6 (19.3±1.3), 18:1ω9 (14.57±0.2) 20:1ω9 (16.26±0.2), 22:1ω9 (3.25±0.2) and 20:2ω6 (1.99±2.1). Fatty acids with 18-carbon atoms are abundant than those with 20 carbon atom except 20:1ω9 whose content is similar to 18-carbon atoms fatty acids. Fig 2 show that camelina oils (%) have more triglycerides

(52.3±0.1), moderate amount of free fatty acids (17.5±0.2) and phosolipids (10.8±0.6) and low cholesterol (6.5±0.2).

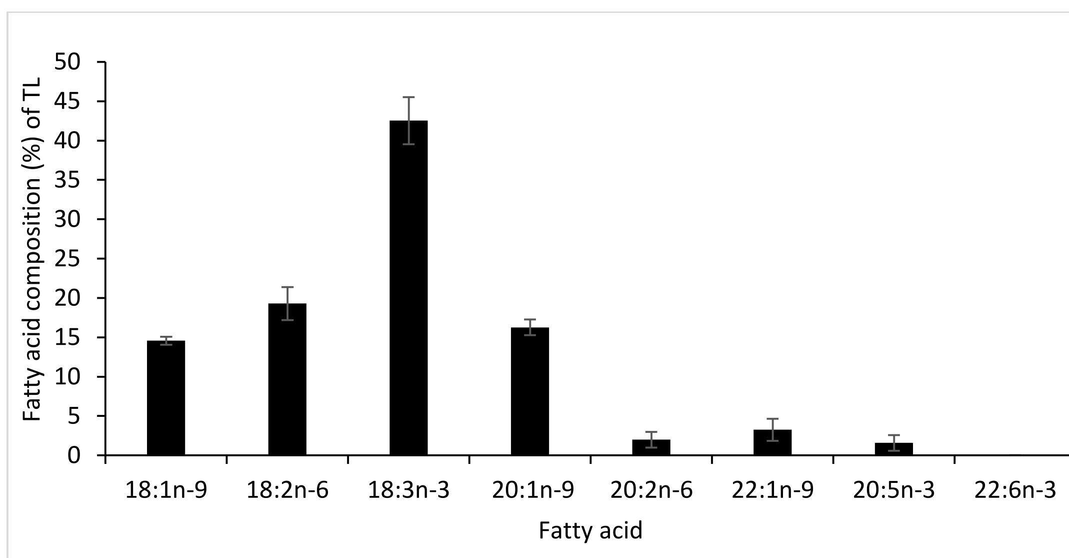


Figure 1: Fatty acid composition (%) of *Camelina sativa* oil (mean ± SD; n = 3)

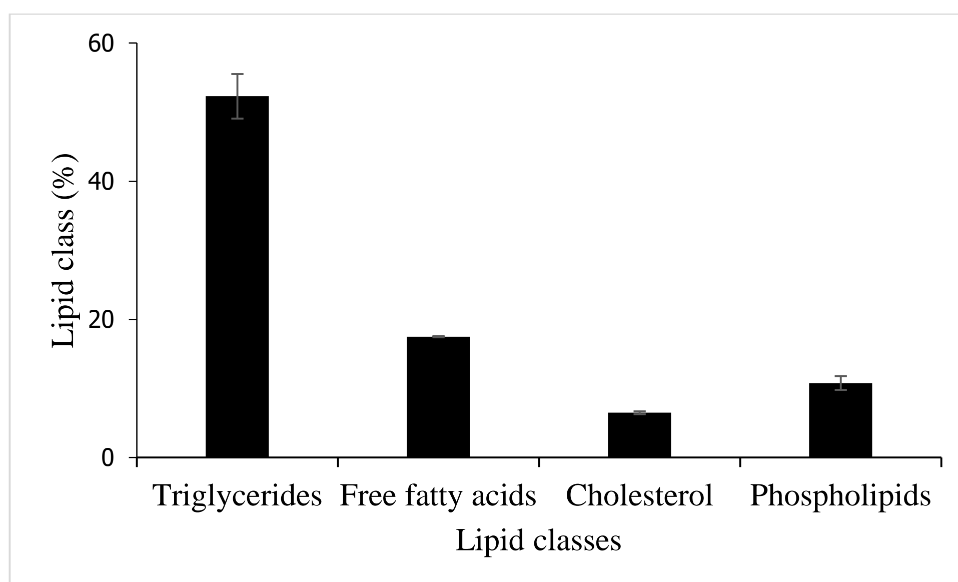


Figure 2: Lipid classes (%) of *Camelina sativa* oil (mean ± SD; n = 3)

2.3.2. Growth response, feed utilization and survival

Table 2 shows the effects of different dietary SM levels on growth performance parameters of red sea bream after 56 days of feeding trial. Results reflect a gradual decrease in weight gain (WG), percentage weight gain (WG%), feed intake (FI), and visceral somatic index (VSI) with inclusion of camelina oil replacing fish oil. On the other hand, feed conversion ratio (FCR) show an inverse relationship with marginal camelina oil addition substituting fish oil. WG was significantly higher($P<0.05$) in diets control, CO10% and CO30% compared to diet CO50% and CO90%. FI was significantly higher in control than all diets while diets CO10%, CO30% and CO50% were significantly higher ($P<0.05$) than diet CO90%. FCR for diets control and CO10% were significantly lower ($P<0.05$) to FCR diets CO30%,CO50% and CO90%, while diets CO10%, CO30%, and CO90% displayed statistically lower ($P<0.05$) FCR compared to FCR for diet 5. VSI were not significantly different but showed downward numerical spiral. Survival rate (SR%) results show that Control, CO30%,CO50% and CO90% are significantly different ($P<0.05$) from diet CO10% abelt having skewed trend.

Table 2: Growth performances of red sea bream (*Pagrus major*) fed with different camelina based diet.

	Test diets				
Growth parameter	control	CO10%	CO30%	CO50%	CO90%
Initial weight(g/fish)	15.82	15.84	15.92	15.83	15.81

Final weight(g/fish)	$48.1^{c}\pm0.0$	$44.71^{b}\pm0.40$	$44.14^{ab}\pm0.10$	$42.52^{ab}\pm0.40$	$42.98^{a}\pm1.10$
SGR[2]	2.26±0.00	2.25±0.01	2.25±0.00	2.24±.00	2.26±0.00
FI(g/fish/56days)[3]	$42.19^{a}\pm0.1$	$40.01^{b}\pm0.1$	$40.8^{b}\pm0.00$	$39.56^{b}\pm0.0$	$35^{d}\pm0.1$
FCR[4]	$1.32^{a}\pm0.01$	$1.42^{ab}\pm0.33$	$1.53^{b}\pm0.00$	$1.72^{b}\pm0.78$	$1.68^{c}\pm0.01$
BWG[5]	203.54±0.2	181.88±0.0	177.27±0.9	168.66±0.0	171.88±0.0
HSI[6]	1.30±0.03	1.40±0.01	1.40±0.05	1.40±0.08	1.40±0.03
VSI[7]	7.12±0.02	7.05±0.05	7.10±0.06	6.96±1.2	6.82±0.17
SR[8]	$100^{b}\pm0.0$	$97\pm^{b}0.20$	$97^{b}\pm1.72$	$86^{a}\pm0.00$	$100^{b}\pm0.00$
CF[9]	2.16±0.10	2.17±0.11	2.26±0.04	2.3±0.09	2.31±0.07

Values[1] are expressed as mean±S.E. (n=3). Data with same alphabets are not significantly different (P ＞0.05).
SGR[2], specific growth rate= 100× (ln final weight- ln initial weight) / (duration).
FI[3], Feed intake (g/fish/56 days) = (total feed intake (g) / number of fishes) in 56 days feeding period
FCR[4], feed conversion ratio = dry feed intake (kg)/weight gain (kg).
BWG [5], body weight gain (%) = 100× (final weight - initial weight) / (initial weight).
HSI[6] , Hepatosomatic index = 100× (liver weight/body weight).
VSI[7], Visceral Somatic Index =100 × (Viscera weight/body weight)
SR[8], survival rate (%) = 100× (initial fish number-dead fish number) / (initial fish number).
CF[9], Condition factor (K)= $100(W/L^3)$, where w is weight and L is length of fish

2.3.3. Carcass composition

Table 3 illustrate the whole body proximate composition red sea bream before and after being fed different levels of CO for 56days (expressed as percentage of wet weight). There were no significant differences ($p > 0.05$) in crude protein, total lipid, moisture and ash compositions among feed groups.

Table 3: Lipid, protein, ash and moisture of whole body red sea bream fed experimental diets (%wet basis)

parameters	Test diet				
	Control	CO 10%	CO 30%	CO 50%	CO 90%
Crude protein	17.08±0.12	16.31±0.30	15.86±0.77	17.4±0.05	16.80±0.34
Total Lipid	11.08±0.12	10.08±0.12	9.08±0.12	10.08±0.12	11.08±0.12

Ash	4.87±0.13	4.72±0.02	4.64±0.03	4.88±0.03	4.79±0.04
Moisture	70.37±0.00	72.9±0.00	72.46±0.00	70.84±0.00	73.12±0.00

2.3.4. Haematocrit and blood chemistry

Table 4 represents the blood parameters of red sea bream after 56 days of feeding trial. Overall, dietary Camelina oil had no significant (P>0.05) effect in blood parameters of fish among different treatments except for those of glucose, plasma total protein and triglyceride (TG). Blood glucose was significantly higher in fish fed control and CO 10% diet than that in CO30%, CO50% and CO 90% groups ($P < 0.05$); however, no significant differences were detected among other groups. Plasma total protein was significantly higher in fish fed diets Control, when compared with other groups ($P < 0.05$), although no differences were detected among all later fish groups. TG of fish fed diet was significantly different without any trend diet ($P < 0.05$). Integration of glutamyl oxaloacetic transaminase and glutamic pyruvate transaminase examined the health of liver, an indicator of overall body health. Results show that both parameters were not significantly different among different fish groups.

Table 4: Hematocrit and blood chemistry parameters in juvenile red sea bream fed test diets for 56 days

	Test diets				
Parameter	Control	CO 10%	CO 30%	CO 50%	CO 90%

HCT (%)[b]	47.33±1.3	42±4.3	44±0.57	44±4.04	45.33±2.02
Glu (mg/dl)[c]	123.33±15.89[b]	90.67±8.66[a]	82.33±16.27[a]	76.33±8.66[a]	69.67±7.88[a]
TBIL(mg/dl)[d]	0.08±0.15	0.30±0.72	0.63±0.28	0.30±0.05	0.30±0.03
Tpro(mg/dl)[e]	4.5±0.23[b]	4.03±0.03[ab]	4.3±0.58[ab]	3.9±0.24 [ab]	3.93±0.12[a]
TCHOg/dl)[f]	302±48.94	296.33±78.59	237.33±8.1	260±7.2	298.66±8.6
TG(g/dl)[g]	160±13.5 [a]	138±23.43 [ab]	156±6.38[ab]	169±2.51 [ab]	194±6.42[b]
GOT (UI/I)[h]	92.67±34.91	144.33±81.66	148.30±30.31	63.00±13.50	47.33±13.09
GPT (UI/I)[i]	32.33±13.61	59.33±41.12	18±6.66	10±0.00	10±0.00

Values [a] are expressed as mean ± SE from triplicate groups (n=3). Data with same alphabets are not significantly different (P＞0.05).
HCT [b:] hematocrit.
GLU [c]: glucose.
T-BIL [d]: total bilirubin.
T-prone: Total serum
T-CHO [f]: total cholesterol.
TG [g]: triglyceride.
GOT [h]: glutamyl oxaloacetic transaminase.
GPT [i]: glutamic pyruvate transaminase.

2.3.5. Lipid class profile

Table 5 and 6 shows the lipid class analysis of the liver and muscle respectively of red sea bream before and after being fed CO as a lipid source for 56 days. Total lipids (TL) Neutral lipid (NL) in fish fed CO90% and CO50%was significantly higher than the CO30%,CO10% and control diet group($p > 0.05$) . Polar lipids were significantly higher in control and the CO30%, CO10% and control diet group($p > 0.05$). Free fatty acids (FFA), Triglycerides (TGs) and Cholesterol (CHO) significantly increased ($p > 0.05$) in tissue with additional of Camelina oil in diets. Phosphatidylinositol (PI) and

Phosphatidylserine (PS) Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) significantly decreased in tissue with corresponding increase in Camelina oil in diets. The PC/PE ratio was significantly increased in CO50% group, followed by CO90% and CO10% groups.

Table 5: Lipid class in liver before and after being fed different levels of Camelina oil

	Test diets					
Lipid class	Initial	Control	CO 10%	CO 30%	CO 50%	CO 90%
TL	43.1±0.7[a]	41.6±0.1[a]	42.5±0.2[a]	41.2±0.3[a]	44.2±0.4[b]	46.05±0.8[b]
NL	32.5±3.0[a]	32.54±0.4[a]	32.6±0.2[a]	33.2±0.2[a]	35.28±0.3[b]	34.01±0.2[b]
PL	64.3±2.0[a]	66.6±2.1[b]	66.5±0.4[b]	64.2±1.5[a]	63.1±0.5[a]	63.1±0.4[b]
CHO	23.9±0.8[a]	26.94±1.2[b]	24.94±2.8[a]	24.4±0.2[a]	23.2±0.2[a]	21.2±0.1[c]
TG	4.1±0.6	4.9±0.5	4.8±0.2	4.3±0.6	4.2±0.8	4.5±2.2
FFA	0.5±0.2	0.3 ± 0.1	1.0 ± 0.1	1.4 ± 1.0	1.7±0.5	2.5±0.1
PI+PS	7.2±1.0[a]	1.57±0.1[b]	0.3±0.3[c]	0.2±0.2[c]	0.0±0.2[c]	0.1±0.5[c]
PC	12.3±0.6	14.36±0.5	11.6±0.3	13.3±0.2	11.9±1.2	10.3±2.1
PE	10.0±0.3[a]	3.2±0.6[b]	2.1±0.2[b]	1.2±0.4[c]	1.0±0.0[c]	0.88±0.1[c]

Data with same alphabets are not significantly different (P＞0.05). NL: Neutral Lipids; shown as percentage of total lipids; PL: Polar Lipids; as percentage of total lipids; FFA: Free fatty acids (% of NL); CHO: Cholesterol (% of NL); PI: Phosphatidylinositol (% of PL); PS: Phosphatidylserine (% of PL); PC: Phosphatidylcholine (% of PL); PE: Phosphatidylethanolamine (% of PL).

Table 6: Lipid class in muscle before and after being fed different levels of Camelina oil

Lipid class	Test diets					
	Initial	Control	CO 10%	CO 30%	CO 50%	CO 90%
TL	38.02±0.1[a]	29.3±0.5[b]	27.5±0.2[b]	26.5±0.1[b]	24.1±0.8[c]	24.7±0.3[c]
NL	30.5±2.0	34.54±0.4	35.6±0.3	35.2±0.8	33.28±1.3	32.01±0.3
PL	66.4±2.0[a]	64.66±1.3[a]	66.5±0.2[b]	66.9±0.4[b]	64.1±0.5[a]	63.1±0.3[a]
CHO	20.2±0.8	12.94±2	12.02±0.2	10.5±0.3	8.34±0.2a	7.4±0.1[a]
TG	4.0±0.6[a]	3.8±0.7[a]	4.4±0.3[b]	4.7±0.4[c]	4.6±0.1[b]	4.8 ±0.2[c]
FFA	0.5±0.2	3.64±0.2	3.64±0.3	3.64±0.3	3.64±0.7	3.64±0.7
PI+PS	8.3±0.9[d]	28.57±0.8[a]	27.57±1.2[a]	26.1±0.3[ab]	26.4±1.2[ab]	24.2±0.2[c]
PC	14.5±0.6[d]	48.36±0.2[a]	43.26±0.6[b]	42.1±2.1[b]	40.3±0.4[c]	41.2.36±0.8[b]
PE	9.1±0.2[a]	0.9±0.1[b]	0.8±0.3[b]	0.5±0.0[bc]	0.3±0.2[c]	0.3±0.01[c]

Data with same alphabets are not significantly different (P＞0.05). TL: Total Lipid presented as percentage, NL: Neutral Lipids; displayed as percentage of total lipids; PL: Polar Lipids; as percentage of total lipids; FFA: Free fatty acids (% of NL); CHO: Cholesterol (% of NL); TG: Triglycerides (% of NL); PI: Phosphatidylinositol (% of PL); PS: Phosphatidylserine (% of PL); PC: Phosphatidylcholine (% of PL); PE: Phosphatidylethanolamine (% of PL).

Table 7: Fatty acid composition (%total fatty acid) in diets of red sea bream muscle

	Test diets				
Fatty acid	Control	CO10%	CO30%	CO50%	CO90%
14:0	6.34±1.3 [a]	5.54±1.2 [ab]	5.4±0.5 [ab]	5.47±0.1 [ab]	4.44±0.8 [b]
16:00	22.34±0.6 [a]	21.2±1.9 [a]	20.3±0.2 [a]	20.01±0.02 [ab]	18.25±0.4 [b]
18:0	3.54±0.4 [a]	2.9±0.2 [a]	2.8±0.8 [a]	2.8±0.6 [a]	2.7±0.3 [a]
Σ Saturated	32.22±2.3[a]	29.64±2.1[a]	28.5±1.0[a]	28.28±0.8[a]	25.39±0.7[c]
161n-7	8.5± 0.5 [a]	6.0±0.4 [b]	4.03±0.2 [bc]	2.1[c]±0.1[c]	2.03[c]±0.2[c]
18:1n-9	10.7 ±05 [a]	12.3±1.3 [b]	18.3[c]±2.1 [a]	19.0[c]±0.6 [a]	19.19[c]±0.2 [a]
20:1n-11	1.9±0.9 [a]	1.6[a]±0.5 [a]	1.5±0.04 [a]	0.8±0.1 [b]	0.6[b]±0.1 [a]
20:1n-9	8.0±1.8 [a]	10.32±2.1 [a]	11.21±1.4 [b]	11.01±4.1 [b]	11.04±0.6 [b]
20:2n-9	2.1±0.2 [a]	2.0±0.4 [a]	1.8±0.4 [a]	1.8±0.2 [a]	1.8±07 [a]
22:1n-9	0.2±0.7	0.2±0.01	0.8±0.7	0.6±0.1	0.8±05
Σ Monoenes	32.2±1.3 [a]	32.45±3.2 [a]	34.64 ±3.1 [ab]	35.31±0.2 [b]	35.46±2.1[b]
20:3n-6	0.1±0.01	0.1±0.01	0.2±0.06	0.2±0.01	0.2±0.01
20:4n-6	0.01±0.1	0.1±0.04	0.1±0.1	0.1±0.01	0.1±0.03
18:2n-6	12±0.1 [a]	16±1.6 [b]	17.3±0.1 [a]	17.5±0.9 [b]	17.9±4.1 [b]
18:3n-6	0.01±0.2	0.2±0.02	0.9±0.4	1.0±0.05	1.0±0.6
22:4n-6	2.0±0.1 [a]	1.0±0.01 [a]	0.8±0.4 [ab]	0.8±0.1 [ab]	0.3±0.2 [b]
Σn-6	13.12±0.5 [a]	17.72±2.6 [b]	19.43±0.7 [bc]	19.1±2.1 [bc]	19.5±2.6 [bc]
18:3n-3	1.03[a]±09	4.3[b]±0.9 [b]	4.5[b]±0.6 [b]	4.9[b]±1.7	5.5[a]±0.8
18:4n-3	1.6±0.4	0.6±0.1	0.8±0.5	0.9±0.6	0.1±0.7
20:3n-3	0.1±0.1	0.1±0.02	0.1±0.01	0.2±0.1	0.2±0.1
20:4n-3	Nd	Nd	Nd	Nd	nd
20:5n-3	8.0[a]±06	5.2[a]±0.4	4.2[ab]±1.3	4.0[ab]±0.5	3.05[b]±1.1
22:5n-3	2.01[a]±0.7	0.5[b]±0.06	0.2[b]±0.08	0.2[b]±0.02	0.1[b]±0.9
22:6n-3	13.38±0.4 [a]	9.0±1.2 [ab]	6.52±1.6 [b]	6.1±1.4 [b]	6.06±1.2 [b]
Σn-3	26.12[a]±1.0 [a]	20.5[a]±5.2 [a]	16.3±0.3 [ab]	16.3±0.5 [ab]	15.0±0.5 [b]
ΣPUFA[3]	39.24±1.3 [a]	38.22±1.3 [a]	35.75±1.8 [b]	35.4±1.3 [b]	34.5±1.5 [b]
Σn-3/n-6 ratio[4]	1.9±0.3	1.2±0.4	0.8±0.4	0.8±0.5	0.7±0.6
ΣEPA+DHA[5]	21.38±0.3	14.2±0.2	10.7±0.3	10.1±0.4	9.06±0.3

1 Values are expressed as means ±S.E. (n=2). Same superscripts are not significant different (P＞0.05).
2 nd = Not detected.
3 Total PUFA is expressed as sum of total n-3 fatty acids and total n-6 fatty acids.
4 n-3/n-6 ratio is expressed as total n-3 PUFA divided by total n-6 PUFA.
5 Sum of eicosapentaenoic Acid (C20: 5n-3) and docosahexanoic acid (C22 : 6n-3) as essential fatty acids

Table 8: Fatty acid composition (%total fatty acid) in neutral lipid fraction of red sea bream liver

	Test diets				
Fatty acid	Control	CO10%	CO30%	CO50%	CO90%
14:0	2.94±1.7[a]	0.92±1.8[b]	1.45±1.1[b]	1.07±1.5[b]	1.04±1.8[b]
16:0	21.52±1.0[b]	19.2±0.2[a]	18.87±1.04[ab]	16.94±4.2[b]	16.3±1.3[b]
18:0	4.5±1.7[a]	2.6±8.5[ab]	2.4±0.7[ab]	2.05±1.2[ab]	1.54±0.4[b]
Σ saturated	28.9±1.2[a]	23.64±0.5[b]	22.72±2.1[b]	20.06±0.8[bc]	18.88±0.2[c]
161n-7	7.6±.04[a]	5.62±1.6[b]	4.8±0.4[b]	3.94±0.6[c]	3.1±1.2[c]
18:1n-9	18.6±0.4	21.31±0.2	21.07±0.2	21.42±0.9	22.67±1.1
20:1n-11	0.82±0.1	0.55±0.6	0.82±6.0	0.55±0.02	1.2±0.2
20:1n-9	2.8±0.1	2.8±0.2	3.8±5.5	5.890.04	8.5±0.4
20:2n-9	3.8±0.6	3.8±0.3	3.8±1.	3.89±0.9	0.15±0.6
22:1n-9	1.18±0.7	0.1±0.4	0.54±0.1	1.18±0.4	0.1±0.2
Σ Monoenes	35.8±0.4	34.68±0.4	34.92±0.9	36.87±0.5	35.72±0.1
20:3n-6	0.1±1.2	0.1±0.2	0.1±0.00	0.1±0.01	0.02±005
20:4n-6	0.82±0.4	0.55±0.3	0.82±0.4	0.55±0.8	0.4±0.09
18:2n-6	9.03±.1.0	9.84±0.5	10.65±0.1	11.71±0.6	11.42±0.1
18:3n-6	0.21±0.2	0.05±0.6	0.08±0.07	0.06±0.1	0.05±0.8
22:4n-6	3.1+1.2	1.3±0.1	1.0±0.6	0.11 ±	0.1± 24
Σn-6	13.08±1.0	13.44±0.1	14.55±1.4	15.32±1.7	18.07±0.5
18:3n-3	2.28±0.5[a]	6.35±0.1[a]	6.65±0.1[ab]	5.54±1.8[a]	9.57±0.2[ab]
18:4n-3	0.19±0.4	0.19±0.1	0.19±3	5.31±2.8	0.53±0.21
20:3n-3	1.08±0.1	1.08±0.6	1.08±7	0.29±3.9	0.66±0.3
20:4n-3	0.49±1.2	0.49±2.0	0.49±1.0	0.07±0.4	0.12±0.6
20:5n-3	9.92±0.9[a]	4.47±0.6[ab]	4.75±0.2[ab]	4.68±1.1[b]	4.04±0.5[ab]
22:5n-3	3.38±0.6	3.66±0.8	3.58±0.1.	3.54±1.3	3.31±2.5
22:6n-3	10.9±0.1[a]	7.72±0.02[ab]	6.15±0.9[ab]	5.56±0.2[b]	5.31±1.6[b]
Σn-3	28.04±0.2	23.9±0.3	22.89±0.1	24.99±0.4	23.9±0.2
ΣPUFA[3]	41.12±0.1	37.34±0.4	37.44±0.2	40.31±0.4	41.97±0.6
Σn-3/n-6 ratio[4]	2.1±0.2	1.7±0.1	1.5±0.00	1.6±0.00	1.3±0.4
ΣEPA+DHA[5]	20.82±0.8[ab]	12.19±0.3[ab]	10.9±0.4[b]	10.2±0.5[ab]	9.3[b]±1.3[ab]

1 Values are expressed as means ±S.E. (n=2). Same superscripts are not significant different (P＞0.05).
2 nd = Not detected.
3 Total PUFA is expressed as sum of total n-3 fatty acids and total n-6 fatty acids.
4 n-3/n-6 ratio is expressed as total n-3 PUFA divided by total n-6 PUFA.
5 Sum of eicosapentaenoic Acid (C20: 5n-3) and docosahexanoic acid (C22 : 6n-3) as essential fatty acids

Table 9: Fatty acid composition (%total fatty acid) in neutral lipid fraction of red sea bream muscle

	Test diets				
Fatty acid	Control	CO10%	CO30%	CO50%	CO90%
14:0	0.98±0.1	0.8±0.1	0.7±0.5	0.5±0.01	0.3±0.1
16:0	25.36±0.5	23.63±0.3 [ab]	23.87±0.1 [ab]	20.9±0.7	21.9[b]±0.1 [ab]
18:0	2.56±0.1	3.8±0.2	3.6±0.2	3.1±0.12	3.2±0.4
Σ Saturated	28.9±0.6[a]	28.23±0.4[a]	27.2±0.9[a]	24.5±0.4[b]	21.37±0.2[c]
161n-7	7.6±0.3[a]	5.62±0.5[b]	4.89±0.4[b]	3.94±0.4[b]	3.1±0.4b[c]
18:1n-9	11.06±0.1[a]	12.47±0.6[a]	15.04±0.5[b]	15.16±0.6[b]	17.35±0.2[c]
20:1n-11	0.6±0.04	0.5±0.4	0.2±0.6	0.2±0.3	0.2±0.5
20:1n-9	6.58±0.1	8.66±0.9	8.46±0.1	8.87±0.4	9.20±0.2
20:2n-9	0.20±0.4	0.20±0.1	0.20±0.9	0.2+0.6	0.10±004
22:1n-9	0.3±0.3	0.34±0.4	0.5±0.4	0.71±0.03	1.760.6
Σ Monoenes	26.34±0.5 [a]	27.79±0.6[a]	29.29±0.4 [ab]	29.07±0.1 [ab]	31.55±0.4 [b]
20:3n-6	0.49±0.3	0.36±0.5	0.4±06	0.41±0.02	0.35±0.5
20:4n-6	Nd	Nd	Nd	Nd	Nd
18:2n-6	12.9±0.6	11.52±0.7	12.14±0.4	13.29±0.5	13.67±0.4
18:3n-6	0.21±0.8	0.05±0.02	0.08±0.7	0.06±0.5	0.05±0.8
22:4n-6	6.7±0.01 [a]	0.2±0.01 [b]	0.45±0.3[b]	0.6±0.7 [b]	0.9±0.4 [b]
Σn-6	19.5±0.1	12.02±0.1	13.07±0.1	14.36±0.1	15.97±0.5
18:3n-3	4.28±0.60	6.35±0.5	6.65±00.5	5.54±0.2	6.57±0.3
18:4n-3	0.19±0.5	0.19±0.6	0.19±0.4	0.31±0.8	0.53±0.10
20:3n-3	0.1±0.6	0.1±0.01	0.5±00.4	0.6±0.4	0.5±0.1
20:4n-3	0.49±0.6	0.49±0.2	0.49±03	0.07±0.2	0.12±0.2
20:5n-3	1.47±0.8	1.7±0.0.2	1.92±0.1	1.56±0.1	1.68±0.01
22:5n-3	1.38±0.1	0.63±0.8	0.69±0.4	0.5±0.3	0.610.002
22:6n-3	24.89±0.7 [a]	22.03±0.6 [a]	22.22±09[ab]	21.26+0.1 [b]	20.33[b]±0.9
Σn-3	29.34±0.5	31.49±0.6	32.66±0.1	29.84±0.2	30.34±0.1
ΣPUFA[3]	48.48±0.4	43.51b±1.3	45.73±1.4	44.2±1.0	46.31b±0.4
Σn-3/n-6 ratio[4]	1.5±0.3	2.6±0.4	2.4±0.1	2.07±0.3	1.8±0.4
ΣEPA+DHA[5]	26.36±2.3 [a]	23.73±1.2 [ab]	24.14±0.7 [ab]	22.82±0.9[b]	22.01±1.4 [b]

1 Values are expressed as means ±S.E. (n=2). Same superscripts are not significant different (P＞0.05).
2 nd = Not detected.
3 Total PUFA is expressed as sum of total n-3 fatty acids and total n-6 fatty acids.
4 n-3/n-6 ratio is expressed as total n-3 PUFA divided by total n-6 PUFA.
5 Sum of eicosapentaenoic Acid (C20: 5n-3) and docosahexanoic acid (C22 : 6n-3) as essential fatty acids

Table 10: Fatty acid composition (% fatty acid) in polar lipid fraction of red sea bream liver

	Test diets				
Fatty acid	Control	CO10%	CO30%	CO50%	CO90%
14:0	4.87±1.2 [a]	3.3±1.6 [a]	3.1±1.0 [a]	3.07±0.6 [a]	2.3±0.4 [a]
16:0	26.8±1.6 [a]	26.5±2.1 [a]	24.7±1.8 [a]	24.2±1.7 [b]	23.5±1.6 [b]
18:0	5.5±1.0 [a]	2.4±0.5 [b]	2.3±0.6 [b]	2.2±1.5 [b]	1.8±1.3 [b]
Σ Saturated	37.17±1.2[a]	32.2±0.4[b]	30.1±0.6[b]	29.5±12[bc]	27.6±0.6[c]
161n-7	2.7±0.1	2.5±0.2	1.8±0.2	2.01±0.02	1.3±0.4
18:1n-9	10.6±1.2 [cb]	12b±0.6 [a]	12.5±1.9 [ab]	13.6±0.3 [a]	14.2±2.1 [a]
20:1n-9	2.23±0.6 [a]	2.5±1.3 [a]	3.4±1.3 [a]	3.8a±0.2 [a]	5.3a±2.1 [a]
22:1n-9	4.18±0.5	2.4±0.01	2.4±0.4	2.3±0.3	2.2±0.2
Σ Monoenes	17.01±1.0	19.4±1.5	20.1±1.3	21.71±1.6	23±1.4
20:3n-6	1.1±0.4	1.3±0.7	1.2±0.4	1.3±0.9	1.0±0.9
20:4n-6	5.17±2.1	1.65±0.4	1.2±0.04	1.03±0.4	1.2±0.1
18:2n-6	14.83±1.0	16.5±0.9	17.45±1.2	18.25±1.6	21.04±2.0
Σn-6	21.1±0.6 [a]	19.45±2.3 [a]	19.85±0.6 [a]	20.58±0.7 [a]	23.24±2.2 [b]
18:3n-3	1.60.3 [a]	3.7b±0.3 [a]	3.9±0.6 [b]	4.2±0.5 [b]	4.4±0.4 [b]
18:4n-3	0.6±0.02	0.6±0.8	0.5±0.02	0.4±0.08	nd
20:4n-3	0.3±0.04	0.5±0.02	0.3±0.01	0.3±0.2	0.4±0.02
20:5n-3	2.9±2.1 [a]	1.5±0.9 [ab]	1.5[b]±0.5 [ab]	1.1b±1.9 [a]	1.1±0.4[b]
22:5n-3	3.6±0.2	3.5±1.2	3.4±0.6	3.3±0.2	3.0±0.2
22:6n-3	10.5±1.6 [a]	8.2±1.6 [ab]	8.6±0.9 [ab]	8.1±1.6[b]	7.2±1.4[b]
Σn-3	19.5±1.2 [a]	18.1[a]±1.3	18.2±1.2 [a]	17.4[b]±0.8 [a]	16.1±1.3 [a]
ΣPUFA[3]	40.6±1.4	37.55±5.3	38.05±0.1	37.98±2.4	39.34±1.3
Σn-3/n-6 ratio[4]	0.92±1.3	0.93±0.7	0.91±0.04	0.84±0.3	0.69±0.04
ΣEPA+DHA[5]	13.4±0.3 [a]	9.7±0.4 [ab]	10.1±0.1 [ab]	9.2±1.6 [b]	8.3±0.2 [a]

1 Values are expressed as means ±S.E. (n=2). Same superscripts are not significant different (P＞0.05).
2 nd = Not detected.
3 Total PUFA is expressed as sum of total n-3 fatty acids and total n-6 fatty acids.
4 n-3/n-6 ratio is expressed as total n-3 PUFA divided by total n-6 PUFA.
5 Sum of eicosapentaenoic Acid (C20: 5n-3) and docosahexanoic acid (C22 : 6n-3) as essential fatty acids

Table 11: Fatty acid composition (% total fatty acid) in polar lipid fraction of red sea bream muscle

	Test diets				
Fatty acid	Control	CO10%	CO30%	CO50%	CO90%
14:0	1.5±0.5	2.1±0.4	2.5±0.02	2.4±1.6	2.06±0.4
16:00	21.1±0.6	20.2±0.1	20.71±0.2	19.8±1.3	18.5±2.0
18:0	1.0±0.6	1.8±0.5	2.1±1.2	2.8±1.0	2.7±0.2
Σ Saturated	22.6±0.3^{a}	24.1±0.5^{b}	25.3±0.5^{bc}	25.0±0.2^{bc}	23.26±0.9^{a}
161n-7	4.67a±03	2.02b±0.02	2.08b±0.1	1.8bc±0.6	1.9±0.7
18:1n-9	18.55±2.3 ^{a}	20.78±1.4 ^{ab}	23.2±1.5^{c}	25.98±1.8 ^{cd}	27.55±0.8^{d}
20:1n-9	6.58±1.5	8.66±0.6	8.46±1.7	8.87±1.3	8.74±1.7
22:1n-9	0.3±0.01	0.5±0.08	0.5±0.02	0.6±0.01	0.6±0.03
Σ Monoenes	31.08±1.2 ^{a}	32.96±1.3 ^{a}	35.79±1.6 ^{a}	38.25±1.2 ^{c}	38.79±1.4 ^{c}
20:3n-6	1.1±0.4	1.3±0.7	1.2±0.4	1.3±0.9	1.0±0.9
20:4n-6	5.02±0.7 ^{a}	4.1±0.9 ^{a}	3.2±0.2 ^{a}	3.0 ±1.6 ^{ab}	2.1±0.4 ^{b}
18:2n-6	4.3±0.9	10.13±1.3	11.1±2.7	11.2±1.6	12.3±1.6
Σn-6	10.42±1.2 ^{a}	15.5±1.2^{b}	15.5±1.2^{b}	15.5±0.5^{b}	15.45±0.8^{b}
18:3n-3	1.2±0.2	2±1.9	2±2.1	1.36±	1.1±0.1
18:4n-3	0.2±0.1	0.6±0.6	0.5±1.6	0.2±1.0	0.2±1.6
20:4n-3	0.1±0.1	0.2±0.04	0.5±0.07	0.6±0.01	0.9±0.07
20:5n-3	7.2±2.3^{a}	5.1±1.2 ^{ab}	5.8±0.07 ^{ab}	5.8±0.7 ^{ab}	4.7±1.6 ^{b}
22:5n-3	2.05±1.6 ^{a}	1.2±0.6 ^{b}	1.3±0.7 ^{b}	1.47±0.3 ^{b}	1.1±0.9 ^{b}
22:6n-3	13.6±2.3 ^{a}	11.2±1.4 ^{a}	11.2±3.5 ^{ab}	10.2±1.3 ^{ab}	10.2±2.1 ^{ab}
Σn-3	24.35±1.3^{a}	21.3±1.3^{b}	21.5±1.2 ^{ab}	19.63±1.4 ^{bc}	18.2 ±1.4 ^{c}
ΣPUFA[3]	34.77±1.2	36.8±0.6	37±0.7	35.13±0.6	33. 65±0.00
Σn-3/n-6 ratio[4]	2.3±0.1	1.3±0.3	1.4±1.3	1.2±0.5	1.1±0.3
ΣEPA+DHA[5]	20.8±1.3^{a}	16.3±0.5^{ab}	17±1.6^{a}	16±0.6^{ab}	14.9±0.1^{b}

1 Values are expressed as means ±S.E. (n=2). Same superscripts are not significant different (P＞0.05).
2 nd = Not detected.
3 Total PUFA is expressed as sum of total n-3 fatty acids and total n-6 fatty acids.
4 n-3/n-6 ratio is expressed as total n-3 PUFA divided by total n-6 PUFA.
5 Sum of eicosapentaenoic Acid (C20: 5n-3) and docosahexanoic acid (C22: 6n-3) as essential fatty acids

2.4. Discussion

Preliminary exploration into the nutritional profile of Camelina oil suggest that dominant fatty acids are 18:3ω3, 18:2ω6, 18:1ω9 and 20:1ω9 (Fig 1). The comparable PUFA and ω3 compositions between camelina and fish oil gives camelina oil potential as a lipid source in diets for farmed red sea bream. Results of our present trial reflect that up to 30% of camelina oil could replace pollock liver oil in diets of red sea bream without significantly compromising growth responses, whole body proximate composition and overall health fish health. The results from our trial are consistent with previous research conducted using vegetables and Pollock liver oils blend influences on red sea bream growth performance. Several different types of vegetable oils and blends have been used to replace fish oil for red sea bream such as palm oil Kolumus et al. (2008), canola oil Huang et al. (2007), which showed significant differences reduction in growth performance with increasing vegetable oil in the dietary treatments. There is a strong likelihood that red sea bream juvenile growth was gradually hampered with marginal addition of dietary Camelina oil, notwithstanding the absence of significant differences among control, CO10% and CO30%. The diminishing growth pattern is likely to have emanated from declining essential polyunsaturated fatty acids more especially eicosapentaenoic acid (EPA) and less likely) from diets Docosahexaenoic acid (DHA). Secondly, the growth pattern with increasing camelina is associated with poor feed intake that shows that CO addition reduced acceptability of the feed hampering growth in the end. Our test diets in the present trial satisfied the 0.5% DHA but not the 1% EPA requirement (Table 1). EFA requirements of juvenile red sea bream are met when the diet contains 1% EPA, 0.5% DHA or 2.8% n-3 HUFA Takeuchi et al. (1990), when dietary lipid is at the optimal level of 15% (Takeuchi et al. 1990; Takeuchi et al. 1991; Takeuchi, et al., 1992 Huang et al. ,2007). Therefore, findings of our present trial contradict the

concept that DHA has higher efficacy than EPA in red sea bream (Takeuchi et al., 1990) and thus provides the notion that both EFA had similar efficacy to influence growth performance. Although such is the case, we propose long term nutritional evaluation to substantiate the allowable dietary CO concentration reported in the present study benchmarked against reports by previous authors.

Marine fin fish naturally possess low or absent Δ6- and Δ5-desaturase enzymes, Mourente et l. (2007), capable of converting short chain fatty acids like 18:3n-3 and 18:2n-6 to long chain poly unsaturated fatty acids (n-3 LC-PUFA) in vivo through 20:4n-6 pathway. Changes in proportions of fatty acids compositions for red sea bream observed in this current study (Tables 8,9,10 and 11) is manifestation they are influenced by diet lipid sources of fish oil and Camelina oil blend. In addition, fatty acid levels in tissue show the efficacy of red sea bream of its inherent ability in oxidation of energy from fatty acids. Previous studies revealed that the red sea bream is similar to other marine finfish species which showed little ability to utilize saturated fatty acids for energy purposes and also inability to convert short chain FA to n-3 LC-PUFA. In our current trial muscle fillet concentrations in both lipid classes of saturated fatty acid in red sea bream were strongly related to the dietary fatty acid composition. Furthermore, oleic acid (18:1n-9), eicosanoid (20:1n-9), linoleic (18:2n-6) alpha linoleic acid (18:3n-3) show increasing trends in both muscle fillets and hepatic tissues for both neutral and polar lipid (Tables 8,9,10 and 11) with corresponding addition of camelina oils in diets. Conversely, EPA (20:5n-3) and DHA (22:6n-3) exhibits an inverse relationship with increase in camelina vegetable oils in both tissues in both polar and neutral lipid classes (Tables 8,9,10 and 11). Both scenarios were also observed prior in studies conducted by Kolumus . (2008), Haugh et al. (2007), Seong et al . (2019); Seong et al. (2020) on red sea bream fed palm oil, canola

oil, microalgae *Schizochytrium* sp respectively as well as Hixson et al., (2013); Hixson et al. (2014a) working on salmon and cod fed camelina oils based diets.

However, proportions of EPA appear to diminish more rapidly between diets and tissues as observed by low EPA in the latter while DHA appear to accumulate between diets and tissue as observed by higher proportions especially in neutral lipid liver tissue than in muscle and diets (Tables 8, 9,10,11). We therefore deduce that, in our present feed trial EPA were preferentially oxidised for energy production in the red sea bream. In addition, from the present study another theory that we submit is that EPA might have been bio actively converted to DHA (22:6n-3). Tables (8,9,10,11,) show that EPA level is a mirrored the diets or lowered in tissues than in diets, whilst DHA proportions modified in tissues compared with fatty acid composition diets fed fish.

The lipid class profile of the feeds differed with respect to changes in on camelina inclusion. The diets were formulated to be isolipidic, thus total (43.2%), neutral (32.1%) and (64.5%) polar lipids were uniform among diets which also comprised of main lipid classes TAG (%) and PL (%). Lipid deposition and storage differed in liver and muscle tissues, corresponding to on the lipid composition of the diet. In the both muscle and liver, red sea breams the lipid significantly increased total lipid, neutral lipid, TAG and CHO with increasing camelina inclusion while in polar lipid, Phospholipids PC, PI, PE decreased with marginal addition of camelina oil. It worthy to note that total lipids and neutral lipid classes were more deposited and stored in liver than in muscle while polar lipid fraction were more concentrated and incorporated in muscle than in liver. The HSI

was the maintained among groups (1.3–1.4%), which is a threshold range for farmed cred seam Kolumus. (2008), suggesting that the excess neutral lipids stored in fish livers of fish did not affect the overall liver weight. Marine fish are known for inherently to store excess dietary lipids in the form of TAG in the liver (Hixon et al., 2013). Neutral lipids like TAG and CHO are rapidly susceptible to respond to changes in dietary lipid compared to polar lipids like PC and PE (Higgs and Dong., 2000).

Although the diets in this study did not vary statistically in the proportion of lipids neutral, CHO and TAG, the composition of diets varied somewhat in respective diets as different levels of Camelina oil were gradually added to the diets and that affected digestion, absorbsoption and metabolism (Jobling et al., 2008). Evidence exist that fish fed vegetable oils store more neutral lipids than fish that consume solely fish oils (Wijekoon, 2012). Salmon fed vegetable oil blend containing 80 % camelina oil had significantly higher lipid deposition and increased TAG content (Hixson et al., 2014b). Our present study show that high amounts of camelina oil in the diet are stored as TAG in the liver rather than metabolized for energy compared to the control that may have affected growth performance. Therefore, feeding the fish a camelina incorporated diet must be with limit to avoid lipid accumulation and hepatic and adipyotic steatosis. Cholesterol also showed Inverse relationship trend with addition of camelina oil is a key component of sterol in animals for transport of lipoprotein and integral part of cell membrane while some if it is stored as neutral lipid (Moss et al., 2018)

Plant seed Camelina oil is likely to possess phytosterols that impinge cholesterol synthesis and bioavailability Pickova and Mørkøre (2007). Study by Hixson et al., (2013) displayed reduced CHO with concomitant camelina oil addition, a pattern observed in the present

study for the similar oil seed *Camelina sativa* cholesterol is a precursor of steroid hormones, (Teshima and Kanazawa, 1971) an essential component in reproductive maturation in animals, it is imperative to investigate appropriate vegetable oils for diet inclusion to offset spawning success

Haematological data are important indices for metabolism, deficiencies, environmental and nutritional influences and stress reflecting overall health of animals. The blood is the chief medium that plays a significant role in many metabolic processes of nutrients. Aminotransferases such as GOT and GLT, are important and critical for the Kreb's cycle, are often considered as an indicator for health and metabolism (Peres et al., 2013). In the present study, the proportions of GLT and GOT in the plasma of fish fed with camelina oils incorporated diets were typical for red sea bream reported in previous work.

Proportions of glucose and triglycerides were on the other hand significantly affected, the trends show addition of camelina oil steadily increased the levels of blood glucose whilst increased blood TAG, suffice to report these levels were within the normal range of values reported for healthy juvenile of red sea bream (Dawood et al., 2017; Dossou et al., 2018a; Hossain et al., 2016). There is no plausible justification for such differences among fish groups and this requires chronic feed trials to explain the mechanism. In a conclusion, results of our feed trial confirm that camelina oils is a potential lipid source to substitute of fish oil for juvenile red sea bream diet with fair amounts of lipid and fatty acids including n-3 LCPUFA which are indispensable fatty acid for marine fish. The study has also confirmed that TAG are stored forms of lipid in neutral lipid and that slight cell morphology can degenerate with use of camelina oils. Besides, camelina oil diets did not alter haematological parameters but caused dismal salinity stresses that may not impinge

growth and health. We propose long term feed trial using camelina oil focussing on DNA, bio- molecular investigation and immunological reactions to show gene expressions responsible for the mechanisms of the results obtained.

CHAPTER 3 : Effect of Substituting Fish Oil with Camelina Oil on Growth Performance, Fatty Acid Profile, Digestibility, Liver Histology, and Antioxidative Status of Red Seabream (*Pagrus major*)

Abstract

A 56-day feeding trial to evaluate the responses of red seabream (initial weight: 1.8 ± 0.02 g) to the substitution of fish oil (FO) with camelina oil (CO) at different ratios was conducted. The control diet formulated at 46% CP (6F0C) contained only FO without CO; from the second to the fifth diet, the FO was substituted with CO at rates of 5:1 (5F1C), 4:2 (4F2C), 3:3 (3F3C), 2:4 (2F4C), and 0:6 (0F6C). The results of the present study showed that up to full substitution of FO with CO showed no significant effect on growth variables BW = 26.2 g–28.3 g), body weight gain (BWG = 1275.5–1365.3%), specific growth rate (SGR = 4.6–4.7), feed intake (FI = 25.6–27.8), feed conversion ratio (FCR = 1.0–1.1), biometric indices condition factor (CF = 2.2–2.4), hepatosomatic index (HSI = 0.9–1.1), viscerasomatic index (VSI = 7.5–9.5), and survival rates (SR = 82.2–100) with different FO substitution levels with CO. Similarly, there were no significant differences ($p < 0.05$) found in the whole-body composition except for the crude lipid content, and the highest value was observed in the control group (291 g/kg) compared to the other groups FO5CO1 *(*232 k/kg), FO4CO2 (212 g/kg), FO2CO4 (232 g/kg) and FO0CO6 (244 g/kg). Blood chemistry levels were not influenced in response to test diets: hematocrit (36–33%), glucose (Glu = 78.3–71.3 mg/dL), total protein (T-pro = 3.1–3.8 g/dL), total cholesterol (T-Chol = 196.0–241 mg/dL), blood urea nitrogen (BUN = 9.0–14.6 mg/dL), total bilirubin (T-Bil = 0.4–0.5 mg/dL), triglyceride (TG = 393.3–497.6 mg/dL), alanine aminotransferase test (ALT = 50–65.5 Ul/L), aspartate aminotransferase test (AST = 38–69.3 Ul/L*)*. A remarkable modulation was observed in catalase (CAT)

and superoxide dismutase (SOD) activities in the liver, as CAT and SOD values were lower with the complete FO substitution with CO (0F6C), and the highest values were observed in the control and (4F2C). This study indicates that red seabream may have the ability to maintain LC-PUFAs between tissues and diets, and CO substitution of FO could improve both lipid metabolism and oxidation resistance as well as maintain digestibility. In conclusion, dietary FO can be replaced up to 100% or 95% by CO in the diets of red seabream as long as n-3 HUFA, EPA, and DHA are incorporated at the recommended level

3.0. Introduction

Highly unsaturated fatty acids (HUFAs), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are certified components of human health with neurological, immunomodulatory, pathological, cardiovascular, and carcinogenic benefits (Fenton et al ., 2013). Fish are a major source of EPA and DHA (Emery et al., 2016) Globally, aquaculture is grappling with a low supply of fish oil (FO) as the main lipid source for fish feed (Turchini et al., 2009; Dawood et al., 2021a). To augment FO fluctuations, the search for an alternative lipid source in aqua feeds has seen terrestrial vegetable oils at the epicenter of various flagship aqua-feed research (Dawood et al., 2021b).

Dietary de novo oilseed *Camelina sativa* (L. Crantz) is an ancient crop that originated in Germany around 600 B.C. and is cultivated traditionally in central Europe as an oil crop (Betancor et al., 2015). Camelina production plummeted in the Middle Ages (5th to 15th centuries), but still evolved as a weed with flax, and has in modern days been coined the "false flax." Camelina is a member of the Cruciferae (Brassicaceae) family, together with mustards, rapes, canola, radish, turnip, broccoli, cabbage, collards, cauliflower, rutabaga,

Brussels sprouts, kohlrabi, and many weeds (Burdin et al.,1995) .Utilization of camelina oil (CO) has been reported in various carnivorous fish species; up to 100 % of added dietary FO was replaced by CO without adverse effects on growth performance, nutrient utilization, and proximate carcass composition of rainbow trout (*Oncorhynchus mykiss*) (Lu et al ., 2020). In addition, Hyuben et al. (2020) demonstrated that up to 40% of FO can be replaced with CO without negative effects on growth performance, fillet fatty acid profile and gut microbiome of gilthead sea bream (*Sparus aurata*).

Elsewhere, supplementation of genetically modified (GM) camelina oil diets of European sea bass (*Dicentrarchus labrax* L.) triggered a metabolic up regulation of both β-oxidation (cpt1a) and fatty acid transport (fabp1) (Betancor et al., 2021). The authors opinioned that GM camelina oil is an excellent source of EPA and DHA and thus an ideal substitute for FO in diets of marine carnivorous species, contributing to bridging the gap between supply and demand for n-3 LC PUFA while also maintaining or increasing tissue n-3 LC PUFA contents (Betancor et al., 2021).

Novel camelina oil (CO) contain high contents of 18:2 n-6 (linoleic acid), 18:1 n-9 (oleic acid) omega 3 α-linolenic acid (ALA, C18:3n-3), which is an essential fatty acid for fish (Hixson et al.,2013; Hixson et al., 2014a; Hixson et al.,2014b; Betancor et al., 2016). Previous studies have shown that FO substitution by appropriate proportions of CO in the feed maintains fish growth response as well as fish health (Hixson et al., 2014a). Total replacement of FO with camelina oil in diets for tilapia could be a suitable alternative for culture, since the growth performance of fish fed total camelina oil diets was not affected compared to a total FO diet, as well as a typical commercial diet. Fatty acid concentrations were significantly modified after 8 weeks of trial, and although camelina oil not enriched tilapia fillet with EPA + DHA at the level of the FO, it efficiently maintained an n-3/n-6

ratio within the recommendation for the prevention of cardiovascular diseases (Toyes-Vargas et al., 2020). Camelina oil is less susceptible to oxidative stress because it contains a high amount of γ-tocopherol, the most potent antioxidant tocopherol isomer (Ni Edhin et al., 2003).

Red seabream (*Pagrus major*), an exclusively carnivorous marine fish, is commonly cultured in Japan and other countries (Dawood et al.,2016a; Dawood et al.,2016b). Several works have been performed on the impact of dietary vegetable oil and algal lipid source feeding on red seabream growth responses and fatty acids (Seong et al., 2019;Seong et al., 2020; Komilus et al., 2008). Studies have also reported the effects of vegetable and algal lipid supplementation in diets of red seabream on blood function, immunity, growth response, oxidative status, and nutrient digestibility, but effects of dietary oils on tissue histology of red seabream are limited and sporadic. In fact, research on the utilization of CO in red seabream diets is limited. The objective of our study was to determine the effect of FO replacement with CO on the growth performance, fatty acid profile, blood health, fatty acid digestibility, histology of the liver, and oxidative status of red seabream.

3.1. Materials and Methods

3.1.1. Experimental Diets

Five experimental diets had homogeneous nutrient contents of energy (2590 kJ/g), crude protein (506.6 g/kg), and lipids (143 g/kg) formulated with different ratios of FO and CO. The control diet (FM: 46%) (6F0C) contained FO without CO, and the other four experimental diets were formulated by gradually substituting FO with CO at rates of 5:1 (5F1C), 4:2 (4F2C), 3:3 (3F3C), 2:4 (2F4C), and 0:6 (0F6C) (Tables 1 and 2). The main

sources of protein in the feed were fishmeal and soybean meal. A blend of soybean lecithin, FO, CO, DHA, and EPA were used as lipid sources. Dextrin was used as a carbohydrate source. Activated gluten was added to the mixture as a binder to improve pellet cohesion and avoid pellet leaching. Dried ingredients were ground, sieved through

a uniform mesh to maintain a homogenous size, and mixed in a food mixer for 15 min. The liquid form ingredients were homogenized in a sonicator (CA4455Z, Kaijo us Corporation, Tokyo, Japan) before mixing with dry ingredients. Water was added to the feed ingredients to form a dough that was pelleted (1.2–2.2 mm in diameter) using a mincer (ROYAL Inc., Tokyo, Japan). Feed pellets were dried at 60 °C for 120 min, and the dried pellets were stored in plastic bags at –20 °C until use.

Table 12. Experimental diets formulation and proximate composition

Ingredient, g/kg DM	**Test Diets**				
	6F0C	**5F1C**	**4F2C**	**2F4C**	**0F6C**
Brown fish meal [a]	460	460	460	460	460
Soybean meal [b]	205	205	205	205	205
Fish oil [c]	60	50	40	20	-
Camelina oil [e]	-	10	20	40	60
Soybean Lecithin [d]	30	30	30	30	30
EPA [f]	50	50	50	50	50
DHA [g]	50	50	50	50	50
Dextrin [h]	50	50	50	50	50
Activated gluten [i]	80	80	80	80	80
Mineral mix [j]	40	40	40	40	40
Vitamin mix [k]	40	40	40	40	40
Stay C [l]	0.8	0.8	0.8	0.8	0.8
α-Cellulose [m]	24.5	24.5	24.5	24.5	24.5
Proximate composition					
Crude Protein	498 ± 3	513 ± 4	508 ± 5	506 ± 0	508 ± 9
Crude Lipid	150 ± 9	144 ± 8	143 ± 13	135 ± 2	144 ± 10
Moisture	101 ± 1	93 ± 1	100 ± 4	90 ± 00	91 ± 5
Ash	101 ± 1	106 ± 00	101 ± 3	104 ± 3	103 ± 5
Energy (kJ/g) [n]	2580 ± 6	2612 ± 14	2603 ± 14	2590 ± 4	2591 ± 2

[a] Defatted brown fish meal. [b] J-OIL MILLS, Inc, Tokyo, Japan [c] Riken Vitamin, Tokyo, Japan. [d] Kanto Chemical Co., Inc. Tokyo. [e] Biopure oil, Box 194, Fort Qu Appele, SK SOG ISO. [f] Riken Vitamin, Tokyo, Japan. [g] Riken Vitamin, Tokyo, Japan. [h, i] Glico Nutrition Company Ltd. Osaka, Japan. Commercial name "A-glu SS. [j] Mineral mixture (mg/kg diet): $MgSO_4$ (5.07), Na_2HPO_4 (3.23), K_2HPO_4 (8.87), Fe citrate (1.1), Ca lactate (12.09), $Al(OH)_3$ (0.01), $ZnSO_4$ (0.13), $MnSO_4$ (0.03), $Ca(IO_3)_2$ (0.01), and $CoSO_4$ (0.04). [k] Vitamin mixture (mg/kg diet): β-carotene (0.10), vitamin D_3 (0.01), menadione $NaHSO_3 \cdot 3H_2O$ (K_3) (0.05), dl-α-tocopherol acetate (E) (0.38), thiamine-nitrate (B_1) (0.06), riboflavin (B_2) (0.19), pyridoxine-HCl (B_6) (0.05), cyanocobalamin (B_{12}) (0.0001), biotin (0.01), inositol (3.85), niacin (nicotinic acid) (0.77), Ca pantothenate (0.27), folic acid (0.01), choline chloride (7.87), p-amino benzoic acid (0.38), and cellulose (1.92). [l] Stay-C: ascorbyl-2-monophosphate-Na/Ca (DSM Nutrition Japan K. K.). [m] Nippon Paper Chemicals, Tokyo, Japan. [n] Calculated using combustion values for protein, lipid, and carbohydrate of 23.6, 39.5, and 17.2 kJ/g.

Table 13: Fatty acid composition (mg/g lipids) in experimental diets.

Fatty Acid Type	Test Diet				
	6F0C	**5F1C**	**4F2C**	**2F4C**	**0F6C**
14:0	14.0 ± 1.8	13.8 ± 0.6	14.3 ± 0.3	11.2 ± 0.2	7.2 ± 0.2
16:0	51.1 ± 0.9	64.7 ± 0.6	86.0 ± 3.0	95.8 ± 50.0	114.0 ± 14.5
18:0	82.0 ± 2.0	35.0 ± 0.7	43.9 ± 3.9	37.4 ± 0.5	23.8 ± 4.3
∑Saturated	147.1 ± 3.6	132.5 ± 1.4	144.2 ± 2.1	144.4 ± 5.4	145 ± 4.1
16:1n-9	55 ± 1.8	50.8 ± 0.6	54.2 ± 0.7	32.9 ± 1.5	31.1 ± 0.1
18:1n-5	1.7 ± 0.5	1.1 ± 0.9	1.7 ± 0.1	2.7 ± 0.2	2.2 ± 0.6
18:1n-9	64.3 ± 1.2	86.1 ± 3.1	120.4 ± 2.0	137.1 ± 1.3	155.2 ± 2.6
20:1n-9	20.8 ± 4.0	17.0 ± 3.5	20.5 ± 1.3	11.0 ± 0.1	9.5 ± 0.3
22:1n-9	33.5 ± 3.5	17.8 ± 0.2	8.9 ± 0.4	7.9 ± 1.6	2.7 ± 1.5
∑MUFA	175.5 ± 0.9	172.8 ± 5.8	205.7 ± 4.1	191.6 ± 6.5	200.7 ± 2.8
18:2n-6	41.1 ± 3.5[a]	52.7 ± 4.1	72.5 ± 3.6	84.8 ± 0.	103.4 ± 11.0
18:3n-6	6.4 ± 0.2	2.2 ± 0.1	1.6 ± 0.2	1.3 ± 0.1	0.4 ± 0.0
20:4n-6	8.2 ± 6.2	7.8 ± 4.6	6.4 ± 0.4	3.8 ± 0.2	2.7 ± 0.5
22:4n-6	2.1 ± 0.2	1.7 ± 0.1	1.4 ± 0.1	0.5 ± 0.0	0.0 ± 0.0
∑n-6 fatty acids	57.8 ± 8.3	64.4 ± 2.5	81.9 ± 7.6	90.4 ± 1.6	106.5 ± 1.6
18:3n-3	8.7 ± 1.6	10.0 ± 0.0	10.7 ± 0.7	12.3 ± 0.1	14.1 ± 8.8
18:4n-3	6.5 ± 0.2	3.1 ± 0.1	2.2 ± 0.1	1.8 ± 0.3	0.0 ± 0.0
20:3n-3	2.4 ± 0.5	1.4 ± 0.1	1.2 ± 0.1	0.5 ± 0.5	0.2 ± 0.2
20:4n-3	10.5 ± 0.2	6.5 ± 0.1	3.3 ± 0.3	3.5 ± 0.1	1.5 ± 0.4
20:5n-3	58.1 ± 20.2	57.0 ± 20.0	31.0 ± 2.0	21.3 ± 1.8	17.1 ± 1.8
22:5n-3	18.5 ± 4.5	14.2 ± 1.0	10.2 ± 0.7	9.0 ± 1.9	7.3 ± 2.0
22:6n-3	144.9 ± 23.9	129.0 ± 30.0	115.7 ± 11.1	101.0 ± 1.5	107.5 ± 2.5
∑n-3 fatty acids	249.6 ± 0.2	221.2 ± 2.6	174.3 ± 6.1	149.4 ± 2.3	146.5 ± 0.5
∑PUFA	307.4 ± 12.4	285.6 ± 21.0	256.2 ± 19.2	239.8 ± 21.7	253. ± 46.2
∑-3HUFA	234.4 ± 1.7	208.1 ± 16.2	161.4 ± 8.7	139.3 ± 0.4	133.6 ± 0.4
∑n-3/n-6 ratio	4.3	3.4	2.1	1.7	1.4
∑EPA+DHA	203 ± 21.1	186.1 ± 11.4	146.7 ± 2.1	122.3 ± 2.5	124.6 ± 1.2

Values are expressed as mean ± standard error (n = 2). Absence of superscript letters refers to non-significant differences between treatments ($p > 0.05$). Total PUFA is expressed as the sum of total n-3 fatty acids and total n-6 fatty acids. Total n-3HUFA is expressed as the sum of n-3 fatty acids in carbons of more than 20. The n-3:n-6 ratio is expressed as total n-3 PUFA divided by total n-6 PUFA. The sum of

eicosapentaenoic acid (EPA; C20:5n-3) and docosahexaenoic acid (DHA; C22:6n-3) are essential fatty acids.

3.1.2. Husbandry

Red seabream juveniles were procured and transported from Tawaki Suisan Ltd., Kumamoto Prefecture, Japan, to Kamoike Marine Production Laboratory, Faculty of Fisheries, Kagoshima University, Japan. Fish were stocked indoors in 500 L polyethylene tanks for a week to acclimatize to laboratory conditions while they were fed a commercial diet (45% crude protein; Higashimaru Foods Ltd., Kagoshima, Japan). At the onset of the feeding trial, 20 fish (1.8 g ± 0.02) per tank (three tanks per treatment) were randomly stocked into fifteen polyethylene tanks with 100 L capacity (filled with 80 L of water) in a flow-through seawater system (2.5 L/min) and continuous aeration under a 12 h light/12 h dark photoperiod regime. Water parameters of rearing tanks through the experimental period were 26.4 ± 1.2 °C, 32.1 ± 0.5 g/L of salinity, 6.31 ± 0.1 mg/L of dissolved oxygen, and 7.4 of pH 70% of the water in culturing tanks was exchanged with new sea water every day to maintain favorable conditions for growth and survival of cultured fish. Fish were manually fed twice daily (08:00 and 16:00 h) until apparent satiation and uneaten diets were collected, dried, and weighted to determine the actual feed intake.

3.1.2.1. Performance Variables and Biometric Indices

At the end of the feeding trial, the fish weight and length were measured individually. Growth indices, feed utilization, and survival rates were calculated using the following formulae:

$$\text{Weight Gain (WG \%)} = \frac{W_{56d} - W_{0d}}{W_{0d}} \times 100 \qquad (1)$$

$$\text{Specific Growth Rate (SGR \% /day)} = \frac{\text{Ln } W_{56d} - \text{Ln } W_{0d}}{T} \times 100 \quad (2)$$

$$\text{Feed Intake (FI, g /fish/ 56 d)} = \frac{\text{Dry diet given - Dry uneaten diet recovered}}{\text{No. of fish}} \quad (3)$$

$$\text{Feed Conversion Ratio (FCR)} = \frac{\text{FI (g)}}{\text{WG (g)}} \quad (4)$$

$$\text{Survival rate (SR \%)} = \frac{N_{56d}}{N_{0d}} \times 100 \quad (5)$$

$$\text{Condition factor (CF)} = \frac{W}{L^3} \times 100 \quad (6)$$

where W56d = final body weight at 56 days, W0d = initial body weight, T = the experimental period in days (d), N0d = initial

number of fish, N56d = final number of fish, W = total fish weight (g), and L = total fish length (cm).

Nine fish were collected randomly per treatment, anesthetized (2-phenoxyethanol, 200 µl/L) and the liver and viscera were eviscerated on the ice surface for hepatosomatic (HSI) and viscerasomatic (VSI) indices. Portions of the collected liver were used for histological studies and hepatic antioxidant analysis.

$$\text{HSI} = \frac{\text{Liver weight, g}}{\text{Fish body weight, g}} \times 100 \quad (7)$$

$$\text{VSI} = \frac{\text{Viscera weight, g}}{\text{Fish body weight, g}} \times 100 \quad (8)$$

3.1.3.

Samples of feed and fish (four fish per tank) were used for proximate composition determination according to standard procedures (AOAC, 2012). Briefly, moisture content was obtained after drying in an oven at 135°C for 5 h. Ash was determined after incineration at 550°C for 6 h. The crude protein content was obtained by determining the nitrogen content (N × 6.25) using automated Kjeldahl analysis (Tecator Kjeltec Auto 2100 analyzer, Foss, Sweden). Crude lipids were gravimetrically determined using a Soxhlet apparatus.

$$\text{Dry matter digestibility} = 100 - (\text{cholestane in diet} / \text{cholestane in faeces}) \times 100 \quad (9)$$

$$\text{Nutrient digestibility} = 100 - ((\text{cholestane in diet} / \text{cholestane in faeces}) \times (\text{nutrient in diet} / \text{cholestane in diet}) \times 100) \quad (10)$$

Total lipids were extracted using a chloroform methanol solution and measured by gravimetry after nitrogen drying. To determine the fatty acid composition of total lipids, fatty acid methyl esters (FAMES) in samples were prepared by transesterification using boron trifluoride in methanol and dichloromethane (Ackman,1998). To determine the fatty acid composition in diets and tissues, FAMEs were identified by comparison of the equivalent chain length (ECL) value and quantified standards (C23:0 methyl esters) determined by a Shimadzu GC 2010 equipped with a flame ionization detector (Supelco, Inc., Japan), and the chromatogram peak areas of total lipids, 5α-cholestane, and fatty acids in the feed were compared directly to those of total lipid, 5α-cholestane and fatty acids in the feces of fish. Digestibility was calculated using the equation described by Sigurgisladottir, et al. (1992).

3.1.4. Blood Hematological Parameters

Heparinized disposable syringes (1600 IU/mL) were used to collect blood from 5 fish/tank. The hematocrit was determined using the micro-hematocrit technique. Then, plasma was obtained by centrifuging the blood samples at 3000g for 15 min under 4 °C, and then stored at −20 °C until analyses. Glucose (Glu), total cholesterol (T-Chol), blood urea nitrogen (BUN), total bilirubin (T-Bil), alanine aminotransferase test (ALT) aspartate aminotransferase test (AST), total protein (T-pro), and triglyceride (TG) levels

were measured using an automated analyzer (SPOTCHEM EZ model SP-4430, Arkray, Inc. Kyoto, Japan)

3.1.5. Antioxidants Activity

First, liver and muscle samples were homogenized in cold iced 0.86% NaCl solution and centrifuged at 4°C and 12,000 rpm for 10 min. The supernatant of liver samples and blood plasma were determined using a microplate reader (Multiskan GO; Thermo Fisher Scientific, K. K., Tokyo Japan). SOD activity was determined using the Kit-WST assay (Dojindo Molecular Technologies, Inc.) at 450 nm. The catalase activity (CAT) assay was performed using spectrophotometric determination of hydrogen peroxide (H_2O_2) which forms a stable complex with ammonium molybdate that absorbs at 405 nm.

3.1.6. Hepatic Histopathological Assessment

Liver samples were cut into small pieces and immersed in Bouin solution for 12 h. The fixed tissues were processed routinely in alcohol and rinsed every 24 h until clear. Tissues were embedded in paraffin blocks, sectioned, deparaffinized, and rehydrated using standard techniques. Sagittal sections (5 µm thickness) were obtained using a rotary microtome (RM 2135, Leica, Nussloch, Germany), placed on glass slides, rehydrated, and stained with hematoxylin and eosin. Finally, the slide was permanently mounted (Entellan, EMD Millipore, Billerica, MA, USA) and examined under a light microscope (BX41, Olympus, Tokyo, Japan).

3.1.6. Statistical Analysis

Data on all parameters were pooled and subjected to verification for normality and homogeneity of variances using Shapiro–Wilk and Levene tests, respectively. One-way analysis of variance (ANOVA) was performed on all data. Significantly different mean data groups were located using Fisher's least significant differences (LSD) test. All statistical analyses were performed using Super ANOVA 1.11, Abacus Concepts, Berkeley, California, USA. Data sets are presented as mean ± standard error of the mean (SEM, n).

3.2. Results

3.2.1. Growth Performance Variables

Table 3 shows the growth, feed utilization, biometric indices, and survival rates of red seabream fed the experimental diets for 56 days. The average initial body weight of red seabream juveniles was 1.8 g. After the 8-week feeding trial, no significant differences were observed in body weight (BW = 26.2–28.3 g), body weight gain (BWG = 1275.5–1365.3%), specific growth rate (SGR = 4.6–4.7), feed intake (FI = 25.6–27.8), feed conversion ratio (FCR = 1.0–1.1), condition factor (CF = 2.2–2.4), hepatosomatic index (HSI = 0.9–1.1), viscerasomatic index (VSI = 7.5–9.5), and survival rate (SR = 82.2–100) of fish fed with different FO substitution levels with CO.

Table 14: Performance variables and biometric indices of red seabream (*Pagrus major*) fed the test diets for 56 days.

Parameters	Test Diets				
	6F0C	5F1C	4F2C	2F4C	0F6C
BW_0 (g/fish)	1.8	1.9	1.8	1.9	1.9
BW_{56d} (g/fish)	26.2 ± 0.1	27.1 ± 0.2	27.3 ± 0.6	28.3 ± 0.1	26.5 ± 0.1
BWG (%)	1306.2 ± 47.9	1326.3 ± 6.1	1364.4 ± 27.2	1365.3 ± 49.1	1275.5 ± 25.4
SGR	4.7 ± 0.1	4.7 ± 0.0	4.7 ± 0.0	4.7 ± 0.1	4.6 ± 0.0
FI	25.6 ± 0.2	26.7 ± 0.4	27.8 ± 1.2	27.0 ± 0.8	27.2 ± 1.1
FCR	1.1 ± 0.0	1.1 ± 0.0	1.1 ± 0.1	1.0 ± 0.0	1.1 ± 0.0
HSI	1.1 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	1.1 ± 0.4	1.1 ± 0.1
VSI	9.1 ± 0.7	9.5 ± 0.3	7.5 ± 0.6	8.5 ± 0.7	8.9 ± 0.7
CF	2.3 ± 0.0	2.3 ± 0.1	2.4 ± 0.1	2.2 ± 0.0	2.5 ± 0.2
SR	82.2 ± 5.8	97.7 ± 2.2	88.8 ± 8.0	100 ± 0.0	86.6 ± 6.62

Values are expressed as the mean ± standard error (n = 3).BW_{56d} = body weight at 56 day; BWG = body weight gain; SGR = specific growth rate; FI = feed intake; FCR = feed conversion ratio; HSI = hepatosomatic index; VSI = viscerasomatic index; CF = condition factor; SR = survival rate (%).

3.2.2. Proximate Composition of Fish Whole Body

Table 4 shows the proximate composition of the red seabream whole body after 56 d of the feeding period. There was no significant alteration in the composition of the red seabream body, except for the crude lipid content. The basal diet (6F0C = free of CO) showed the highest lipid content (291 g/kg) compared to the other groups 5F1C *(*232 k/kg), 4F2C (212 g/kg), 2F4C (232 g/kg) and 0F6C (244k g/kg).

Table 15: Carcass proximate analysis (g/kg, dry matter basis) of red seabream (*Pagrus major*) fed the experimental diets for 56-days.

Parameter	Test Diets				
	6F0C	5F1C	4F2C	2F4C	0F6C
Moisture	704 ± 4.8	694 ± 4.5	702 ± 11.8	697 ± 6.9	700 ± 4.7
Crude Protein	546 ± 6	512 ± 00	544 ± 45	539 ± 4	531 ± 2
Crude Lipid	291 ± 3 ^{a}	232 ± 1 ^{b}	212 ± 4 ^{bc}	232 ± 1 ^{b}	244 ± 9 ^{b}
Ash	157 ± 2	154 ± 3	161 ± 2	155 ± 1	155 ± 1

Values are expressed as the mean ± standard error (n = 3). Absence of superscript letters refers to non-significant differences between treatments ($p > 0.05$) and presence of different superscript letters refers to significant differences between treatments ($p < 0.05$).

Table 16: Fatty acid composition (mg/g lipid) in the liver of red seabream (*Pagrus major*) fed the experimental diets 56-days.

Fatty Acid Type	Test Diet				
	6F0C	5F1C	4F2C	2F4C	0F6C
14:0	27.1 ± 0.3	29.1 ± 0.2	33.5 ± 0.6	32.0 ± 0.1	26.1 ± 0.2
16:0	123.2 ± 0.1^{a}	109.5 ± 0.1^{a}	102.5 ± 0.7^{b}	96.0 ± 0.3^{b}	85.9 ± 0.1^{b}
18:0	67.5 ± 0.0^{a}	57.5 ± 0.2^{a}	46.1 ± 0.6^{ab}	36.0 ± 0.6^{bc}	26.3 ± 0.5^{bc}
∑Saturated	217.8 ± 0.0^{a}	195.6 ± 0.3^{a}	182.1 ± 0.3^{ab}	164.0 ± 0.9^{b}	138.3 ± 0.1^{bc}
16:1n-9	61.5 ± 0.5^{a}	44.8 ± 0.2^{a}	37.7 ± 0.9^{a}	32.9 ± 0.7^{ab}	27.0 ± 0.7^{b}
18:1n-5	4.9 ± 0.2^{a}	2.0 ± 0.1^{a}	3.0 ± 0.1^{a}	1.7 ± 0.01^{b}	0.0 ± 0.7^{c}
18:1n-9	70.1 ± 0.01^{a}	98.3 ± 0.5^{b}	124.6 ± 0.8^{c}	148.4 ± 0.1^{c}	168.6 ± 1.0^{c}
20:1n-9	51.0 ± 0.2^{a}	44.2 ± 0.2^{a}	36.8 ± 0.5^{b}	37.5 ± 0.0^{b}	31.2 ± 2.1^{c}
22:1n-9	34.5 ± 0.3^{a}	31.0 ± 0.01^{a}	23.0 ± 0.0^{a}	20.6 ± 0.5^{ab}	13.6 ± 0.1^{b}
∑MUFA	222.0 ± 0.6	219.5 ± 0.1	223.1 ± 0.2	241.1 ± 0.6	240.2 ± 2.1
18:2n-6	84.0 ± 0.1	86.1 ± 0.2	99.2 ± 2.7	108.9 ± 1.5	113.2 ± 4.6
18:3n-6	5.8 ± 0.2^{a}	3.7 ± 0.1^{a}	3.1 ± 0.2^{a}	2.5 ± 0.8^{ab}	1.5 ± 0.0^{b}
20:4n-6	9.6 ± 0.5^{a}	5.3 ± 0.6^{ab}	3.6 ± 0.0^{b}	3.0 ± 0.0^{b}	3.2 ± 0.0^{b}
∑n-6 fatty acids	99.6 ± 0.2	95.1 ± 0.0	105.3 ± 0.2	114.4 ± 1.3	117.7 ± 3.2
18:3n-3	15.5 ± 0.4^{a}	18.6 ± 0.4^{ab}	20.2 ± 0.6^{b}	25.1 ± 2.1^{bc}	27.9 ± 0.0^{bc}
18:4n-3	12.5 ± 0.1^{a}	9.1 ± 0.2^{ab}	9.6 ± 0.2^{ab}	7.9 ± 0.0^{b}	3.9 ± 0.0^{c}
20:3n-3	7.4 ± 0.3^{a}	3.6 ± 0.1^{b}	1.7 ± 0.0^{bc}	0.6 ± 0.0^{c}	0.9 ± 0.4^{c}
20:4n-3	6.3 ± 0.5^{a}	3.7 ± 0.02^{b}	2.3 ± 0.01^{b}	2.6 ± 0.00^{b}	0.0 ± 0.0^{c}
20:5n-3	80.5 ± 1.6^{a}	59.6 ± 0.2^{b}	55.7 ± 0.3^{b}	47.4 ± 0.8^{b}	52.5 ± 2.4^{b}
22:5n-3	23.0 ± 0.6^{a}	14.5.0 ± 0.2^{b}	7.1 ± 0.5^{c}	6.7 ± 2.0^{c}	6.5 ± 0.1^{c}
22:6n-3	159.0 ± 0.1	145 ± 0.8	142.2 ± 0.5	128.4 ± 0.7	120.2 ± 0.0
∑n-3 fatty acids	304.2 ± 2.6^{a}	254.9 ± 2.9^{ab}	238.8 ± 0.0^{ab}	218.7 ± 0.9^{ab}	211.9 ± 2.1^{ab}
∑PUFA[1]	403.8 ± 0.9	350 ± 5.3	344.1 ± 0.0	333.1 ± 1.1	329.6 ± 0
∑-3HUFA[2]	276.2 ± 5.9	226.4 ± 2.5	209.0 ± 2.9	185.7 ± 4.30	180.1 ± 5.2
∑n-3/n-6 ratio[3]	3.1	2.7	2.3	2.0	1.8
∑EPA + DHA[4]	239.5± 0.1^{a}	204.6 ± 0.0^{a}	197.9 ± 0.0^{a}	175.8 ± 0.0^{ab}	172.7 ± 0.3^{ab}

Values are expressed as mean ± standard error (n = 2). Absence of superscript letters refers to non-significant differences between treatments ($p > 0.05$) and presence of different superscript letters refers to significant differences between treatments ($p < 0.05$). [1] Total PUFA is expressed as the sum of total n-3

fatty acids and total n-6 fatty acids. [2] Total n-3HUFA is expressed as the sum of n-3 fatty acids in carbons of more than 20. [3] The n-3: n-6 ratio is expressed as total n-3 PUFA divided by total n-6 PUFA. [4] The sum of eicosapentaenoic acid (C20:5n-3) and docosahexaenoic acid (C22: 6n-3) are essential fatty acids.

Table 16: Fatty acid composition (mg/g lipid) in the muscle of red seabream (*Pagrus major*) fed the experimental diets for 56-days.

Fatty Acid Type	Test Diet				
	6F0C	5F1C	4F2C	2F4C	0F6C
14:0	26.8 ± 0.1^{a}	17.1 ± 0.2^{a}	12.4 ± 0.2^{ab}	11.6 ± 0.4^{abc}	8.6 ± 0.1^{c}
16:0	73.0 ± 0.2	75.2 ± 0.1	82.4 ± 0.7	99.6 ± 0.3	108.2 ± 0.1
18:0	96.3 ± 0.0^{a}	53.2 ± 0.2^{ab}	45.5 ± 0.6^{ab}	30.1 ± 0.6^{bc}	27.9 ± 0.5^{bc}
∑Saturated	196.1 ± 5.6	145.5 ± 0.3	140.3 ± 0.3	141.3.4 ± 0.9	144.7 ± 0.1
16:1n-9	83.6 ± 0.4^{a}	65.7 ± 0.2^{ab}	44.0 ± 0.9^{ab}	31.4 ± 0.7^{ab}	29.9 ± 0.5^{b}
18:1n-5	1.4 ± 0.2	0.2 ± 0.1	0.2 ± 0.1	- ± -	- ± -
18:1n-9	76.6 ± 2.5^{a}	101.3 ± 0.5^{a}	126.2 ± 0.8^{ab}	145.8 ± 0.1^{ab}	164.2 ± 1.0^{b}
20:1n-9	21.2 ± 0.0^{a}	13.5 ± 0.2^{b}	14.2 ± 0.5^{b}	13.6 ± 0.0^{b}	11.0 ± 1.2^{b}
22:1n-9	35.5 ± 0.5	26.0 ± 0.01	23.5 ± 0.0	20.3 ± 0.5	20.9 ± 0.1
∑MUFA	218.3 ± 1.6	206.7 ± 0.1	208.1 ± 0.2	211.1 ± 0.6	226 ± 2.1
18:2n-6	63.2 ± 0.1^{a}	80.9 ± 0.2^{ab}	118.4 ± 2.7^{ab}	128.8 ± 1.5^{ab}	144.0 ± 1.3^{b}
18:3n-6	6.1 ± 0.2^{a}	1.7 ± 0.1^{b}	0.1 ± 0.2^{b}	0.9 ± 0.8^{b}	0.0 ± 0.0^{c}
20:4n-6	8.6 ± 0.5^{a}	6.2 ± 0.6^{a}	4.3 ± 0.0^{a}	1.0 ± 0.0^{b}	0.0 ± 0.0^{c}
∑n-6 fatty acids	77.9 ± 0.2	88.8 ± 2.1	122.8 ± 0.2	130.7 ± 1.3	144.0 ± 3.2
18:3n-3	10.6 ± 0	10.1 ± 0.4	12.4 ± 0.6	13.7 ± 2.1	14.7 ± 0.0
18:4n-3	4.1 ± 0.02^{a}	1.3 ± 0.2^{b}	1.2 ± 0.2^{b}	1.2 ± 0.0^{b}	1.2 ± 0.0^{b}
20:3n-3	4.5 ± 0.2^{a}	1.2 ± 0.1^{b}	0.6 ± 0.0^{c}	0.6 ± 0.0^{c}	1.1 ± 0^{b}
20:4n-3	5.3 ± 0.3^{a}	2.1 ± 0.02^{b}	2.2 ± 0.2^{b}	2.2 ± 0.0^{b}	0.0 ± 0.0^{c}
20:5n-3	64.4 ± 1.1^{a}	60.0 ± 1.6^{a}	24.0 ± 3.1^{b}	24.9 ± 0.8^{b}	19.8 ± 0.8^{b}
22:5n-3	4.0 ± 0.0^{a}	0.2 ± 0.2^{b}	0.1 ± 0.5^{b}	0.3 ± 0.03^{b}	0.1 ± 0.01^{b}
22:6n-3	147.2 ± 5.3	133.7 ± 2.8	123.3 ± 1.5	112.4 ± 7.0	100.0 ± 0.0
∑n-3 fatty acids	237.8 ± 2.6	209.6 ± 1.7	161.8 ± 3.0	152.3 ± 1.1	131.9 ± 3.1
∑PUFA[1]	315.7 ± 2.1	298.4 ± 5.3	284.6 ± 0.0	283.3 ± 1.1	275 ± 2.3
∑-3HUFA[2]	225.4 ± 0.5	197 ± 2.5	150.2 ± 2.9	140.4 ± 4.30	121.0 ± 5.2
∑n-3/n-6 ratio[3]	3.0	2.3	1.3	1.2	1.0
∑EPA + DHA[4]	211.6 ± 1.3^{a}	193.7 ± 0.0^{a}	147.3 ± 0.0^{a}	137.3 ± 0.0^{ab}	119.8 ± 0.3^{b}

Values are expressed as mean ± standard error (n = 2). Absence of superscript letters refers to non-significant differences between treatments ($p > 0.05$) and presence of different superscript letters refers to significant differences between treatments ($p < 0.05$). [1] Total PUFA is expressed as the sum of total n-3 fatty acids and total n-6 fatty acids. [2] Total n-3HUFA is expressed as the sum of n-3 fatty acids in carbons of more than 20. [3] The n-3: n-6 ratio is expressed as total n-3 PUFA divided by total n-6 PUFA. [4] The sum of eicosapentaenoic acid (C20:5n-3) and docosahexaenoic acid (C22: 6n-3) are essential fatty acids.

3.2.3. Apparent Nutrient Digestibility

Apparent digestibility of the different diets with varying CO inclusion in relation to chain length for red seabream is shown in Table 7. Generally, n-3 fatty acids (95–97%) exhibited higher apparent digestibility, and saturated fatty acids (SFA) showed lower

apparent digestibility percentages (88–93%). There was no significant difference *($p > 0.05$)* in the apparent digestibility of MUFA among the experimental groups. Fish fed the 5F1C diet showed lower digestibility values of saturated, n-6 (88.1%), and n-3 fatty acids (92.1%), and the highest values were in the 4F2C group (93.6% saturates, 97% MUFA; 97.7 %; n-6 and 99%; n-3).

Table 17: Apparent digestibility (%) of fatty acids.

Fatty Acid Type	Test Diet				
	6F0C	5F1C	4F2C	2F4C	0F6C
14:0	91.2 ± 3	92.5 ± 2	94.1 ± 6	96.0 ± 1	96.6 ± 2
16:0	94.3 ± 1	86.4 ± 1	91.7 ± 07	92.3 ± 3	90.7 ± 1
18:0	95.3 ± 0	85.1 ± 2	95.2 ± 6	88.6 ± 6	85.5 ± 5
∑Saturated	93.6 ± 0	88.0 ± 3	93.6 ± 3	92.3 ± 9	90.9 ± 1
16:1n-9	93.5 ± 1	93.7 ± 1	98.2 ± 1	96.9 ± 13	94.1 ± 0.1
18:1n-5	95.0 ± 2	97.5 ± 1	92.3 ± 1	97.7 ± 0.1	95.9 ± 7
18:1n-9	92.7 ± 0.1	89.6 ± 5	98.2 ± 8	96.8 ± 1	95.1 ± 1
20:1n-9	92.1 ± 2	92.1 ± 2	98.2 ± 5	96.1 ± 0	94.6 ± 21
22:1n-9	99.1 ± 2	99.4 ± 1	98.2 ± 2	95.3 ± 2	97.0 ± 1
∑MUFA	94.5 ± 6	94.5 ± 1	97.0 ± 2	96.6 ± 6	95.3 ± 21
18:2n-6	93.6 ± 3	84.4 ± 3	98.5 ± 6	97.3 ± 3	96.1 ± 1
18:3n-6	94.1 ± 2	97.5 ± 4	94.6 ± 0.1	97.7 ± 2	96.3 ± 0
20:4n-6	93.6 ± 5	83.0 ± 6	99.0 ± 0	97.0 ± 0	96.0 ± 00
22:4n-6	94.2 ± 0.1	95.5 ± 1.0	98.6 ± 0.1	97.6 ± 0.0	94.7 ± 0.2
∑n-6 fatty acids	93.9 ± 0.1	90.1 ± 2.1	97.7 ± 0.2	97.4 ± 2.6	95.8 ± 0.3
18:3n-3	95.1 ± 0.6	81.2 ± 0.9	99.2 ± 0.1	98.5 ± 4.1	97.9 ± 0.0
18:4n-3	95.5 ± 0.01	97.0 ± 0.2	99.4 ± 0.0	99.0 ± 0.0	97.1 ± 0.0
20:3n-3	95.3 ± 0.2	88.6 ± 0.8	99.4 ± 0.5	98.8 ± 2.0	96.7 ± 0.3
20:4n-3	95.1 ± 0.01	94.4 ± 0.1	98.6 ± 0.3	98.6 ± 0.1	97.4 ± 0.5
20:5n-3	95.7 ± 0.3	95.2 ± 0.5	98.4 ± 0.4	98.5 ± 0.1	97.8 ± 0.1
22:5n-3	98.7 ± 0.1	94.8 ± 0.7	99.8 ± 0.1	98.9 ± 0.3	97.2 ± 0.4
22:6n-3	94.6 ± 0.2	93.5 ± 1.5	99.6 ± 0.0	97.6 ± 0.9	96.6 ± 0.1
∑n-3 fatty acids	95.7 ± 0.01	92.1 ± 1.7	99.2 ± 0.0	98.6 ± 0.5	97.2 ± 0.4
∑PUFA[1]	94.8 ± 0.2	91.1 ± 3.0	98.4 ± 0.0	98.0 ± 0.2	96.5 ± 0.4
∑-3HUFA[2]	95.9 ± 0.4	93.3 ± 0.1	99.2 ± 0.04	98.5 ± 0.2	97.1 ± 0.1
∑n-3/n-6 ratio[3]	1.02	1.02	1.01	1.0	1.04
∑EPA + DHA[4]	95.2 ± 0.2	94.3 ± 0.1	99.0 ± 0.2	98.0 ± 0.1	97.2 ± 0.2

Values are expressed as mean ± standard error (n = 2). Absence of superscript letters refers to non-significant differences between treatments ($p > 0.05$). [1] Total PUFA is expressed as the sum of total n-3 fatty acids and total n-6 fatty acids. [2] Total n-3HUFA is expressed as the sum of n-3 fatty acids in carbons of more than 20. [3] The n-3: n-6 ratio is expressed as total n-3 PUFA divided by total n-6 PUFA. [4] The sum of eicosapentaenoic acid (C20:5n-3) and docosahexaenoic acid (C22: 6n-3) are essential fatty acids.

3.2.4. Blood Chemical Parameter

The hematocrit and plasma chemical parameter results are tabulated in Table 8. Substitution of FO with CO at increasing rates showed no significant differences ($p < 0.05$) in blood composition in terms of hematocrit (36–33%), glucose (Glu = 78.3–71.3 mg/dL), total protein (T-pro = 3.1–3.8 g/dL), total cholesterol (T-chol = 196.0–241 mg/dL), blood urea nitrogen (BUN = 9.0–14.6 mg/dL), total bilirubin (T-Bil = 0.4–0.5 mg/dL), triglyceride (TG= 393.3–497.6 mg/dL), alanine aminotransferase test (ALT = 50–65.5 Ul/L), aspartate aminotransferase test (AST= 38–69.3 Ul/L).

Table 18: Blood health of red seabream (*Pagrus major*) fed the experimental diets for 56-days.

Parameters	**Test Diets**				
	6F0C	5F1C	4F2C	2F4C	0F6C
Haematocrit (%)	36.0 ± 1.1	37.3 ± 3.1	36.3 ± 3.1	36.6 ± 1.2	33.0 ± 4.2
Glucose (mg/dL)	72.3 ± 4.0	71.3 ± 14.3	78.3 ± 10.5	72.3 ± 19.0	88 ± 5.5
Serum total protein (g/dL)	3.1 ± 0.1	3.4 ± 0.2	3.8 ± 0.4	3.3 ± 0.0	3.4 ± 0.2
Total Cholesterol (mg/dL)	213.6 ± 8.9	238.0 ± 24.2	241.6 ± 6.9	196.0 ± 8.0	229.6 ± 21.6
BUN (mg/dL)	11.3 ± 2.0	8.3 ± 2.0	13.0 ± 3.2	14.6 ± 1.2	9.0 ± 1.1
T-Bil (mg/dL)	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.0	0.5 ± 0.0	0.5 ± 0.0
Triglyceride (mg/dL)	393.3 ± 62.6	476.6 ± 23.3	437.6 ± 62.3	415.0 ± 58.0	497.6 ± 2.3
ALT (UI/L)	52.3 ± 11.3	65.5 ± 15.4	64.3 ± 7.6	50 ± 1.3	53.0 ± 3
AST (UI/L)	43.0 ± 10.5	44.0 ± 29.1	38.0 ± 4.8	42.3 ± 9.5	69.3 ± 16.3

Data represent the mean ± SEM (n = 3). Absence of letters indicates no significant difference between treatments ($p > 0.05$). Alanine aminotransferase test; ALT, aspartate aminotransferase test; AST, blood urea nitrogen; BUN, total bilirubin, T-Bil.

3.2.5. Antioxidants Capacity

Figures 1 and 2 show CAT and SOD activities in the liver, muscle, and plasma of red seabream after 56 days of the experimental period. No remarkable alterations in CAT and

SOD levels were observed, except in the liver. Replacing FO with CO in a ratio of 4:2 (4F2C) did not cause any difference in the values of the liver CAT and SOD, while a remarkable reduction occurred with the complete substitution of FO with CO (0F6C), and the highest values were observed in the control and (4F2C).

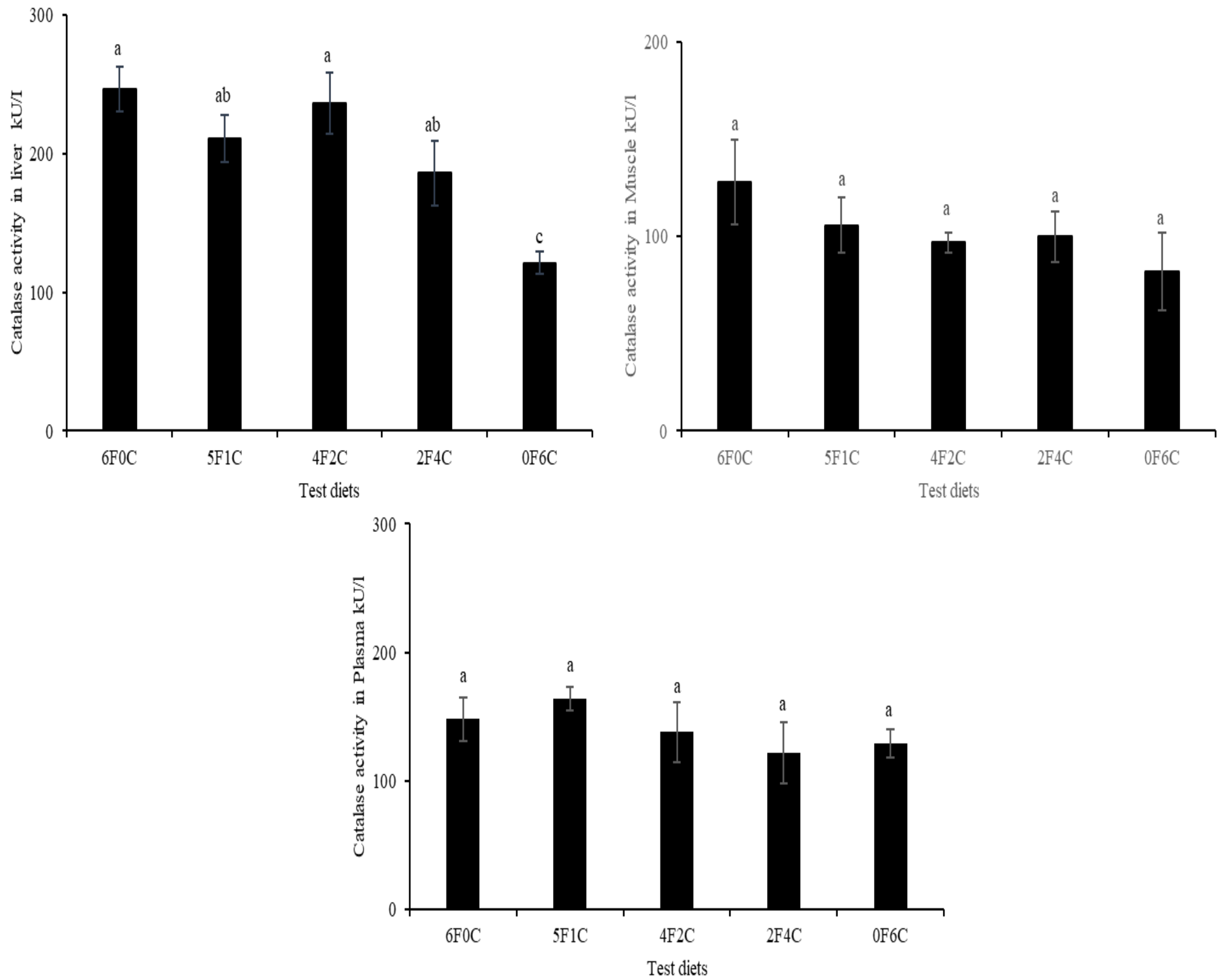


Figure 3: Catalase activities in liver, muscle, and plasma of red seabream after 56 days of the experimental period. Absence of superscript letters refers to non-significant differences between treatments ($p > 0.05$) and presence of different superscript letters refers to significant differences between treatments ($p < 0.05$).

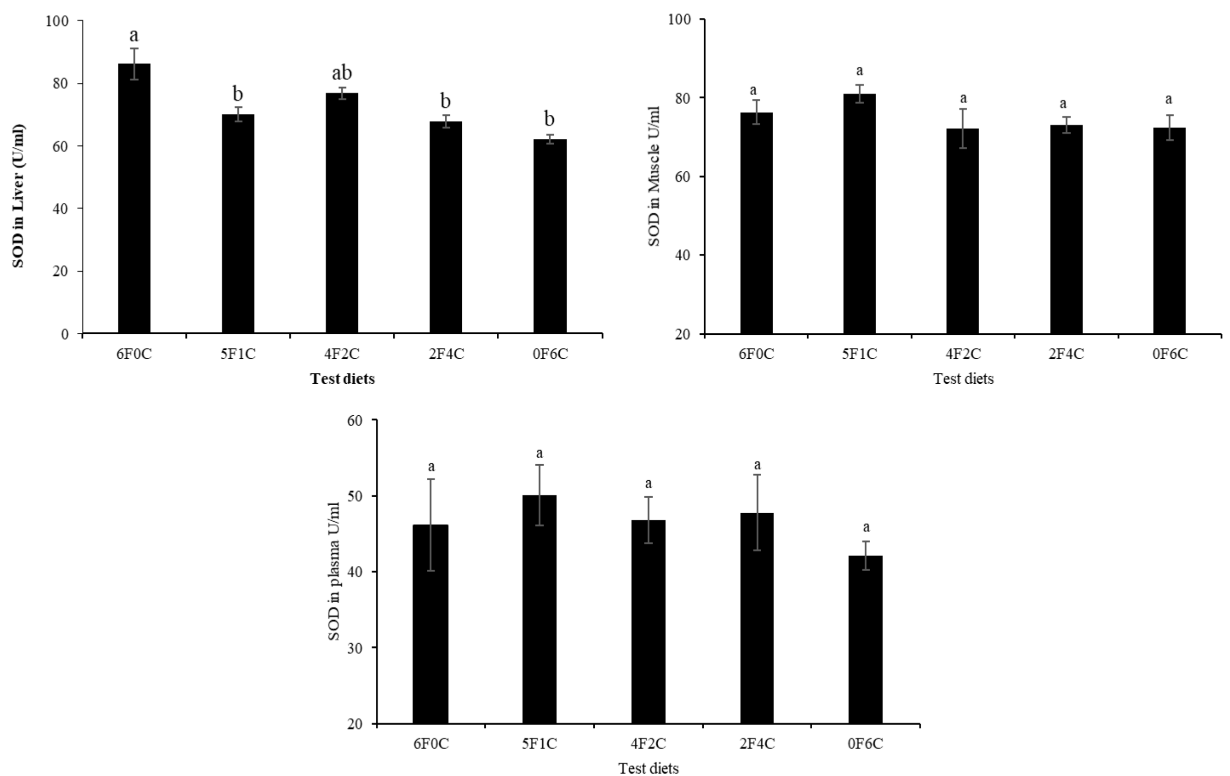


Figure 4: Superoxide dismutase (SOD) activities in liver, muscle, and plasma of red seabream after 56 days of the experimental period. Absence of superscript letters refers to non-significant differences between treatments ($p > 0.05$) and presence of different superscript letters refers to significant differences between treatments ($p < 0.05$).

3.2.6. Hepatic Histological Examination

Cross-sections of the liver of red seabream fed experimental diets for 56 days are shown in Figure 3. Small lipid droplets and lipid vacuoles of the hepatocytes were clear in fish groups fed on 6F0C and 2F4C, and more pronounced in the fish fed the 0F6C diet than in those fed the 5F1C and 4F2C diets.

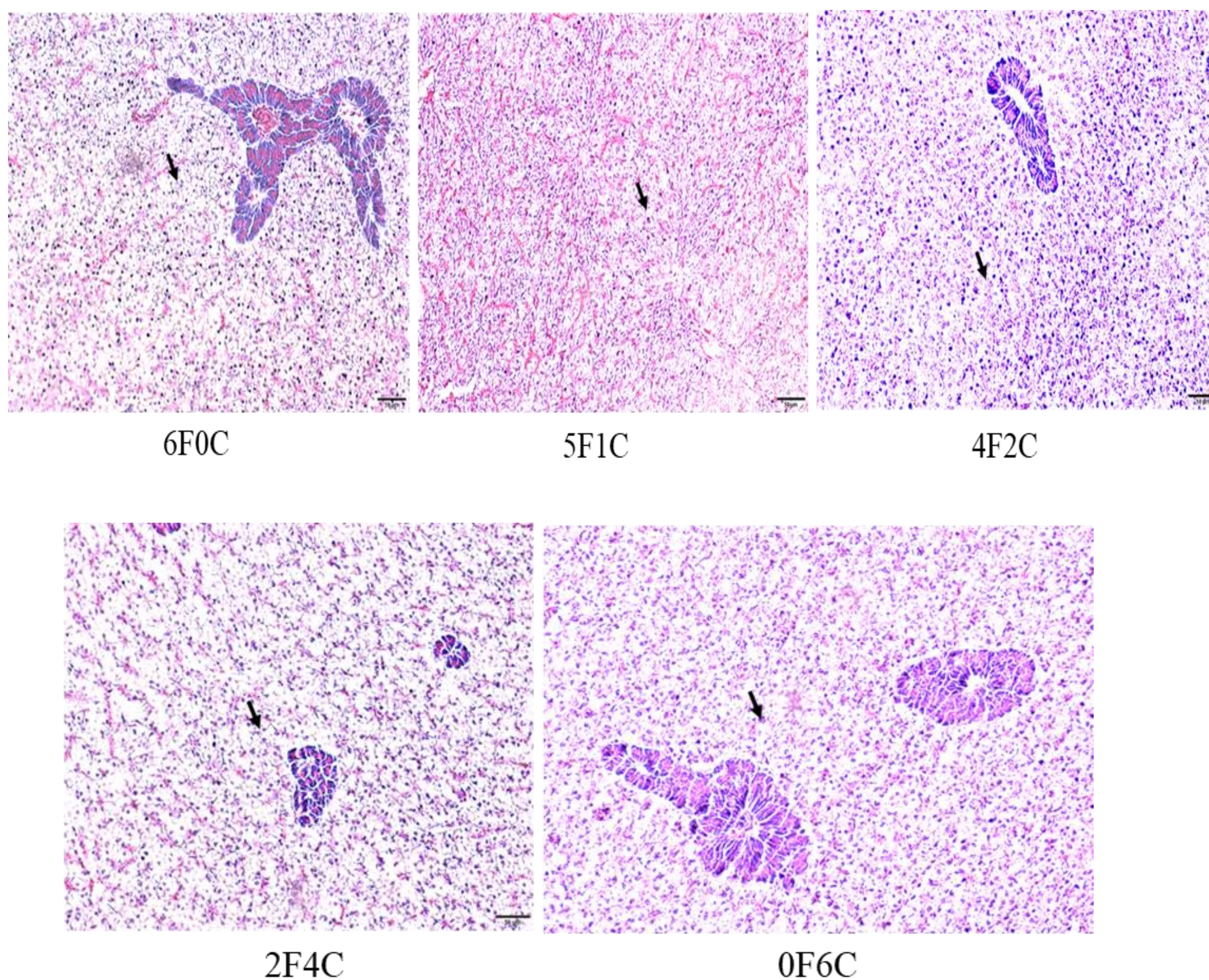


Figure 5: Cross-section in liver of red seabream fed experimental diets for 56 days. Arrows indicate small lipid droplets and lipid vacuoles of the hepatocytes. Hematoxylin and eosin (H&E) Staining, scale = 50 µm).

3.3. Discussion

3.3.1. Growth Performance and Nutrient Utilization

The results of the present trial demonstrated that diets containing CO could completely replace FO without negatively affecting the growth performance and health status of red

seabream. Previous studies have shown that fish oil (FO) can be partially substituted by vegetable canola oil (Haung et al., 2007) and palm oil (67%) (Komilus et al., 2008) in the red seabream diet. Growth performance of red seabream juveniles was gradually impeded by incremental dietary palm oil, and the decline in growth could have been caused by the lowering of EPA and DHA in diets (Komilus et al., 2008). FO can be completely (100%) substituted with vegetable oil to satisfy the requirements of n-3 HUFA (Koshio 2002). Koshio (2002) recommended inclusion of 5–10 g/kg of limited essential n-3 highly unsaturated fatty acids EPA and DHA in the diet, respectively, for juvenile red seabream. The present study is consistent with studies by Betancor et al. (2021) on gilthead sea bream and Hixson et al. (2014b) on rainbow trout (*Oncorhynchus mykiss*), which did not show significant differences in growth response to dietary FO full substitution with CO. We attribute the growth maintenance to proportions of EPA and DHA incorporated in diets as well as fish meal, which might have compensated for the low LC-PUFA in CO.

In the present feed trial, the addition of dietary CO did not completely alter the body composition of red seabream. Although the present study showed a slight increase in crude lipid in FO control compared to all other diets, it did not warrant changes in overall red seabream performance and health across all red seabream groups. Similarly, red seabream-fed canola oil diets exhibited a uniform chemical proximate composition in all parameters (Haung et al., 2007). The fatty acid compositions of vegetable oils and FO are inherently different. Therefore, substituting FO with vegetable oils inevitably influences ARA, EPA, and DHA (Wei et al., 2018). In the present study, the contents of EPA, DHA, and Σn-3 PUFA in juvenile red seabream decreased with increasing CO replacement levels, and C18:1n-9, C18:2n-6, and C18:3n-3 PUFA increased with the corresponding

increase in FO replacement by CO. The results reflected the changes in fatty acid profiles of their respective diets to a great extent, indicating that dietary fatty acid profiles strongly influenced fatty acid composition in the muscle and liver of red seabream. It is worth highlighting that ARA, EPA, and DHA in the liver and muscle of red seabream were mirror images of those detected in the respective diets. Thus, our present study has shown that the fatty acid elongase and fatty acid oxidase enzymes in the liver and muscle of juvenile red seabream were not expressed when dietary FO were replaced by CO. We speculate that the almost exclusively similar levels of ARA, EPA, and DHA in diets and tissues indicate that red seabream has limited capacity to biosynthesize DHA and EPA from short chain fatty acids, including ALA. Bell et al. (2006) reasoned that marine fish have a poor ability to synthesize long-chain PUFAs. The n-3: n-6 ratio between diets and tissues was constant, indicating efficient bioconversion of n-3 PUFA from CO. High levels of n-3 LC-PUFA in CO are vital for effective replacement of FO in diets for marine species, such as sea bream, that have limited capacity to endogenously produce DHA and EPA and depend on their inclusion in the diet (Hyuben et al., 2020). Therefore, it is worth noting that CO has an important twin effect as it maintains EPA and DHA levels in the liver and filets, while at the same time fostering a better n-3: n-6 ratio that plays great immunomodulatory and pathological roles in fish and human health. Transgenic camelina oil, at full replacement of FO, was effective substitute for FO as a dietary lipid source of n-3 LC-PUFA in diets for rainbow trout. Fish fed high levels of transgenic dietary camelina oil enriched with EPA and DHA had FA profiles that were generally similar to those of fish fed FO (Osmond et al.,2021).

3.3.2. Fatty Acid Digestibility

The apparent digestibility estimations (ADC fatty acid) obtained in the present trial were generally high and consistent with those reported previously for red seabream (Ishikawa et al., 1997) as well as those of other species. CO is highly digestible (95.9%) and is utilized as an energy source by Atlantic salmon (Ye et al., 2016). Double bonds are fundamental determinants of the unsaturation of fatty acids. Highly unsaturated fatty acids with more carbon molecules are easily melted and easily diffuse (Morais et al., 2012). Therefore, highly unsaturated fatty acids are likely to be easily digested because of their high melting points (Turchini et al., 2005). In this analysis, n-3 was found to be the most easily digested fatty acid, followed by n-6, saturated fatty acids, and monounsaturated fatty acids, which were the least digestible. Ultimately, the combination degree of unsaturation and the melting point of individual fatty acids resulted in the apparent digestibility of PUFA < MUFA < SFA and short-chain < longer-chain fatty acids, as reported extensively for several species (Turchini et al., 2005). Our present study shows a slight modification to the previously reported PUFA < SFA < MUFA, but similarly maintains that PUFAs are more easily digested than saturated and mono-unsaturated fatty acids.

3.3.3 Blood Chemistry

Blood parameters are reliable indicators of fish health and are dependent on environmental cues such as temperature, season, and nutritional status (Dossou et al., 2018b; El Basuini et al., 2016). The results of the present trial show that all blood parameters and hematocrit mirrored those previously reported for red seabream (El Basuini et al. (2016), and there were no statistical differences among different red seabream groups fed different diets.

3.3.4. Lipid Peroxidation

Previous research has shown that dietary ingredients, including vegetable oils, have an influence on lipid peroxidation. The present trial investigated the impact of dietary de novo CO on oxidative stress stability as an important parameter in the nutritional evaluation of FO alternatives. Polyunsaturated fatty acids (PUFAs) in aquafeeds, particularly EPA and DHA, are highly susceptible to reactive oxygen species (ROS) and reactive nitrogen species (RNS), which form peroxides and free radicals (Arab-Tehrany et al., 2012). Peroxidation is deleterious because it damages tissue cells and subsequently alters physiological and biochemical processes. The first line of the oxidative defense mechanism is enzymatic, including catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), and glutathione peroxidase (GPx), as well as low molecular weight substances such as thiorbaburtic acid reactive substances (TBARS), vitamins (C and E), glutathione (GSH), bilirubin, and flavonoids (Jin et a.,2017; Zuo et al., 2013; Radovanovic et al., 2010).Oxidative stress occurs when excess free radicals are produced in the body, including ROS and RNS (Shourbela et al., 2021; Abdel-Warith et al., 2021). When the oxidation level exceeds the removal of oxides, cells and tissues are damaged (Kohen et al., 2002; Dawood et a1, 2021c). SOD and CAT enzymes are fundamental for defense against oxygen radicals, thereby preventing a chain of reactions triggered by superoxide radicals TBARS, Abele et al. (2004); Pham-Huy et al. (2008) are non-enzyme low molecules that play a similar role in scavenging free radicals (Mourente et al.,2007; Iheanacho et al., 2020). Changes in the activities of these enzymes within the antioxidant system often denote oxidative disturbance that may promote stress and usually orchestrated by surpassed generation of free radicals/ ROS against antioxidants/enzymes

functions in the cell or tissues (Richard et al., 2008). Additionally, the imbalance (in the antioxidant system) created can trigger oxidative condition, leading to cellular damage and predisposes the organisms to multiple disease conditions (Richard et al.,2008; Dawood et al.,2021d) .The results of the present study show that no remarkable differences in CAT and SOD were observed except for in the liver, as a remarkable reduction occurred with the complete substitution of FO with CO (0F6C), and the highest values were observed in the control and (4F2C).The overarching trend shows that the red seabream oxidation resistance were enhanced after CO substitution of FO. CAT is an essential enzyme in biological defense systems. However, the levels of CAT in this experiment were not significantly different (Bhagat et al., 2016; Yu et al., 2016). The results of the present study correspond with earlier observations by Long et al. (2019) who reported that the activities of total antioxidant capacity SOD and CAT showed a declining trend with increasing dietary FO replacement level, indicating that increased blended vegetable oil levels in the diets may have reduced the oxidation level in the hemolymph of the crabs.

3.3.5. Histomorphology of Liver

The results show the increase in lipid accumulation and hepatic adipose infiltration in the liver of fish fed the 6F0C, 2F4C, and 0F6C diets compared to those fed 5F1C and 4F2C diets. Several reports have shown that lipid infiltration in cell vacuoles becomes pronounced as vegetable oil replaces FO. We opinion that feeding with alternative plant oil sources did not impair the liver and intestine tissues probably by alleviating the role of ROS via improved antioxidative and proinflammatory responses (El-Asely et al., 2020). However, in the present study, it did not damage the cells. Hepato- and viscera

somatic indices represent the proportion of liver and viscera weights to whole-body weight, respectively, and can be used as indices of the nutritional status of red seabream (Dawood et a., 2015) (FM: 28%) because the liver and viscera are energy storage organs. Results showed no significant changes in the HSI and VSI between red seabream groups, indicating that CO did not cause liver impairment. Previous studies on FO replacement using alternative lipid sources have reflected histomorphology alterations in tissues. For instance, supranuclear accumulation of lipid droplets was observed in the intestinal cells of some of the groups fed diets supplemented with vegetable oils (FM: 30%) (Caballero et al.,2002). Similarly, livers from these groups showed large amounts of lipid droplets within the hepatocytes. In contrast, Bell et al. (1995) reported a high degree of vacuolization due to lipid deposition in the livers of turbot fed marine FO . This was not observed in fish fed diets containing borage oil. Accumulation of lipid droplets in enterocytes from the pyloric caeca and midgut has been observed in *Arctic charr* fed linseed oil (Olsen et al., 2000). Tissue histopathological interpretation of ultramphological alterations is difficult but can provide basic information for a thorough understanding of the metabolism of nutrients, including fatty acids, in various lipid sources. In conclusion, the results of the present study suggest that FO can be replaced with CO in the feeds of farmed red seabream without compromising growth, blood chemistry, digestibility, and overall histological morphology. It is important to establish that alternative dietary lipids to FO are not only supplied in the correct proportions and balance for optimal growth and feed conversion but can maintain optimal health function and sustain overall biochemical and physiological responses. The present study shows that normal growth and overall health can be more successfully attained if dietary FO is replaced by CO, which provides a more physiologically balanced biochemical

composition. In the future, bimolecular studies would provide interesting information on the mechanisms for the utilization of CO in the diets of marine species.

CHAPTER 4: Growth Performance, Growth Related Genes, Digestibility, Digestive Enzyme Activity, Immune and Stress Responses of *de novo* Camelina Meal in Diets of Red Sea Bream (*Pagrus major*)

Abstract

A 60-day experiment was designed to assess the effect of different ratios of fish meal (FM): camelina meal plant protein (CM) on growth response and relative gene expression of growth-promoting factors, feed utilization potency, digestive enzymes activities, apparent digestibility (ADC), stress response, non-specific immunity of *Pagrus major*. Four isonitrogenous (490.7 g/kg of crude protein) and isolipidic (91.5 g/kg total lipid) experimental diets were formulated and designated as camelina meal, CM0, Soyabean meal ,SBM20.5, CM20.5, and CM33 based on protein contents. At the end of the feed trial, significantly higher ($p < 0.05$) weight gain, specific growth rate, and feed intake but lower feed conversion ratio were recorded in fish fed CM0, SBM20.5, and CM20.5 than fish fed CM33. The lowest growth, feed utilization, enzyme activity, and digestibility were recorded in fish fed CM33. Significantly higher pepsin, amylase, and protease activities were observed in fish fed CM0, SBM20.5, and CM20.5 diets than fish fed CM33. The highest ADC of protein was recorded in fish fed CM0, SBM20.5, and CM20.5 diets. Hematocrit levels were depressed CM33 while total serum protein, total cholesterol,

triglyceride, blood urea nitrogen, total bilirubin, aspartate aminotransferase, and alanine aminotransferase were not significantly changed by the inclusion of CM. Non-specific immune variables (lysozyme activity, peroxidase activity in serum and nitro blue tetrazolium) in fish fed CM0, SBM20.5, and CM20.5 were significantly higher than in fish fed CM33 diet. The superoxide dismutase of fish fed CM20.5 was not significantly different from CM0 and SBM20.5 ($p > 0.05$). Catalase and low salinity stress test show that CM0, SBM20.5, and CM20.5 were not significantly ($p > 0.05$) different, while CM33 was significantly lower than the rest of the diets. TBARs show that CM20.5 and CM33 diets were significantly different ($p < 0.05$), but CM20.5 was not significantly different from SBM20.5. Significantly higher hepatic *IGF-1* and *IGF-2* mRNA expression was found in fish-fed diet groups CM0, SBM20.5, and CM20.5 than fish fed CM33. The present study indicated that the addition of CM up 205 g/kg to diet maintains growth, digestive enzymes, nutrient digestibility, immunity, stress resistance, and feed utilization efficiency of red sea bream.

4.0. Introduction

Fish meal (FM) is a primary protein ingredient in aquaculture enterprises. Availability and accessibility of FM have been plummeting due to unprecedented demands for various uses in the wake of the global population boom. (Wei et al., 2020). Low FM supply has, in turn, caused prices to soar (Ido et al., 2015). Food and Agricultural Organization FAO data released in 2016 projects that there will be a widening gap between accessible FM proportions and its usage in the ever-increasing global aquaculture dispensation (Biachi et al., 2020). Therefore, it is imperative to explore alternative protein sources in the

aquafeed sector. For the past decade and a half, there has been a surge in research identifying alternatives to the finite FM. Numerous proteins have been examined and recommended for use in aquafeed to lessen the inclusion of FM while maintaining the growth performance and health of cultured animals Dawood et al., (2021a) *Hermetia illucens* prepupae meal in practical diets of rainbow trout (*Oncorhynchus mykiss*) Cardinalletti et al. (2019) Housefly pupae (*Musca domestica*) Ido et al.(2015) soy milk (Biswas et al.(2017), fermented rapeseed meal Dossou et al.(2018b) microalgae *Schizochytrium* species Seong et al.(2019);Seong etal.(2020) are among the plant or other proteins that can partially replace FM in cultured red sea bream. Scientific evidence shows that considerable quantities of FM in diets of Atlantic salmon can be substituted with plant protein without showing deleterious growth response and nutrient use Espe et al. (2006). Conversely, other feed trials have presented the detrimental ramifications of using enormous proportions of plant protein in place of FM Jiang (et al.,2019). In addition, plant proteins have high proportions of anti-nutritional factors (ANFs) and fiber that diminish palatability, feed intake, and nutrient digestibility (Dawood et al., 2020a). However, plant protein meals have a positive influence if incorporated in adequate quantities in animal diets. Notably, Sinigrin molecules present in plant meals have high antioxidative and immune efficacy that improve health status in foodstuffs (Mazumder et al., 2016). Plant-based camelina meal (CM) is pegged at $260/metric ton compared to FM, which fetches $1385/metric ton Glencross et al. (2020) and therefore, the former is a viable and affordable alternative for the latter. Meanwhile, novel oilseed *Camelina sativa* has just been re-introduced in Canadian agriculture as an alternative source of biofuel, especially jet fuel (Mudalkar et al., 2014). Cruciferous (Brassicaceae) *Camelina sativa* thrives under low fertile and highly salty grounds; it is highly resilient to insect

attack and does well in frost (freeze and thaw cycles) after they emerge late in cold and cool to warm climate (Onyilagha et al., 2003) Furthermore, the literature confirms the potential of camelina oil as a lipid source in aquaculture and livestock sectors (Hixson et al., 2014c; Betancor et al., 2018; Toyes-vargas et al., 2020). A recent study by Mzengereza et al., (2021) show that complete substitution of fish oil by camelina oil in red seabream (*Pagrus major*) maintains growth performance and sustains health as far as limiting essential fatty acids, ecopentanoic acid (EPA), and docosahexanoic acids (DHA) are incorporated in diets of red sea bream. However, there is a handful of documented information on the efficacy of using the CM in aquaculture diets. Bullerwell, et al. (2016) reported that there were desirable feed utilization efficiencies, intestinal morphology, proximate content of carcass, and growth performance in rainbow trout (*Oncorhynchus mykiss*) fed diets supplemented with up to 200 g/kg CM in its early trials and proposed further studies to determine optimum inclusion. Preliminary nutritional evaluation conducted by Hixson et al. (2016) show that CM has 38% crude protein and considerable proportions of methionine, lysine, phenylalanine, threonine, leucine, isoleucine, and valine. The residual crude lipid in the meal (16%) is rich in α-linoleic acid (18-3n-3) and linoleic acid (18 = 2n-6) fatty acids (Agency C.F.I 2017). In addition, CM contains phytochemicals including phytic acid, total tannins, sinapines, and glucosinolates that interfere with animals' chemical and physiological processes (Matthas,1997) Alterations to the dietary composition of feedstuffs require prior knowledge of the nutrient utilization index of each ingredient, such as the apparent digestibility coefficients (ADCs) of the nutrients and digestive enzyme activities to avoid adverse repercussions on the overall performance of the cultured animals (Dossou et al., 2021) in invertebrates, stress response comprises many physiological processes regulated by the hypothalamus-pituitary-adrenal

axis (Olivotto et al., 2002). In fact, with teleost fish, it has been established that the inducement of both the hypothalamus and the pituitary controls cortisol release (Olivotto et al., 2002). In particular, the adrenocorticotropic hormone (ACTH) is considered the most critical molecule that regulates the synthesis and the release of cortisol from the interrenal cells in the head kidney. Olivotto et al. (2002) reported that cortisol, glucocorticoid receptor, and *HSP70* are essential markers in fish acute stress response. Mzengereza et al. (2021) reported that superoxide dismutase and catalase are the first line of defense enzymes that reflected the effects on the antioxidative state of red seabream after being fed diets incorporated with camelina oils.

Bio-molecular variables have gained credibility for aquaculture studies to supplement already available husbandry practices and evaluate farmed fish responses to various stimuli in their environment (Panserat et al., 2007). Evidence exists that biotechnology items (relative growth-related gene particularly insulin-like growth factor–I (*IGF-I*) and insulin-like growth factor-I (*IGF-II*) have been utilized as a bio-molecular tool to assess growth response in finfish experimentation Picha et al. (2008) on the one hand and to examine the effectiveness of commercial diets in the short term on the other hand. In addition, *IGF-I* is an important metabolic pathway biomarker for molecular tissue in finfish growth; modifications in the *IGF-I* gene indicator can partially lead to alterations in growth performance (Duan 1998). Furthermore, *IGF-I*, mainly produced in the liver, is an essential parameter in the protein anabolism of fish (Luo et al., 2012). The current feed trial was designed to determine the optimum supplementation level of CM for the red seabream diet and its ramifications on growth performance, expression of growth-

promoting genes, feed utilization, body biochemical variables, stress tolerance capacity, and immune response.

4.1. Materials and Methods

4.1.1. Camelina Meal and Test Diets

Camelina meal (CM) was purchased from Tokai Seapro Co., Ltd. (Fukuoka, Japan) and finely ground into powder form using a blender. Four diets were formulated with uniform crude lipid and crude protein contents. Dietary treatments were graded as follows: control diet (fish meal, CM0) and experimental diets containing 205 g/kg soybean meal (SBM20.5), 205 g/kg CM (CM20.5), and 330 g/kg CM (CM33). The diets were formulated in line with the nutritional requirements of finfish enshrined in NRC (2011) (Table 1). Dry ingredients were mixed for 45 min using an RM-G10 food mixer (Remacom Co., Ltd. Shizuoka, Japan) by slowly adding lipid sources initially premixed in a sonicator (USK-3RA, As One Corp., Osaka, Japan) and 30–40% water. The dough was extruded through a grinder with 1.2–2.2 mm in diameter to produce air-dried pellets in a DK 400 mechanical convection oven (Yamato Scientific, Tokyo, Japan) at 60 °C for 2 h. The diets with 10% moisture content were stored under −20 °C until and during the feeding trial. The feedstuffs, diets, and whole fish body were analyzed for moisture, crude protein, and ash using standard methods (AOAC,2012) Total lipid (TL) contents of test diets and whole fish body were determined according to Bligh and Dyer (1959). The total amino acid (TAA) profile of the diet was determined using the liquid chromatography technique (HPLC, Shimadzu Corporation, Kyoto, Japan) following the procedure reported in Teshima et al. (1986) and Kader, et al. (2010) (Table 2). Firstly, 2 mg of sample was mixed with a known concentration of norleucine as internal standard, to this mixture, 4 N methanesulfonic acid was added and hydrolyzed for 22 h at 110 °C. Finally,

the pH of the hydrolysate was adjusted to 2.2 ± 0.05, then filtered and stored at 4 °C. The separation and chromatography assessment of the amino acid content was conducted using an HPLC ion exchanging resin column

Table 19: The proportion of ingredients (g/kg) and chemical composition (%) of experimental diets.

Ingredient (g/kg DM)	CM0	SBM20.5	CM20.5	CM33
Brown fish meal	59.0	45.0	45.0	31.0
Soybean meal	-	20.5	-	-
Camelina meal	-	-	20.5	33.0
Casein	1.0	1.0	1.0	6.0
Squid meal	5.0	5.0	5.0	5.0
Krill meal	4.0	4.0	4.0	4.0
Soybean lecithin	3.0	3.0	3.0	3.0
Pollack liver oil	2.0	2.0	2.0	2.0
Wheat flour	1.4	1.4	1.4	1.4
Activated gluten	5.0	5.0	5.0	5.0
Mineral mix	3.0	3.0	3.0	3.0
Vitamin mix	3.0	3.0	3.0	3.0
Stay C [m]	0.08	0.08	0.08	0.08
Alpha-cellulose	13.52	7.02	7.2	4.00
Methionine	0	0.1	0.2	0.5
Lysine	0	0.09	0.29	0.65
Taurine	0	0.17	0.18	0.29
Total	100	100	100	100
Proximate constituents				
Crude protein	49.6 ± 0.4	49.9 ± 0.2	48.2 ± 0.9	48.6 ± 0.1
Total lipid	9.5 ± 0.1	9.4 ± 0.0	9.0 ± 0.0	8.7 ± 0.0
Moisture	11.2 ± 1.0	10.86 ± 0.3	10.95 ± 0.6	11.26 ± 0.5
Ash	12.4 ± 0.3	12.5 ± 0.3	11.6 ± 0.3	9.5 ± 0.1

[a–n] were previously detailed by Mzengereza, et al. [20]. The experimental diets fed to red seabream for 60 days formulated by replacing fish meal with camelina meal at 0 g/kg for reference diet (CM0), 205 g/kg with soybean meal (SBM20.5), 205 g/kg with camelina meal (CM20.5) and 330 g/kg camelina meal (CM33).

Table 20. Amino acid content (AA g/100 g diet, dry matter basis) (mean ± standard error, n = 3) of the experimental diets fed to red seabream for 60 days formulated by replacing fish meal with camelina meal at 0 g/kg for reference diet (CM0), 205 g/kg with soybean meal (SBM20.5), 205 g/kg with camelina meal (CM20.5) and 330 g/kg camelina meal (CM33).

Amino Acids	CM	CM0	SBM20.5	CM20.5	CM33
Essential					
Arginine	3.24	2.53	2.57	2.54	2.85
Histidine	0.91	2.02	2.12	2.37	2.21
Isoleucine	1.64	1.89	1.95	1.87	1.96
Leucine	2.67	3.64	3.18	3.61	3.52
Lysine	1.83	4.08	4.14	3.67	3.75
Methionine	0.64	1.38	1.28	1.38	1.49
Phenylalanine	1.76	2.45	2.32	2.53	2.65
Threonine	1.68	2.12	2.12	2.3	2.2
Tryptophan	0.42	0.25	0.32	0.28	0.33
Valine	1.98	2.17	2.14	2.23	1.94
Non-essential					
Taurine	nd	0.77	0.75	0.8	0.9
Aspartic acid	3.24	4.65	4.35	4.26	4.25
Glutamic acid	6.56	8.03	8.12	8.22	8.08
Serine	2.01	2.04	2.25	2.3	2.2
Proline	2.21	3.40	2.93	3.39	3.38
Glycine	2.01	2.38	2.28	2.51	2.47
Alanine	1.09	2.70	2.37	2.56	2.24
Tyrosine	1.23	2.67	2.68	2.61	2.65

4.1.2. Fish Husbandry and Sampling Methods

A feeding trial was performed on 120 red seabream *(Pagrus major)* (6.5 g average individual initial weight). The trial was done at the Kamoike marine production facility of the Kagoshima University Faculty of Fisheries (Kagoshima, Japan). Fish were

procured from a commercial fish hatchery in Miyazaki prefecture in Japan. Fish were acclimatized for four weeks to dissolved oxygen concentration between 6.6–6.1 mg/L and water temperatures range of 16.3–18.2 °C. Red seabream juveniles were unbiasedly stocked into twelve 100 L polyethylene circular tanks. Triplicate tanks per diet were stocked with 10 fish each in a flow-through system at natural photoperiod (12 h light/12 h darkness). Fish were fed twice daily to apparent satiation (8:00 am and 3:00 pm) per day for 60 days. After a 60-day feeding period, all fish were starved for 24 h before sampling was done. The fish were inoculated with eugenol (4-allylmethoxyphenol, Wako Pure Chemical Ind., Osaka, Japan) as an anesthetizer. All fish were weighed individually and counted for the evaluation of the growth performance and survival. Further, the whole-body length was detected for the calculation of the condition factor. Three fish per group (1 fish/tank) were collected and kept at −20 °C for proximate analysis using the standard method mentioned earlier. Nine fish per treatment (three fish/tank) were used for blood collection while their intestines, livers were dissected and kept for intestinal digestive enzymes, liver antioxidative capacity, and gene expression. The livers of fish were weighed to calculate the hepatosomatic index (HSI). Four fish per tank were used in the low water salinity stress (triplicates). The remaining fish per tank were collected and divided into two replicates per treatment to run the digestibility trial:

4.1.3. Determination of Antinutrients Contents in Camelina Meal

$$\text{Weight gain (\%)} = (\text{Weight}_{60\ Days} - \text{Weight}_{0\ Day})/\text{Weight}_{0\ day} \times 100; \quad (11)$$

$$\text{Specific growth rate (SGR \%/day)} = ((\text{Ln Weight}_{60\ Days} - \text{Ln Weight}_{0\ Day})/60) \times 100; \quad (12)$$

$$\text{Survival (\%)} = (\text{Fish No.}_{60\ Days}/\text{Fish No.}_{0\ Day}) \times 100; \quad (13)$$

$$\text{Feed intake (g/fish/60 days)} = (\text{dry diet provided} - \text{dry uneaten diet retrieved})/\text{no. of fish}; \quad (14)$$

$$\text{Feed efficiency ratio (FER)} = \text{Fish live weight gain (g)/dry feed intake (g)}; \quad (15)$$

$$\text{Condition factor (CF)} = \frac{W}{L^3} \times 100 \quad (16)$$

$$\text{HSI} = \frac{\text{Liver weight, g}}{\text{Fish body weight, g}} \times 100 \quad (17)$$

Antinutrients were determined using procedures proposed in AOAC [32]. Briefly, trypsin inhibitors were assayed using casein solution added to all samples as substrate. Absorbance was read at 380 nm. Tannin standard solution (tannic acid) was prepared ranging (10–30 mg/L) to determine tannin. The absorbance of the standard solution and samples was monitored at 500 nm. Phytic acid was evaluated by 0.3% ammonium thiocyanate solution added into each sample as a marker and was titrated with iron (III) chloride solution (0.00l95 g iron/mL). The ultimate result is a somewhat brownish yellow color that lasts for 5 min.

Protease inhibitors were measured using egg albumin as substrate (2% in phosphate buffer, pH 7), and 1 mL of the Bromelain enzyme solution (1% in phosphate buffer, pH 7) were incubated for 10 min at 55 °C. To stop the reaction, 5 mL of 10% trichloroacetic acid (TCA) was applied. The samples were read at 280 nm. All samples' absorption was measured in the spectrophotometer (Spectronic 200, Thermo Fisher Scientific K.K., Tokyo, Japan). The results are displayed in Table 3.

Table 21: Anti-nutritional factors content in the milled camelina meals (mean ± standard error, n = 3).

Anti-Nutrient Factor	**g/kg**
Tannin	34.2 ± 0.2
Phytic acid	40.7± 0.0
Trypsin inhibitor	20.3 ± 0.0
Protease inhibitor	12.0 ± 0.1

4.1.4. Digestive Enzyme Assay

Fish were starved for 24 h before sample collection and collected intestines and stomach. The fish were inoculated with an anesthetizer (Eugenol, 4-allylmethoxyphenol, Wako Pure Chemical Ind.) before dissection. The sample was washed with 2% sucrose buffer and weighed storage at −81 °C until use. Intestine (wet wt. 2 g) were mixed with 5 mL of ice-chilled homogenization buffer (20 mM Tris-HCl, 1 mM EDTA, 10 mM $CaCl_2$, pH 7.5) [32]. Samples were homogenized using a pellet pestle cordless motor (Sigma-Aldrich, St. Louis, MO, USA). 400 µL of Tris buffer was added and centrifuged for 30 min at 1700× *g*, 4 °C. The supernatant was preserved at −81 °C until enzymatic assay.

4.1.4.1. Protease Activity

Bradford's method employing protein assay Coolmassie Brilliant Blue (CBB) solution (Nacalai Tesque, Inc., Kyoto, Japan) was done for protein content quantification. Protease activity was measured by Qing and Xing (1997) method with some modifications using 0.5% casein as a substrate. The assay was performed as follows: 100 µL of 0.04 mol/L EDTA-Na, 2 mL of 0.5% casein, 400 µL of Tris buffer, 200 µL of enzyme extract sample of the intestine, and 800 µL distilled water were mixed and incubated for 15 min. The

reaction was quenched by adding 1 mL of 30% cold TCA. The mixture was centrifuged at 2000× *g* for 20 min at 4 °C. One mL of the supernatant was mixed with 1 mL of Folin reagent and 5 mL of 0.55 mol/L Na_2CO_3, gently mixed and incubated for 15 min. OD was measured using a microplate reader (Multiskan GO, Thermo Fisher Scientific K. K., Tokyo, Japan) at 680 nm using distilled water as blanks. One unit was measured as the hydrolysis of casein that liberated 1 µg of tyrosine per min.

4.1.4.2. Pepsin

Pepsin activity was performed according to Natalia et al. (2004) using 2% hemoglobin in 0.06 NH_4Cl as substrate. Five hundred µL of 2% haemoglobin in 0.06 N HCl substrate was mixed with 100 µL of crude enzyme extract of the stomach and incubated for 10 minutes at ambient temperature. The reaction was quenched by the inclusion of 1 mL of 5% TCA, and the mixture was incubated for 5 min at ambient temperature. The mixture was centrifuged for 5 min at 12,000× *g* under 4 °C. Optical density (OD)was recorded at 280 nm using a microplate reader (Multiskan GO, Thermo Fisher Scientific K. K.). For blank reading, trichloroacetic acid was used instead of enzyme extract.

4.1.4.2. Lipase

Lipase activity was evaluated following the procedure of Roberts (1985) 4-methylumbelliferyl butyrate (4 MUB) substrate was applied. Sixty microliters of the substrate (0.5 mM 4 MUB, 5 mM egg lecithin, 10 mM sodium taurocholate, and 150 mM NaCl) was mixed with 20 µL of Tris buffer (pH 7.5) and 20 µL of crude enzyme extract. Each sample was divided into two, each placed at 4 °C in an ice bath and 37 °C in a water bath and simultaneously incubated for 10 minutes. Tris buffer (0.2 mL, 1 M, pH 7.5) was added to stop the reaction. Emission was measured at 450 nm (excitation 380 nm) using

a fluorescence spectrophotometer (F-2700, Hitachi High-Tech Corp. Tokyo, Japan). The difference in fluorescence between the readings taken on the 4 °C incubated samples and the 37 °C control samples per assay were the final reading. Basal stock solutions of 4-methylumbelliferone were used as blank.

4.1.4.3. Amylase

Amylase potency was determined using the modified method proposed by Mushirata et al. (2018), where 1% starch solution was used as a substrate. Fifty microliters of enzyme extract, 25 µL 20 mM sodium phosphate buffer (pH 6.9, containing 6.0 mM NaCl), and 25 µL of the substrate solution were mixed and incubated at 37 °C for 60 minutes. Fifty µL of dinitrosalicylic acid reagent (1% dinitrosalicylic acid and 30% sodium potassium tartrate in 0.4 M NaOH) was added to the mixture to quench the reaction and samples were incubated in boiling water for 5 min. The OD was measured using a microplate reader (Multiskan GO, Thermo Fisher Scientific K. K.) at 540 nm using a maltose solution as blank. Total liberated maltose was evaluated by the standard curve. The activity was measured in units of U, which equaled the quantity of maltose released in one minute (mol).

4.1.5. Blood Function Assessment

After the growth experiment, fish were sampled by drawing blood from the caudal vein. Heparinized disposable syringes were used for collecting blood for hematocrit and other plasma bioassays, while non-heparinized disposable syringes were used to collect blood for serum analysis. Plasma and serum samples were centrifuged at 3000× *g* for 15 min at 4 °C using a centrifuge (MX-160; Tomy Seiko Co., Ltd., Tokyo, Japan) and kept at −80

°C for later use. Hematocrit was assayed in the microhematocrit machine using whole blood. Serum parameters were assessed by a dry chemistry analyzer (SPOTCHEMTM EZ model SP-4430, Arkray, Inc. Kyoto, Japan).

4.1.6. Non-Specific Immunological and Antioxidative Assays

Serum total peroxidase was determined by referring to a procedure by Salinas et al. (2008) Lysozyme activity in serum was determined turbidimetrically as stipulated in protocols of (Laygren et al., 1999) . An enzyme activity unit was defined as the amount of enzyme that produced a decrease in absorbance of 0.001/min. The oxidative radical production by neutrophils during respiratory burst was measured by the nitro blue tetrazolium (NBT) assay in whole blood samples (Anderson and Siwicki, 1995) .Biological antioxidant potential (BAP) and reactive oxygen metabolites (d-ROMs) were also measured spectrophotometrically in blood plasma with an automated analyzer (FRAS4, Diacron International s.r.l., Grosseto, Italy) following procedures recommended by the commercial FRAS4 manufacturer, d-ROMs test, and BAP Test, (Wismerll Co., Ltd., Tokyo, Japan). Antioxidant enzyme function was measured in liver tissues after the experiment. Fish were killed in a slurry of ice water, dissected, and liver collected and kept at −80 °C until used for analysis. Liver samples were firstly homogenized in sucrose buffer and centrifuged at 4 °C and 12,000 rpm for 10 min. Superoxide dismutase (SOD) activity was determined using the SOD-WST assay kit (Dojindo Molecular Technologies, Inc. Kumamoto, Japan) and absorbance read at 450 nm. The catalase activity (CAT) assay was performed using spectrophotometric determination of hydrogen peroxide (H_2O_2), which forms a stable complex with ammonium molybdate that absorbs at 405 nm. The

thiobarbituric acid reactive substances (TBARs) concentration was measured using a TBARs assay kit (Cayman Chemical, Miami, FL, USA) at 540 nm. The supernatant of liver samples was determined using a microplate reader (Multiskan GO; Thermo Fisher Scientific, K.K.).

4.1.7. Low Salinity Stress Evaluation

Four fish per tank were placed in 20 L transparent glass tanks at random. The 20 L tanks contained 18 L of dechlorinated freshwater. This test was conducted in triplicate for each experimental treatment. Time taken to reach 50% mortality was calculated by the following equation (Moe et al., 2004)

$$Y = aX + b \tag{18}$$

where Y = log10 (survival), X = time to individual death of fish (min). LT_{50} (X) was obtained when Y = 1.7 as $\log_{10}(50) = 1.7$.

4.1.8. Digestibility Assessment

The digestibility of nutrients (crude protein, crude lipid, and dry matter) was measured by an indirect method using chromium oxide as an inert marker. Before feces collection, juvenile red seabream was accustomed to the diet containing chromic oxide for six days. Feeding schedules to apparent satiation twice daily were maintained. Faecal matter siphoned from the bottom and collecting them into the small fishnet. Faeces were pooled and stored at −20 °C until analysis. Soon before analysis, faeces were freeze-dried in and milled to powder form. Levels of chromium oxide in diets and faeces were quantified by Furukawa (1966) The following formulas were used to computerize the ADCs of the nutrients.

$$ADC_{nutrient} (\%) = 100 - (\% Cr_2O_3 \text{ diet}/\% Cr_2O_3 \text{ faeces} \times \% \text{ nutrient faeces}/\% \text{ nutrient diet}) \quad (19)$$

$$ADC_{drymatter} (\%) = 100 - (100 - (\% Cr_2O_3 \text{ diet}/\% Cr_2O_3 \text{ faeces}) \quad (20)$$

4.1.9. Real Time PCR Analysis

Fish were dissected, and liver samples were obtained. Liver samples were placed in fivefold of RNAlater (Invitrogen; Thermo Fisher Scientific K. K.) liver weight and stored at −80 °C until analysis. The RNeasy Mini Kit 50 (Qiagen; Hilden, Germany) was used for the RNA extraction. Briefly, liver samples (30 mg) were placed in a tube, homogenized, and centrifuged at 12,000 rpm for 15 s. The supernatant was collected and mixed with 70% ethanol. After RNA extraction, cDNA was obtained using the Prime ScriptTM RT Master Mix Kit (Takara Bio Inc. Shiga, Japan), following the manufacturer's protocol. Real-time PCR analysis was performed using SYBR Select Master Mix kit (Thermo Fisher Scientific K. K.) using the following primers as presented in Table 4.

Table 22: Forward (F) and reverse (R) primers were used for growth-related mRNA quantitative real-time PCR.

Name	**Primer Sequence (5′–3′)**	**Accession Number**
β-actin-F	TCTGTCTGGATCGGAGGTC	JN226150.1
β-actin-R	AAGCATTTGCGGTGGACG	
IGF-1-F	TAAACCCACACCGAGTGACA	AB050670.1
IGF-1-R	GCGATGSSGAAAAGCTACGG	
IGF-2-F	CGGCAAACTAGTGATGAGCA	AB360966.1
IGF-2-R	CAGTGTCAAGGGGGAAGTGT	

Where: *β-actin* is the housekeeping gene according to the protocol proposed by Hossan et al. (2016) *IGF-1*: Insulin-Like Growth Factor 1; *IGF-2*; Insulin-Like Growth Factor 2.

Elongation factor (β-actin) was used as the housekeeping gene (Table 4). Amplification was performed using the CFD-3120 Mini Opticon Real-Time PCR System (BIO-RAD,

Singapore) with the following protocol: Initial 2 min denaturation at 95 °C, 40 cycles of 95 °C for 15 s, and 65 °C for 30 s. Each assay was done in triplicate.

4.1.10. Statistical Analysis

Firstly, the normal distribution of the data was verified using Kolmogorov–Smirnov test and the Shapiro-Wilk test. To confirm the homogeneity of variance on data sets, it was subjected to the Levene test. The experimental tanks were arranged in a Completely Randomized Design (CRD) and treatment diets replicated; therefore, the experimental design fulfilled the third assumption of using ANOVA, which demands that observations be independent of each other. Data were then subjected to a one-way analysis of variance (ANOVA). All analysis was performed using Super ANOVA 1.11 (Abacus Concepts, Berkeley, CA, USA). Probabilities of $p < 0.05$ were defined as significant. Significant different means among various treatments were evaluated using the Tukey– Kramer post hoc test. Results are reported as the "mean ± S.E." (S.E referring to the standard error of the mean). To synthesis the data, multivariate analyses were performed on the data to summarize the overall response of red seabream to the test diets. Briefly, data was holistically normalized and plotted using heat map graphical function readily developed in GraphPad Prism version 8.0.1 for Windows (Graph Pad Software, San Diego, CA, USA). To run by principal component analysis (PCA), data was standardized followed by PCA. Data were finally visualized by running theUnweighted Pair Group Method with Arithmetic Mean (UPGMA) hierarchical algorithm using a correlation matrix model. PCA and UPGMA were analyzed using Pa leontological Statistical Software version 3.21, University of Kansas, Kansas, USA. (Hammer et al., 2001).

4.2. Results

4.2.1. Growth and Nutrient Utilization Variables

Information on growth response, feed efficacy indices, and survival of fish were shown in Table 23.

Table 23: Growth variables feed utilization markers, biometric indices, and survival obtained after 60 days feeding period in red seabream fed diets formulated by replacing fish meal with camelina meal at 0% for reference diet (CM0), 205 g/kg with soybean mean (SBM20.5), 205 g/kg with camelina meal (CM20.5) and 330g/kg camelina meal (CM33).

Parameter	CM0	SBM20.5	CM20.5	CM33
Initial weight(g/fish)	6.5	6.5	6.4	6.4
Final weight(g/fish)	29.9 ± 0.7 [a]	28.1 ± 0.9 [a]	28.3 ± 0.8 [a]	24.9 ± 0.3 [b]
SGR[2]	2.52 ± 0.0 [a]	2.42 ± 0.1 [a]	2.45 ± 0.1 [a]	2.2 ± 0.0 [b]
FI(g/fish/56days) [3]	27.6 ± 0.8 [a]	24.7 ± 1.1 [ab]	23.6 ± 1.5 [ab]	20.6 ± 0.9 [bc]
FCR [4]	1.0 ± 0.0 [b]	1.1 ± 0.1 [b]	1.1 ± 0.0 [b]	1.2 ± 0.1 [a]
BWG [5]	353.5 ± 4.9 [a]	327.6 ± 18.5 [a]	334.1 ± 14.3 [a]	275.09 ± 5.8 [b]
HIS [6]	1.8 ± 0.1	1.6 ± 0.1	2.0 ± 0.2	1.9 ± 0.1
SR [7]	100± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0
CF [8]	1.9 ± 0.1	1.9 ± 0.0	1.8 ± 0.1	1.8 ± 0.2

Values are shown as mean ± S.E. (n = 3). Data with the same letters in the column are not significantly different ($p < 0.05$). [2] Specific growth rate (SGR %/day). [3] FI, Feed intake (g/fish/60 days). [4] FCR, feed conversion ratio. [5] BWG, Body Weight gain (%). [6] HSI, Hepatosomatic index. [7] SR, Survival (%).

By the end of the 60-day feeding period, fish served control, SBM20.5, and CM20.5 diets showed significantly higher final live body weight, weight gain, specific growth rate, and feed intake, but lower feed conversion ratio than fish fed CM33 diet. Survival rates, hepatosomatic index, and condition factor rates of fish were not markedly changed by the supplementation of CM in the diets.

4.2.2. Whole Body Proximate Evaluation

Carcass proximate composition of *Pagrus major* juvenile is presented in Table 6. There was no alteration ($p > 0.05$) on ash and lipid contents at the end of 60 days of the feeding regime. The group fed CM33 showed lower whole-body protein levels than all diets, including control ($p < 0.05$).

Table 24: Proximate composition (%) of the whole-body carcass obtained after 60 days feeding period in red seabream fed diets formulated by replacing fish meal with camelina meal at 0% for reference diet (CM0), 205 g/kg with soybean mean (SBM20.5), 205g/kg with camelina meal (CM20.5) and 330g/kg camelina meal (CM33).

Parameter	CM0	SBM20.5	CM20.5	CM33
Crude protein	21.9 ± 0.8 [a]	20.9 ± 0.1 [a]	20.3 ± 1.7 [a]	18.3 ± 0.5 [b]
Total Lipid	16.6 ± 0.4	17.8 ± 2.4	18.1 ± 0.1	18.2 ± 0.2
Moisture	69.6 ± 0.5	68.0 ± 1.2	69.4 ± 0.3	69.1 ± 0.5
Ash	4.2 ± 0.1	5.7 ± 0.1	4.6 ± 0.1	3.2 ± 0.05

Values represent means of triplicate groups ± S.E.M., n = 3 means with different letters are significantly different ($p < 0.05$); means with the same letters are not significantly different ($p > 0.05$).

4.2.3. Digestive Enzyme Activity

Table 7 represented the enzyme activity in the intestine of red seabream fed test diets. Intestinal enzyme activities (protease, lipase, and amylase) and stomach enzyme activity (Pepsin) in fish fed CM33 recorded the lowest values compared to the other group. Except for lipase, CM has an impact on enzyme activities in the intestine of red sea bream.

Table 25: Enzyme activities (U/mg protein) in the intestine obtained after 60 days feeding period in red seabream fed diets formulated by replacing fish meal with camelina meal at 0% for reference diet (CM0), 205 g/kg with soybean mean (SBM20.5), 205 g/kg with camelina meal (CM20.5) and 330 g/kg camelina meal (CM33).

Parameters	**CM0**	**SBM20.5**	**CM20.5**	**CM33**
protease	3.0 ± 0.0 [a]	1.2 ± 0.1 [b]	1.2 ± 0.1 [b]	0. 8 ± 0.0 [c]
Pepsin	1.05 ± 0.2 [a]	0.69 ± 0.0 [a,b]	0.63 ± 0.0 [a,b]	0.55 ± 0.0 [b]
Lipase	0.33 ± 0.1	0.28 ± 0.1	0.3 ± 0.1	0.2 ± 0.0
Amylase	0.6 ± 0.1 [a]	0.14 ±0.4 [b]	0.37 ± 0.0 [b]	0.28 ± 0.3 [b]

Values represent means of triplicate groups ± S.E.M., n = 3 means with different letters in column are significantly different ($p < 0.05$); means with the same letters are not significantly different ($P > 0.05$).

4.2.4. Serum Biochemical Constituents

Table 8 carries the serum biochemistry data of red seabream juveniles after the 60-day feeding period. Hematocrit levels were depressed CM33 ($p < 0.05$) when compared with the control group. Except for the glucose, total serum protein (T-Pro), total cholesterol (T-Cho), triglyceride (TG), blood urea nitrogen (BUN), total bilirubin (T-Bill), and aspartate aminotransferase test (AST) and alanine aminotransferase test (ALT) were not significantly ($p < 0.05$) changed by the inclusion of CM.

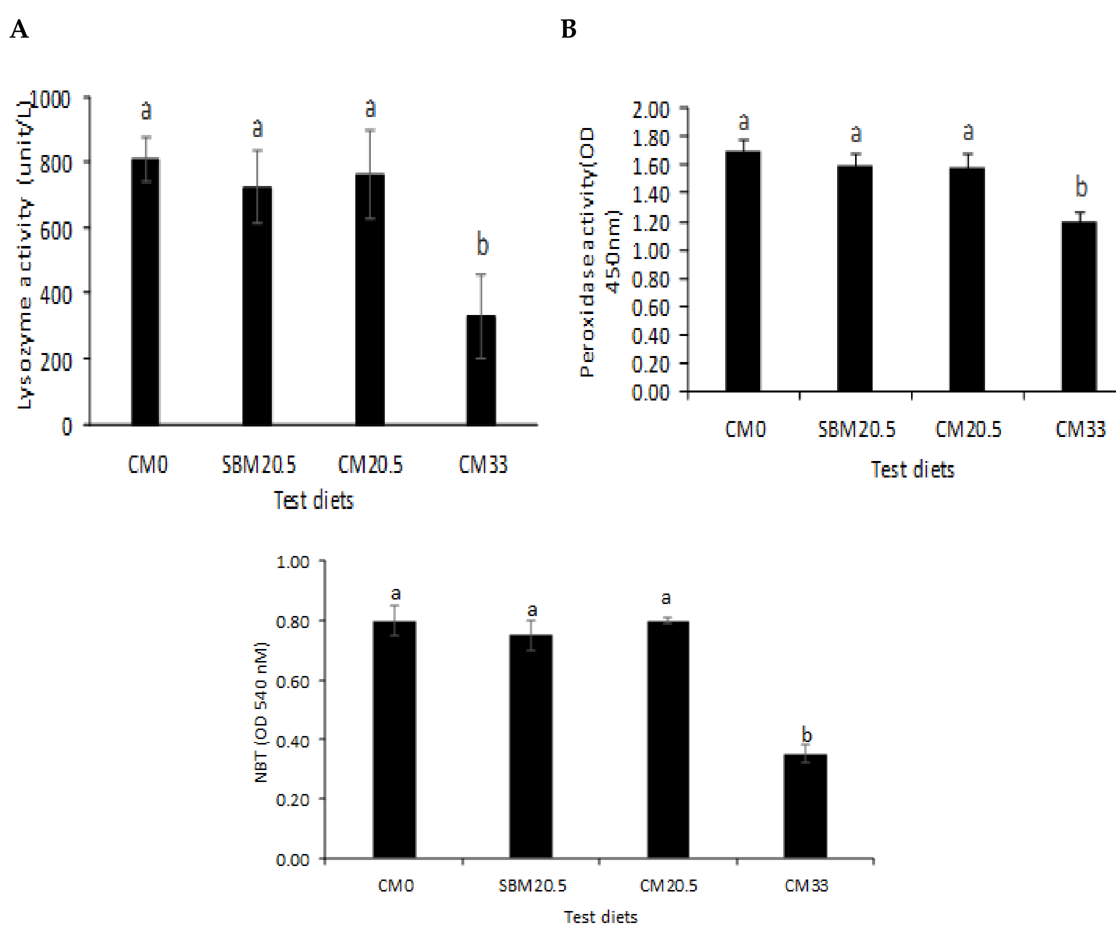
Table 26: Serum biochemistry parameters obtained after 60 days feeding period in red seabream fed diets formulated by replacing fish meal with camelina meal at 0% for reference diet (CM0), 205 g/kg with soybean mean (SBM20.5), 205 g/kg with camelina meal (CM20.5) and 330g/kg camelina meal (CM33).

Parameter	**Control**	**SBM 20.5**	**CM 20.5**	**CM 33**
Hematocrit (%)	35.5 ± 0.5 [a]	37.5 ± 1.5 [a]	35.5 ± 2.5 [a]	24 ± 1 [b]
Glucose (mg/dL)	70.3 ± 5.7 [a]	76.7 ± 4.9 [a]	78 ± 16.7 [a]	108 ± 20.4 [b]
Total bilirubin (mg/dL)	0.3 ± 0.0 [a]	0.3 ± 0.0 [a]	0.3 ± 0.0 [a]	0.3 ± 0.0 [a]
Total protein (mg/dL)	3.2 ± 0.1 [a]	3.4 ± 0.7 [a]	3.2 ± 0.1 [a]	3.7 ± 0.0 [a]
T-Cho g/dL) [2]	178 ± 58.2 [a]	184.3 ± 28.9 [a]	188.3 ± 14 [a]	225 ± 2.6 [a]
TG (g/dL) [3]	181 ± 9.7 [a]	173.6 ± 8.8 [a]	164.3 ± 10.7 [a]	170.6 ± 12.6 [a]
AST (IU/L) [4]	35 ± 3.4 [a]	36.5 ± 6.4 [a]	31.7 ± 3.7 [a]	26.5 ± 11.5 [a]
ALT (IU/L) [5]	33 ± 2.3 [a]	18 ± 3.1 [a]	28 ± 1.8 [a]	10.7 ± 0.6 [a]

Values are expressed as mean ± SE from triplicate groups (n = 3). Data with the same alphabets in the column are not significantly different ($p > 0.05$). [2] T-Cho: total cholesterol. [3] TG: triglyceride. [4] AST: Aspartate transaminase. [5] ALT: Alanine transaminase.

4.2.5. Immunological Response

Figure 1 presents the non-specific immune response data of red seabream juveniles after the 60-day feeding on CM-rich diets.



(C)

Figure 6: (A) Serum lysozyme activity (U/L, $n = 3$); (B); serum peroxidase activity ($n = 3$); (C) nitro blue tetrazolium (%, mean ± standard error, n = 3) of nutrients in red seabream fed diets formulated by replacing fish meal with camelina meal at 0% for reference diet (CM0), 205 g/kg with soybean mean (SBM20.5), 205 g/kg with camelina meal (CM20.5) and 330 g/kg camelina meal (CM33).Values with different letters are significantly different ($p < 0.05$)

Results show that lysozyme activity, peroxidase activity in serum, and nitro blue in whole blood of red seabream fed up to 205 g/kg CM were not significantly different from control (CM0) and soybean meal diet (SBM20.5) ($p < 0.05$). In contrast, fish serum-fed meal camelina supplemented at 330 g/kg (CM33) were significantly lower ($p > 0.05$) than fish groups from the rest of the diets.

4.2.6. Resistance to Oxidative and Salinity Stress

Figure 2 illustrates antioxidant enzymes function and thiobarbituric acid reactive substances (TBARs) data, while Figure 3 shows low salinity challenge results of red seabream juveniles after the 60-day feeding on camelina meal rich diets. Values are presented in triplicates = 3. Central axis based on mean values of d-reactive oxygen metabolites (d-ROMs) and biological antioxidant potential (BAP) from each treatment. Results show that in zone A, high (BAP) and low d-ROMs (good condition) for fish groups CM0, SBM20.5, and CM20.5 and Zone D low BAP and high d-ROMs (stressful condition) for CM33. Results for superoxide dismutase (SOD) and show that red seabream fed up to 205 g/kg CM were not significantly different from control (CM0) and (SBM20.5), but CM20.5 and CM33 were not significant ($p > 0.05$). Catalase (CAT) and low salinity stress test show that CM0, SBM20.5, and CM20.5 were not significantly ($p < 0.05$) different from each other, while CM33 was significantly lower than the rest of the diets ($p > 0.05$). TBARs show that camelina diets CM0, CM20.5, CM33 were all significantly different ($p <0.05$), but CM20.5 was not significantly different from SBM20.5.

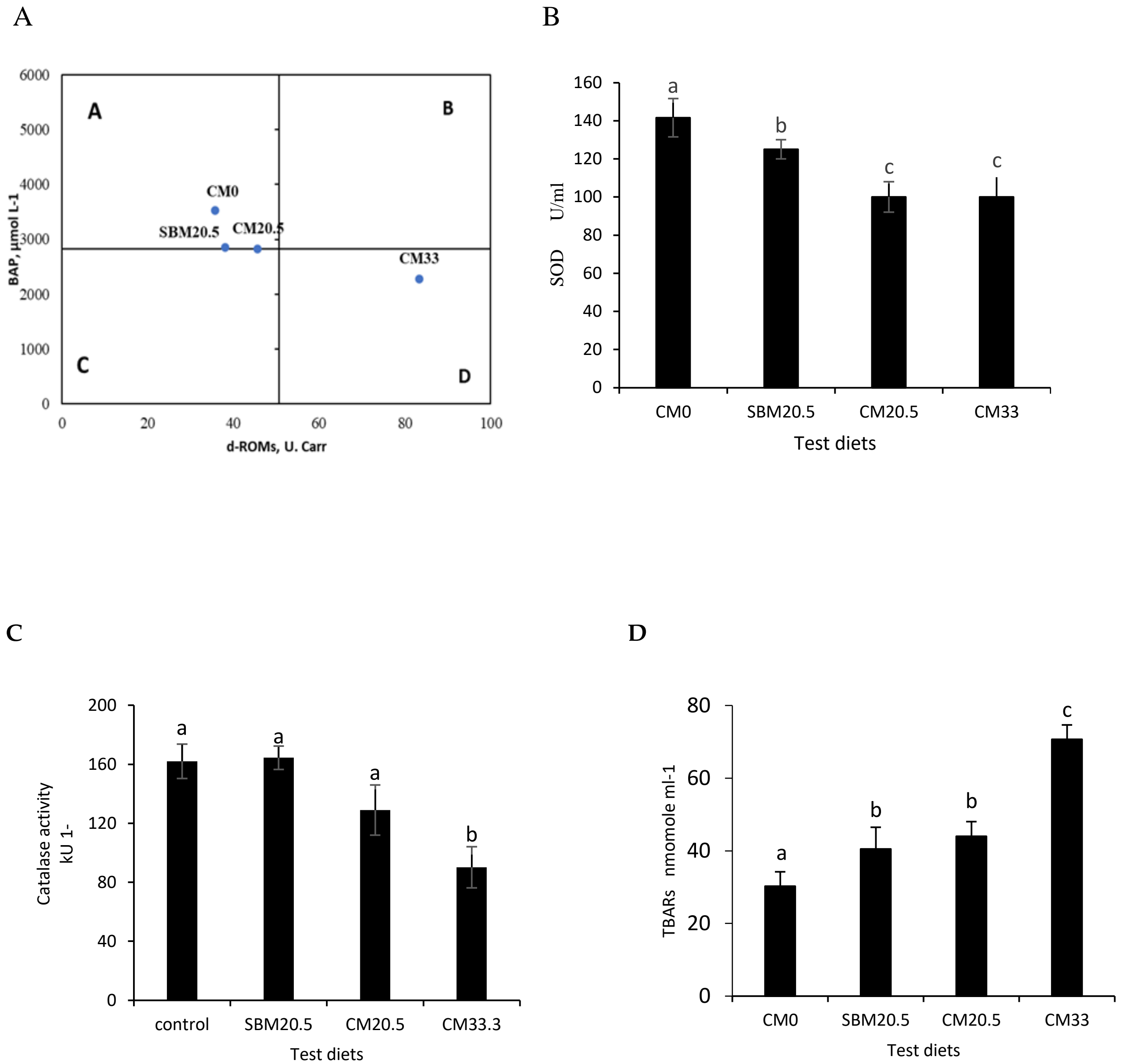


Figure 7: (A) BAP: Biological Antioxidant potential and d-ROM s; Reactive Oxygen Metabolites, (B) SOD; superoxide dismutase, (C) CAT, Catalase (D) TBARs: Thiobarbituric Acid Reactive substances (%, mean ± standard error, n = 3) of nutrients in red seabream fed diets formulated by replacing fish meal with camelina meal at 0% for reference diet (CM0), 205 g/kg with soybean mean (SBM20.5), 205 g/kg with camelina meal (CM20.5) and 330g/kg camelina meal (CM33). Values with different letters are significantly different ($p < 0.05$).

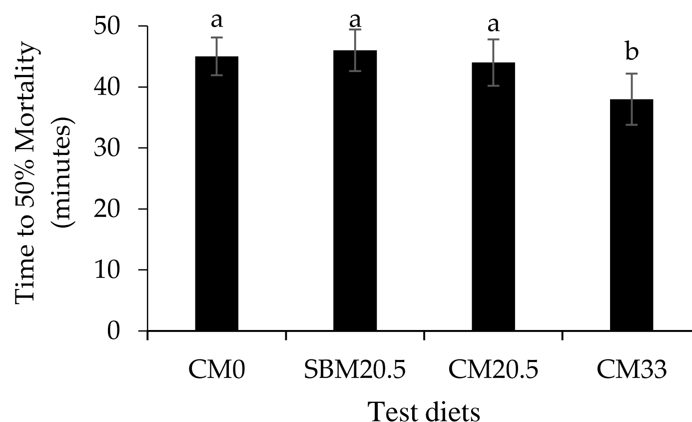


Figure 8: LT_{50} (min) was obtained from the onset of mortality time of red sea bream exposed to freshwater. (%, mean ± standard error, n = 3) of nutrients in red seabream fed diets formulated by replacing fish meal with camelina meal at 0% for reference diet (CM0), 205 g/kg with soybean mean (SBM20.5), 205 g/kg with camelina meal (CM20.5), and 330 g/kg camelina meal (CM33). Values with different letters are significantly different ($p < 0.05$). Values with different letters are significantly different ($p < 0.05$).

4.2.7. Apparent Digestibility Coefficients of Nutrients

The findings of apparent nutrient digestibility showed that there was no significant ($p > 0.05$) differences in total lipid and dry matter among the fish group (Table 9). In CM0, SBM20.5, and CM20.5, similar protein digestibility was observed. However, protein digestibility in CM33 was significantly ($p < 0.05$) lower among the other diets.

Table 27: Apparent digestibility coefficients (%, mean ± standard error, n = 3) of nutrients in red seabream fed diets formulated by replacing fish meal with camelina meal at 0% for reference diet (CM0), 205 g/kg with soybean mean (SBM20.5), 205 g/kg with camelina meal (CM20.5) and 330 g/kg

Parameter	**CM0**	**SBM20.5**	**CM20.5**	**CM33**
Total lipid	87.8 ± 0.7	86.8 ± 0.9	84.9 ± 0.1	84.6 ± 0.6
Dry matter	72.9 ± 2.5	72.4 ± 1.4	70.4 ± 2.5	72.6 ± 0.4
Crude protein	91.9 ± 0.5 [a]	91.2 ± 0.3 [a]	90.8 ± 0.2 [a]	87.6 ± 0.3 [b]

Values are means of triplicate groups ± S.E.M, n = 3. Within a row, means with different letters are significantly different (*p* < 0.05); means with the same letters are not significantly different (*p* > 0.05.

4.2.8. Relative Growth Gene Expression

Relative gene expression of hepatic *IGF-1* and *IGF-2* were presented in Figure 4 A, B. Significantly higher hepatic *IGF-1* and *IGF-2* mRNA expression was found in fish-fed diet groups CM0, SBM20.5, and CM20.5 than fish fed CM33.

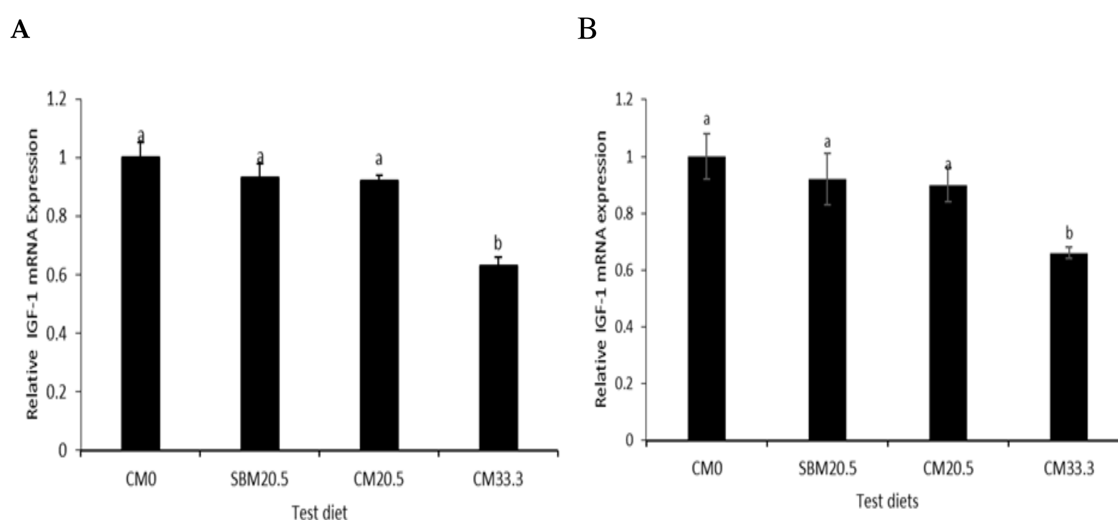


Figure 9: (A,B) qPCR analyses of relative expression of growth-related genes (IGF-1 and IGF-2) in the liver of red seabream fed four different diets by replacing fish meal with camelina meal at 0% for reference diet (CM0), 205 g/kg with soybean mean (SBM20.5), 205 g/kg with camelina meal (CM20.5) and 330 g/kg camelina meal (CM33). Relative mRNA expressions results are presented in triplicated, n = 3 for all treatments. Letters indicate Turkey-Kramer post hoc test and confidence interval of 95% ($p > 0.05$) interpreted as Values represent means of triplicate groups ± S.E.M., n = 3 means with different letters are significantly different ($p < 0.05$); means with the same letters are not significantly different ($p > 0.05$). S.E. represents the standard error of M = mean.

4.2.9. The Heatmap Analysis

In the Head map diagram (Figure 5), there was a marked distribution of response to different parameters in red seabream among four treatments. Comparatively, red seabream fed diet 0 g/kg camelina meal (CM0) without plant protein supplementation responded highly (100–80% mean distribution) in terms of overall growth parameters, oxidative resistance and freshwater stress responses, immune makers, feed utilization, and gene expressions than in red seabream compared with the diets supplemented with soybean meal and camelina meal.

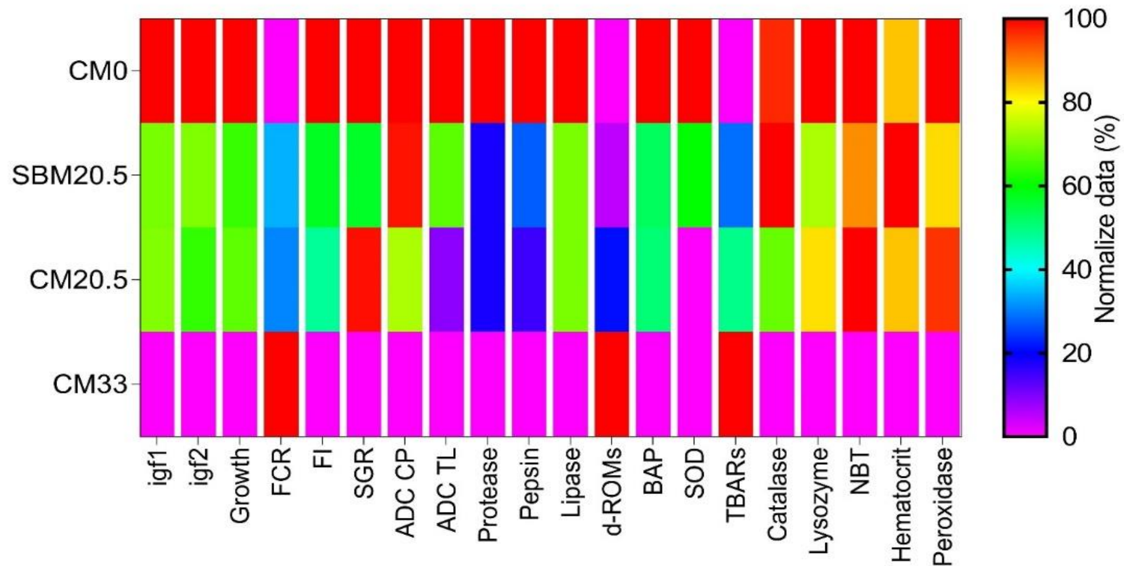


Figure 10: Heatmap diagram of red seabream fed four different diets by replacing fish meal with camelina meal at 0% for reference diet (CM0), 205 g/kg with soybean mean (SBM20.5), 205 g/kg with camelina meal (CM20.5), and 330 g/kg camelina meal (CM33). The horizontal axis showed the parameters being assessed, and the vertical axis showed the dietary groupings. The legend showed the normalized (0–100% scale) mean values of each parameter in the investigation. The red bar and purple bar represent the highest and lowest mean responses, respectively. In the Figure 5 abbreviations in the horizontal axis are define as follows: igf1 and igf2 represent growth factor gene 1 and growth factor gene 2 respectively, Growth: Final weight growth weight, FCR:Feed Conversion ratio FI: Feed intake,SGR: specific growth rate ,ADC CP: Apparent nutrient digestibility of protein ,ADC TL : Apparent nutrient digestibility of Total Lipid, d -ROMs: Reactive Oxygen Metabolites, BAP : Biological Antioxidant Potential, SOD, Superoxide Dismutase, TBARs, Thiobarbutic Reactive Substances, NBT: Nitro blue Tetrazolium

There is a comparable trend in growth performance, immunity, stress resistance, and feed utilization efficacy in red seabream fed diets with 205 g/kg Soybean or 205 g/kg camelina meal (30–80% mean distribution). As shown, supplementation with 330 g/kg camelina meal (CM33) reduced (less than 30% mean distribution the growth performance, digestibility, enzymes efficacy, nutrient utilization index, immunity, and stress capacity (Figure 5).

4.2.10. Principal Components Analysis (PCA)

The principal component analysis (PCA) presented in Figure 6 revealed that PC1 and PC2 components explained 97% (87.7% and 9.04%, respectively) of the total variation. The use of PCA in this study helps to explore existing correlations in relationships between parameters assessed for fish fed different diets. The loading plot helps to show the contribution of each variable to the difference between diets (Table 10).

Table 28: Table of loading for the parameters assessed with principal component analysis. PC1 and PC2 explained the total variation as displayed.

Parameter	PC1	PC2	PC3
igf1	0.24	−0.05	0.08
igf2	0.24	-0.01	0.02
Growth	0.24	−0.02	0.13
FCR	−0.24	0.04	−0.12
FI	0.23	0.14	0.06
SGR	0.21	−0.21	0.44
ADC CP	0.23	−0.12	−0.27
ADC TL	0.20	0.35	−0.35
Protease	0.20	0.4	0.34
Pepsin	0.20	0.41	0.20
Lipase	0.24	−0.05	0.07
d-ROMs	−0.23	0.16	0.20
BAP	0.23	0.13	0.15

SOD	0.20	0.40	−0.33
TBARs	−0.24	−0.08	0.11
Catalase	0.23	−0.11	−0.35
Lysozyme	0.23	−0.14	0.12
NBT	0.22	−0.27	0.05
Hematocrit	0.21	−0.30	−0.28
Peroxidase	0.23	−0.24	0.10

The most significant positive correlations coefficients with the PC1 axis were found in the following parameters: feed intake (FI) growth response (IGF-1, IGF-2, SGR, growth (weight gain)), digestive enzymes (protease, pepsin, lipase), immune response (lysozyme activity, NBT, peroxidase, haematocrit) and antioxidant (catalase, BAP and SOD), apparent digestibility coefficients (ADC total lipid, ADC crude protein). The parameters that correlated most strongly negatively correlated with the PC1 axis were antioxidant (TBARs and d-ROMs) and FCR. The strongest positive correlations with the PC2 axis were found in the parameters: SOD, protease, pepsin, and ADC (TL). The parameter most strongly negatively correlated with the PC2 were SGR, NBT, haematocrit, and peroxidase. These parameters assessed in this investigation were associated with diets (CM0, SBM20.5, CM20.5, and CM0) and feed intake (Figure 6). Antioxidant (BAP, SOD), digestive enzyme (protease, pepsin), digestibility (TL), and growth (BW, IGF-2) enzyme were associated with CM0. Immune (lysozyme, NBT, catalase, peroxidase), blood health (haematocrit), and digestibility (CP) were associated with diet SBM20.5 and CM20.5. The FCR, d-ROMs, and TBARs were associated with CM33 (Figure 6).

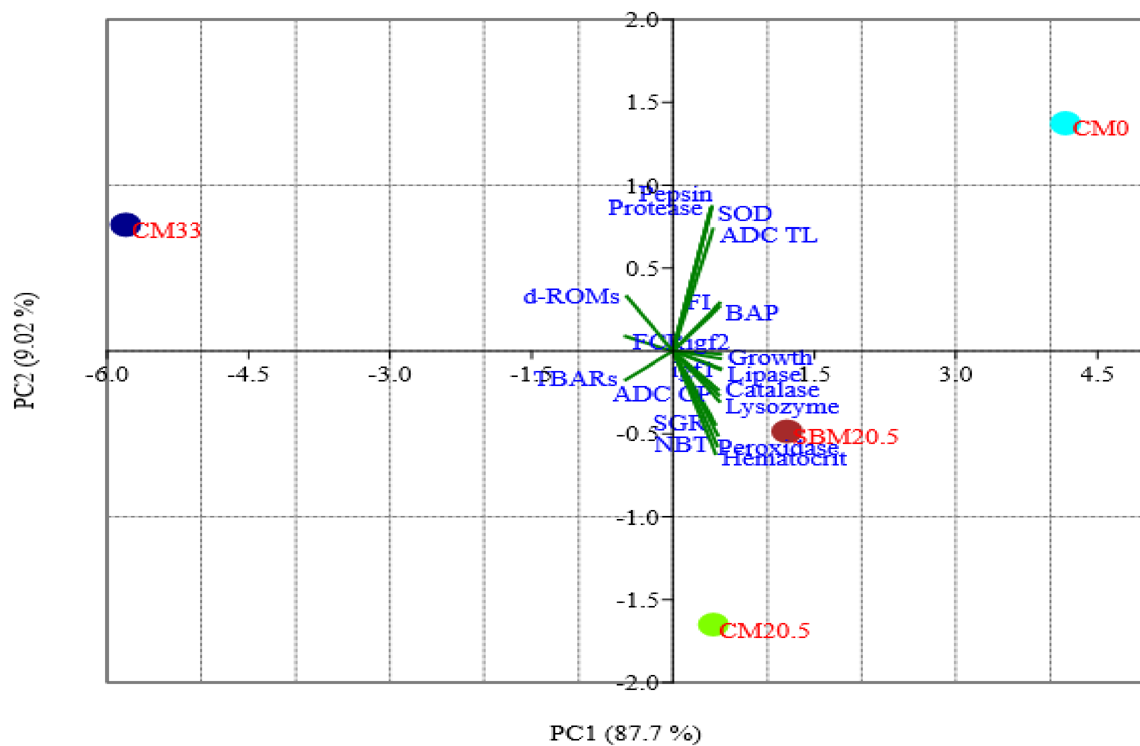


Figure 11: Principal component analysis (PCA) plot (PC: 1:87.7%, PC: 9.04%) correlation of responses of growth, feed utilization indices, immunity markers, stress biomarkers, relative gene expressions, digestibility coefficients, and enzyme activity of red seabream using a covariance, where fours diet treatments formulated by replacing fish meal with camelina meal at 0% for reference diet (CM0), 205 g/kg with soybean mean (SBM20.5), 205 g/kg with camelina meal (CM20.5) and 330 g/kg camelina meal (CM33).

4.2. 11. UPMGA Correlation Matrix

According to Figure 7, the following parameters: pepsin, protease, SOD, FCR, FI, *IGF-1*, *IGF-2*, d-RMOs, growth (weight gain), SGR, ADC (TL), Lipase, BAP, catalase, peroxidase, NBT, SGR had a strong correlation on CM0. Haematocrit, d-ROMs, catalase, ADC (CP) were strongly correlated to SBM20.5. Peroxidase, NBT, SGR are correlated to CM20.5. d-ROMs, FCR, and TBARs, correlated with CM33. The higher the correlation coefficient, the more the influence on the diet.

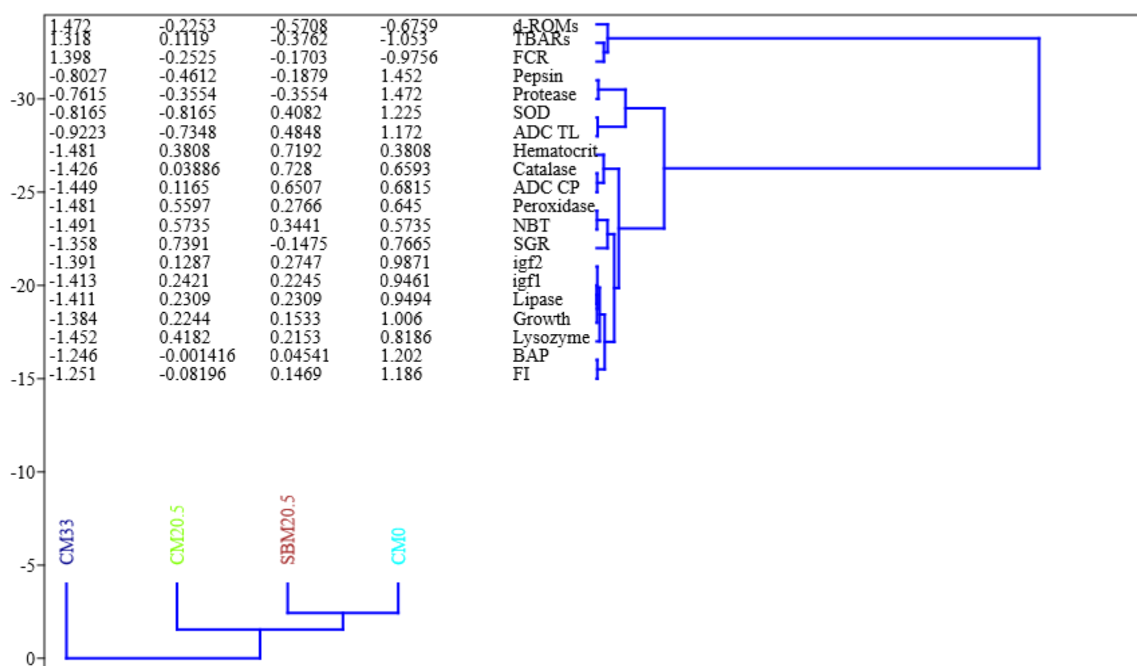


Figure 12: Dendrogram derived from UPMGA analysis of responses of growth, feed utilization indices, immunity markers, stress biomarkers, relative gene expressions, digestibility coefficients, and enzyme activity of red seabream using a correlation matrix model, where fours diet treatments formulated by replacing fish meal with camelina meal at 0% for reference diet (CM0), 205 g/kg with soybean mean (SBM20.5), 205 g/kg with camelina meal (CM20.5) and 330 g/kg camelina meal (CM33).

4.3. Discussion

Feed is a significant contributor to high aquaculture production costs. As a result, there is a global effort to identify affordable inputs to partially substitute expensive aquaculture and animal production feed sources, mainly fish meal (Dawood et al., 2020b). Plant products have recently emerged as potential sources to replace fish meal in aquafeed (Dawood et al., 2020a). The higher inclusion of camelina meals in diets of red seabream decreased growth response, weight gain (WG), and feed intake (FI) while increased feed conversion ratio (FCR) in the present experiment. Inadequate fish growth response observed at CM33 supplementation corroborates earlier work on the supplementation of plant protein in fish feed experimentation (Brown et al., 2016). However, FM replacement with CM up to 205 g/kg positively affected fish final body weight, WG, specific growth rate, or FI. Bullerwell et al. (2016) recommended that the maximum dietary inclusion threshold of camelina meal in juvenile rainbow trout diets is 200 g/kg, although further research will be required to determine more definite values. Pan et al. (2011) found that dietary inclusion levels of high-oil residual camelina meal (HORM) up to 160 g/kg did not negatively affect the growth performance of rainbow trout.

In the present study, despite the experimental diet meeting the amino acid requirement of red seabream, poor growth performance, FI, and FCR were still observed in CM33. In addition, enzyme activities in the intestine and digestibility of nutrients were lowest

among the group. We believe that the dismal growth performance and poor feed utilization obtained in CM33 were caused by high amounts of phytochemicals, which are conventionally referred to as anti-nutritional factors (ANFs). While there are significant nutritional profiles in terms of protein and lipid levels in camelina meal, limitations for its utilization in aquafeed is the presence of ANFs that diminishes feed palatability and digestion of the feed (Matthaus and Zubr,2000) . Previous reports showed that ANFs in camelina include glucosinolates, phytic acid, sinapine, and tannins (Matthaus and Zubr,2000). Bullerwell, et al (2016) reported that a significantly reduced feed intake of rainbow trout fed 300 g/kg camelina may have been due to high glucosinolate levels of this diet. Condition factor and viscero somatic index (VSI) were not affected by dietary CM in red seabream. This result corroborates previous work on plant protein fed to red seabream (Dossou et al.,2021). For utilization of CM on aquaculture feeds, improvement of palatability using attractants and reduction of ANFs in CM by an extruder treatment or fermentation are necessary.

Results of the current study reveal that protein carcass proximate composition was significantly lowered by dietary CM. Thus, carcass compositions were similar to results that have been reported previously in red seabream (Dossou et al., 2018b). Significant influences of dietary rapeseed plant protein lowered crude protein, crude lipid, and energy levels whole body in a study by Kader et al.(2010), similar to the present study. Therefore, we suggest that higher supplementation of CM at CM33 was a critical factor that lowered protein levels and subsequently resulted in poor growth of red sea bream. In contrast, our results agree with Bullerwell et al. (2016) and Pan et al. (2011), who observed that camelina meal inclusion at 200 g/kg and 160 g/kg respectively had a similar carcass composition as control-fed fish.

Endogenous enzymes are fundamental in digesting nutrients in experimental finfish to increase the bioavailability of micronutrients for the sustainability of physiological body needs Adoye et al. (2016) findings of the current feed trial have confirmed that higher proportions of CM (CM33) led to the lowest activity of all digestive enzymes analyzed except lipase activity. Deterioration of enzyme activity was inferred from ANFs, enormous proportions of fiber, and secondary plant metabolites in CM, diminishing nutrient digestion (Hassan et al., 2017; Refstie et al., 2018). Remarkably, the dismal levels of ADC of crude protein in red seabream fed diets with high dietary (CM33) levels may have caused a reduction in pepsin and protease activities caused by protease and trypsin inhibitors present in CM.

The composition of feed ingredients has substantial ramifications on the ADC of various nutrients, notably crude protein, total or crude protein, energy, and dry matter (Dossou et al., 2021). In the present study, diets CM0, SBM20.5, and CM20.5 had significantly superior protein digestibility than diet CM33.3. Similarly, total lipid ADCs were numerically higher for diets CM0, SBM20.5, and CM20.5. No variance was detected in dry matter ADCs in fish fed different camelina diets. These results show that red seabream could not efficiently use increased proportions of CM, leading to growth retardation. The finding in the current feed trial agrees with, Nagel et al. (2017) who reported that rapeseed incorporated diets resulted in low feed ingestion and poor nutritional quality of the diets for juvenile turbot. According to Kokou and Fountoulaki, (2018) plant-based protein sources contain proportions of anti-nutritional forms that lower nutrient digestibility.

Principal component analysis (PCA) (Table 9, Figure 6), Heatmap (Figure 5), UPMGA Correlation matrix (Figure 7) data presented in this manuscript indicate that relative expression of growth-related genes; *IGF-1* and *IGF-2* were higher in CM0, SBM20.5,

and CM20.5 and significantly lower on CM33 diets. This result is consistent with earlier work on the efficacy of liver *IGF-1* and *IGF-2*, which showed that relative expression of *IGF-2* was most significant in the liver of rainbow trout fed the control FM diet and lower in soybean diets, thus asserting that elevated *IGF-2* resulting in higher somatic growth in fish (Eppler et al., 2010; Jiminez-Amilburu et al., 2013). Similarly, there was a sharp downregulation of liver *IGF-I* gene expression in a fish-fed diet with elevated plant protein levels (Bu et al., 2018a; Bu et al., 2018b). Therefore, the present study hypothesizes that there was marked depression of protein synthesis with marginal addition of CM in diets, which resulted in growth retardation of red sea bream. The expression of GH/IGF genes is amplified under optimum nutrients, frequent daily feeding (Hevroy et al., 2011), higher lipid-protein nutrients ratio (Kumar et al., 2011), or the higher inclusions of dietary fishmeal (Gomez-Requeni et al., 2005).

Blood chemistry variables are regarded as the first indicator for the physiology, bio-chemical and pathological status of cultured animals in response to different feeding regimes (Hossain et al, 2016). Hematocrit and plasma glucose levels in CM33 showed significantly lower and higher respectively among the groups. Plasma glucose is generally considered an indication of stress in fish; low glucose levels indicate the low-stress status of fish (Eslamloo et al., 2012). Significantly lower glucose contents in fish-fed diets CM0, SBM20.5, and CM20.5 indicated that red seabream fed these diets displayed better physiological conditions than the group fed CM33. Lower hematocrit levels in CM33 support the previous results that fish groups were in poor health, and in contrast, results show that CM0, SBM20.5, and CM20.5 inclusion of CM in diets of red seabream registered higher hematocrit score thus maintained a normal physiological status.

SBM20.5 and CM20.5 diets groups maintained non-specific immune response, lysozyme activity, nitro blue tetrazolium reduction test (NBT), and peroxidase activity. Conversely, diet CM33 registered significantly lower non-specific immune indicators. Lin and Luo (2011) highlight that high substitution proportions of FM by soybean meal diminished the immune system of tilapia by lowering lysozyme activity in serum. We speculate, therefore, that CM33 leads to the declination of growth performance, among other reasons, due to the poor health of fish.

Our findings show that as dietary CM levels increased, there was a corresponding decrease in liver SOD and CAT activities, while the amount of TBARs in the liver significantly increased. The results show that TBARs content was significantly higher in fish fed the SBM20.5, CM20.5, and CM33 diets than the control diet (CM0). The implication of our findings hypothesizes that high dietary CM and SBM levels may reduce the antioxidant capacity of juvenile red sea bream, thereby breaking the fluctuating cycle of synthesis and deamination of free radicals in organism hepatic oxidative stress induced by the inclusion of high levels of CM. Shen et al. (2020) found that the substitution of cottonseed protein concentrates for a proper amount of fish meal increased SOD activity. The previous antioxidative responses are supported by BAP and d-ROMs results, showing that diets CM0, SBM20.5, and CM20.5 showed high BAP and low d-ROM while CM33 showed low BAP and high d–ROM. The lower antioxidant response in CM33 groups could be the detrimental effects of the increased level of antinutrient levels products present in CM at high supplementation.

In fish farming, growth and stress response management is of primary importance since they affect production and farm income (Piccinetti et al., 2017). Stress indicates hormonal status, nutrient metabolism rate, feed utilization capacity, and overall immunity of

cultured fish (Piccinetti et al., 2017). Acute and lethal stress challenge is used to examine general welfare and health status by measuring the lethal time of 50% mortality (LT_{50}) in the freshwater of the fish (Dawood et al., 2020c). Stress is a precursor for high energy demand in response to stimuli at the expense of anabolic processes, affecting survival and growth performance (Kubilay and Ulukoy , 2002). In the present trial, significantly higher values of LT_{50} obtained in diets CM0, SBM20.5, and CM20.5 indicated high resistance to salinity's acute stress in fish groups fed these diets. On the contrary, fish fed diet CM33 produced lower values of LT_{50}, indicating that a high level of CM supplementation diminished the tolerance of freshwater stress in red seabream. The capacity of fish to repel and adapt in a stressful environment is directly related to enhancing immune capabilities that enable resistance to various stressors and improves growth (Tovar-Ramirez, 2010). Paccinetti et al. (2017) reported that the low cortisol levels detected at the earliest larval stages reflected a stress-coping ability. This feature may protect larvae from the high metabolic demands involved in stress responses, thereby promoting faster growth and survival of Ballan wrasse (*Labrus bergylta*). In a nutshell, the findings of the current study showed that CM protein could replace up to 205 g/kg of FM protein according to growth-related gene expression, growth performance, non-specific immunity, and protein enzyme activity as well as protein ADCs. However, high levels of FM protein substituted by CM protein in diets could decrease the growth performance and hepatic *IGF-I* gene expression level of red sea bream. Results herein provide new insight toward the optimization of alternative plant protein for the proper growth and development of farm-raised fish for human consumption and underscore the ability of camelina protein sources to deliver balanced nutrition. Further studies on protein concentration and the effects of the ANF in

these products may increase the maximum inclusion level of camelina protein products in red seabream feeds.

CHAPTER 5: General Discussion

Recent advances in fish nutrition turned its focus to reduce over-reliance on resources derived from marine environment (Cardinalatte et al., 2019). Modern feed formulations in aquaculture mainly hinge on utilization of alternative feedstuffs to improve the environmental sustainability of the aquaculture sector while making aqua feeds affordable (Cardinalatte et al., 2019; Dossou et al., 2021). Use of alternative feedstuffs require screening to understand the nutritional implications of the feedstuffs on the physiology and performance of tested animals.

Identification of the most expensive components in feed formulations is the first step in reducing aqua-feed costs. Generally, macronutrients; protein and lipids are crucial and more expensive nutrients Dawood et al. (2021a); Dawood et al. 2021b; Dossou et al.(2021) requiring alternative sources in feed-nutritional research. Previous studies underscore that it is important to maintain sufficient supply of protein and lipid in aqua-feed because they are needed for normal and rapid growth as well as energy provision functions (Cardinaletti et al., 2019). Selection of alternative ingredients must focus on nutrients quality of ingredients and composition because these influence the ability of fish to reach their full growth potential. Several alternatives to fish oil and fish meal. The high cost of FO in aquaculture has sparked main research into alternative lipids to replace FO over the past three decades. Our preliminary step of this research was utilization of camelina oil aimed at determing the average inclusion as replacement for FO as reported by (Olsen et al., 2012). Our present research complements the sentiments shared by the FAO which forecasts that the sustainability of the aquaculture enterprise will likely

depend on using terrestrial plant oils for aquaculture feeds rather than exclusively depending on FO as a lipid source (FAO, 2012).

The first part of this study evaluated the nutritional potential of replacing fish oil with camelina oil to ascertain its suitability for utilization in red sea bream diets. It was found out that response in fatty acid content, lipid class composition, fish growth and biochemical blood parameters varied in five different diets formulated by gradually replacing FO with CO. Preliminary results showed increasing camelina oil in diets decreased fish performance. The negative growth may have been caused by decreased amount of Highly Unsaturated Fatty Acids (HUFA); particularly Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA) when high amount of plant oil is added to the fish diets (Haung et al.,2007; Kolumunus et al.,2008, Huyben et al., 2020; Seong et al.,2019, Seong et al., 2020). Our early exploration into CO revealed that abundant fatty acids in camelina oil were ALA, LNA, 18:1n-9 and 20:1n-9, similar fatty acids amount in diets and tissue, corroborating (Hixson et al., 2013; Betancor et al.2021; Osmond et al.,2020). Studies by Hixson et al. (2013) showed that addition of camelina oil increased total and neutral lipids and TAG and lowering polar lipids and PC, PI and PE. In addition, Hixson et al. (2013) reported that high amount of camelina oil were stored in fish tissues as TAG while fish oil were highly oxidised to produce energy necessary for growth.

The blood parameters indicate that physiological state of animals including fish. Camelina oil did not have a pronounced effect on haematocrit and plasma biochemistry of fish in the present experiment and these blood indices for the red sea bream mirrored other previous studies failing within the normal range (Dossou et al., 2018b, Dawood et al, 2021c). Therefore, the study submits that lack of markedly alteration in blood biochemistry indicated maintenance of overall fish health in CO diets.

The second part of the study was designed on the inclusion of soybean lecithin to CO to improve the ingestion of the diets by the fish and its excellent nutritive value. It has been known that marine animals such as prawn*, penaus japonicus* , and *P chinensis*, Japanese flounder, *paralitchys olivaceus* , *plecoglossus altivelis* and red sea bream, *Pagrus major* Kanazawa et al. (1983) require phospholipids like soyabean lecithin for normal growth and growth. It has been reported that soybean lecithin contains high amount phospholipids: phosphatidycholine (PC) and Phosphatidyinisitol (PL) serve as growth promoting factors for marine species (Kanazawa ,1991; Kanazawa et al., 1983). Furthermore, Yoshida (1992) suggested that soyabean lecithin may contain some growth promoting factors apart from the known phospholipids: phosphatidycholine (PC) and Phosphatidyinisitol (PL). Combining soybean lecithin and CO increase the optimum amount of the HUFA, enhance feed intake and improve growth in this trial. Results showed that 100 % substitution of FO with CO growth unaffected. This is attributed firstly, to an efficient feed utilization capacity where feed intake and feed conversation ratios were similar in fish fed both FO and CO diets. Furthermore, the digestibility studies are important indicators of palatability and acceptance of feed in larvae and juvenile that must be considered to highlight how effective the ingredients performed in promoting growth (Dossou et al., 2021). Apparent nutrients digestibility coefficients were similar in all feed groups, suggesting that PUFA in CO is essential to enable efficient catabolism and lipid digestion confirming CO as suitable for FO replacement candidate for aquaculture diets. These results are consistent with previous results which showed that fish fed diets CO replacement for fish oil grew well and showed no negative effects on any performance parameter evaluated Betancor et al. (2015), indicating this CO was also a feasible alternative to fish oil.

Catalase (CAT) and Superoxide dismutase (SOD) are the main antiperoxidation enzymes of the oxidation resistance defense mechanism in fish (Yu et al., 2019). Therefore, CAT and, SOD are major indicators for the oxidation resistance potential. Outcome of antioxidant parameters; SOD and Catalase indicate that CO maintained and modulates resistance to peroxidation tolerance capacity in muscle and plasma with slight alterations at full substitution in liver. Moreover, CO has high proportion of antioxidant Vitamin E (Hixson et al., 2014a, 2014b); thus we alluded that at high CO red sea bream were able withstand stress precursor due to natural synthesis of the most potent tocopherol to regulate oxidation in plasma and muscle. The marginal changes in liver at full substitution might emanate from PUFAs are vulnerable to scavenging radicals that form peroxides responsible for cell injury. CAT have capacity to convert hydrogen peroxides into oxygen and water through decomposition thereby protecting cells from injury by hydrogen peroxides (Bhagat et al., 2016; Yu et al.,2016).

Histological studies reflect impacts feed composition and source on pathology and morphology of the tissue. Liver and viscera are storage organs for lipid especially oil in form of neutral fraction particularly the TAG. The proportion of whole body to liver weight is called Hepatosomatic index (HSI). Since liver is a storage organ for oil, HSI is the measure for the extent of storage and the amount of oil being stored and these reflect nutritional and pathology of the tissues. Results of the present study indicate that there were similaries in the HSI between red seabream groups fed CO and FO diets, confirming that CO did not influence liver damage. The HSI complements histology data where gradual substitution of FO with CO `does not show vacuolisation, but only relatively bigger cell size in 100% FO substitution for CO indicating a considerable amount of

deposited lipid in form oil; but did not warrant any pathological injury to the whole tissues and not did not affect growth performance.

The success and sustainability of aquaculture production is determined by protein sources used in aquaculture feeds (Cardinaletti et al., 2019). In conventional fish feed, major protein sources are mostly animal based particularly fishmeal. Unfortunately, fish meal prices are not affordable by most aquaculture farmers leading to investigation into alternatives. Substituting fish meal with other sources must be tailored at maintaining growth responses and overarching health aspects of aquatic animals. Alternatives such as algal powder and oil, myriad marine by-products and insect meal require specialized, sophisticated technology and infrastructure which is not only economically not feasible for many but also requiring special set of skills to operate. On the other hand, vegetable and plant sources are easily accessible and affordable.

The final part of our study explored the use of camelina meal (CM) replacing fish meal (FM) in diets of red sea bream and observe overall performance. Tacon (2015) underscores that in the last two and half decades there has a been a flurry of research directed at reducing dietary inclusion of FM with plant protein sources that are readily present in the feed market and are comparatively affordable and accessible by fish farmers. Results in this present experiment show that marginal inclusion of CM in practical diets of red sea bream resulted in decreased growth, expression of growth feed utilization and FCR. In addition, higher CM levels impede the growth genes IGf1 and IGF2 which were evidently down regulated in the present feed trial. The findings herein are in agreement with several previous studies by Bullerwell et al. (2016) which found that 20% camelina meal in diets of rainbow trout had similar growth performance to fish fed control diet. Our study indicates that inclusion of 20.5% of camelina meal in diets maintain growth,

diminishes FCR and FI, digestibility and digestive enzyme activity similar to control diet. Digestive enzyme activity and digestibility of nutrients are dependable tools that can be employed as indices of digestive processes and nutritional state of animals (Ueberschär, 1988). Utilization of enzymes is known to catalyse or activate zymogens to enhance digestion and growth responses (Dabrowski, 1979; Lauf and Hoffer, 1984; Kolkovski et al., 1993).Studies by Bullerwell et al. (2016); Hixson et al.(2016) indicate that camelina meal high inclusion levels (80 and 300 g/ kg dietary inclusion) decreased feed ingestion and weight gain in salmon, and lowered weight gain and increased feed conversion ratio in trout, showing a lower utilization of nutrients in tested fish species. Furthermore, camelina meal contains different anti nutritional factors (ANFs), which evidently hampered digestive and absorptive activity fish fed the test diets, a reason several studies reported negative impacts on growth response and tissue histo-morphology at high levels of CM inclusion (Brown et al., 2016; Bullerwell et al., 2016; Hixson et al., 2016)

Stress tolerance potential was determined using Oxidative stress was measured using the free radical analytical system comprised of defensive enzymes SOD and CAT as well as low molecular tool; TBARs. In order to verify the results an assessment of the derivatives of oxidative stress reactive oxygen metabolites (d-ROMs test) and biological antioxidant potential (BAP test) were measured too. Generally, results painted a diminishing stress capacity in red sea bream fed higher levels of CM. The foregoing results are alluded to an imbalance oxidant activity exceeds the neutralizing capacity of antioxidants at high CM inclusion rate in diets (Celi et al., 2010). In recent years, SOD, Catatalse and TBars, d- ROMs and BAP were reported to be reliable parameters for determining the oxidative stress conditions of fish (Gao et al., 2012). Usually stress is a precursor to immunity status as physiological condition indicators in animals. Nonspecific immune parameters:

Lysozyme activity, peroxidase active and nitro blue tetrazolium exhibited depressing trend with addition of CM and were significantly decreased at highest CM inclusion. We suggested that deleterious ANF and other oxidative molecules in CM not only stressed the fish but weakened the overarching immune capability of the fish thereby negatively impacting on growth performance. Secondary plant metabolites(ANF) can be removed, and reduced through natural approaches e.g. fermentation with biological terms (yeast, probiotics, prebiotics, and enzymes), changing to different forms like concentrates and physical methods like soaking in water or subjecting to heat (Dawood et al., 2020b; Dossou et al., 2018a,)

In conclusion, camelina oil completely (100%) replaced fish oil in diet of red sea bream (*pagrus major*) without alterations on growth and health of fish. Camelina meal can replace 20.5% of fish meal in diet of red sea bream (*pagrus major*). Higher camelina meal 33% inclusion diminished growth and health of red sea bream. Long term study utilising camelina oil is highly recommended to establish definitive optimum level to include in diets. Optimisation of camelina meal in its utilization is recommended through fermentation for decreasing antinutritional factors (ANF) and supplementation of function materials (yeast, probiotics, prebiotics, and enzymes).

References

1. AOAC (1998). Association of official analytical chemists. In *Official Methods of Analysis of Official Analytical Chemists International*, 16th ed; Washington, DC, USA, 1998.
2. AOAC. (2012). *Official Methods of Analysis of Aoac International*, 19th ed.; Aoac International: Gaithersburg, MD, USA.
3. Abele, D., Puntarulo, S. (2004). Formation of reactive species and induction of antioxidant defence systems in polar and temperate marine invertebrates and fish. Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 138, 405–415.
4. Ackman, R.G. (1998). Remarks on official methods employing boron trifluoride in the preparation of methyl esters of the fatty acids of fish oils. J. Am. Oil Chem. Soc. 75, 541–545.
5. Adeoye, A.A., Jaramillo-Torres, A., Fox, S.W., Merrifield, D.L., Davies, S.J. (2016). Supplementation of formulated diets for tilapia (*Oreochromis niloticus*) with selected exogenous enzymes: Overall performance and effects on intestinal histology and microbiota. Anim. Feed. Sci. Technol. 215, 133–143.
6. Abdel-Warith, A.W.A., Younis, E.M., Al-Asgah, N.A., Gewaily, M.S., El-Tonoby, S.M., Dawood, M.A.O. (2021). Role of fucoidan on the growth behavior and blood metabolites and toxic effects of atrazine in Nile tilapia *Oreochromis niloticus* (linnaeus, 1758). Animals. 11, 1448
7. Arab-Tehrany, E., Jacquot, M., Gaiani, C., Imran, M., Desobry, S., Linder, M. (2012). Beneficial effects and oxidative stability of omega-3 long-chain polyunsaturated fatty acids. Trends Food Sci. Technol. 25, 24–33.
8. Anderson, D., Siwicki, A. (1995). *Basic Hematology and Serology for Fish Health Programs*; Fish Health Section; Asian Fisheries Society: Manila, Philippines ; p. 18.

9. Bhagat, J., Ingole, B., Singh, N. (2016). Glutathione s-transferase, catalase, superoxide dismutase, glutathione peroxidase, and lipid peroxidation as biomarkers of oxidative stress in snails: A review. Invertebr. Surviv. J. 13, 336–349.
10. Bell, J.G., Strachan, F., Good, J.E., Tocher, D.R. (2006). Effect of dietary echium oil on growth, fatty acid composition and metabolism, gill prostaglandin production and macrophage activity in atlantic cod (*Gadus morhua* L.). Aquac. Res. 37, 606–617.
11. Bell, J.G., Tocher, D.R.; MacDonald, F.M., Sargent, J.R. (1995). Effects of dietary borage oil [enriched in γ-linolenic acid, 18: 3 (n-6)] or marine fish oil [enriched in eicosapentaenoic acid, 20: 5 (n-3)] on growth, mortalities, liver histopathology and lipid composition of juvenile turbot (*Scophthalmus maximus*). Fish Physiol. Biochem. *14*, 373–383.
12. Betancor, M., Sprague, M., Usher, S., Sayanova, O., Campbell, P., Napier, J.A., Tocher, D.R. (2015). A nutritionally-enhanced oil from transgenic *Camelina sativa* effectively replaces fish oil as a source of eicosapentaenoic acid for fish. Sci. Rep. 5, 1–10.
13. Betancor, M.B., MacEwan, A., Sprague, M., Gong, X., Montero, D., Han, L., Napier, J.A., Norambuena, F., Izquierdo, M., Tocher, D.R. (2021). Oil from transgenic *Camelina sativa* as a source of EPA and DHA in feed for European sea bass (*Dicentrarchus labrax* L.). Aquaculture. 530, 735759.
14. Betancor, M.B., Sprague, M., Sayanova, O., Usher, S., Metochis, C., Campbell, P.J., Napier, J.A., Tocher, D.R. (2016). Nutritional evaluation of an EPA-DHA oil

from transgenic *Camelina sativa* in feeds for post-smolt Atlantic salmon (*Salmo salar* L.). *PLoS ONE*, *11*, e0159934.

15. Betancor, M.B., Li, K., Bucerzan, V.S., Sprague, M., Sayanova, O., Usher, S., Han, L., Norambuena, F., Torrissen, O., Napier, J.A. (2018) Oil from transgenic *camelina sativa* containing over 25 % n-3 long-chain pufa as the major lipid source in feed for Atlantic salmon (*Salmo salar*). Br. J. Nutr. 119, 1378–1392.
16. Bianchi, M.C.G., Chopin, F., Farme, T., Franz, N., Fuentevilla, C., Garibaldi, L., Laurenti, A.L.G. (2020). *FAO: The State of World Fisheries and Aquaculture*; Food and Agriculture Organization of the United Nations: Rome, Italy, p. 200.
17. Biswas, A., Araki, H., Sakata, T., Nakamori, T., Kato, K., Takii, K. (2017). Fish meal replacement by soy protein from soymilk in the diets of red sea bream (*Pagrus major*). Aquac. Nutr. 23, 1379–1389.
18. Brown, T.D., Hori, T.S., Xue, X., Ye, C.L., Anderson, D.M., Rise, M.L. (2016). Functional genomic analysis of the impact of camelina (*camelina sativa*) meal on Atlantic salmon (*Salmo salar*) distal intestine gene expression and physiology. Mar. Biotechnol.18, 418–435.
19. Bu, X.-Y.,Wang, Y.-Y., Chen, F.-Y., Tang, B.-B., Luo, C.-Z., Wang, Y., Ge, X.-P., Yang, Y.-H. (2018a) An evaluation of replacing fishmeal with rapeseed meal in the diet of *pseudobagrus ussuriensis*: Growth, feed utilization, non-specific immunity, and growth-related gene expression. J. World Aquac. Soc. 49, 1068–1080.
20. Bu, X., Lian, X., Zhang, Y., Chen, F., Tang, B., Ge, X., Yang, Y. (2018b). Effects of replacing fish meal with corn gluten meal on growth, feed utilization, nitrogen

and phosphorus excretion and igf-i gene expression of juvenile *pseudobagrus ussuriensis*. Aquac. Res. 49, 977–987.

21. Budin, J.T., Breene, W.M., Putnam, D.H. (1995) Some compositional properties of camelina (*Camelina sativa* l. Crantz) seeds and oils. J. Am. Oil Chem. Soc. 72, 309–315.
22. Bullerwell, C.N., Collins, S.A., Lall, S.P., Anderson, D.M. (2016). Growth performance, proximate and histological analysis of rainbow trout fed diets containing *camelina Sativa* seeds, meal (high-oil and solvent-extracted) and oil. Aquaculture.452, 342–350.
23. Bligh, E.G., Dyer, W.J. A. (1959). rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911–917.
24. Caballero, M.J., Obach, A., Rosenlund, G., Montero, D., Gisvold, M., Izquierdo, M.S. (2002). Impact of different dietary lipid sources on growth, lipid digestibility, tissue fatty acid composition and histology of rainbow trout, *Oncorhynchus mykiss*. Aquaculture. 214, 253–271.
25. Canadian Food Inspection Agency (2017). *The Biology of Camelina sativa(L.) Crantz (Camelina). Directive 94–08 (Dir94-08), AssessmentCriteria for Determining Environmental Safety of Plant with Novel Traits*.Retrieved from http://www.inspe ction.gc.ca/plant s/plants-withnovel-trait s/appli cants/ direc tive-94-08/biolo gy-docum ents/camel ina-sativa-l-/eng/13309 71423 348/13309 71509470.
26. Cardinaletti, G., Randazzo, B., Messina, M., Zarantoniello, M., Giorgini, E., Zambelli, A., Bruni, L., Parisi, G., Olivotto, I., Tulli, F. (2019). Effects of graded

dietary inclusion level of full-fat *hermetic illucens* prepupae meal in practical diets for rainbow trout (*Oncorhynchus mykiss*). Animals. 9, 251.

27. Celi, P., Sullivan, M., Evans D. (2010). “The stability of the reactive oxygen metabolites (d-ROMs) and biological antioxidantpotential (BAP) tests on stored horse blood,” The Veterinary Journal. 183; 2, 217–218, 2010.
28. Dabrowski, K. (1984) The feeding of fish larvae: present (state of the art) and perspective. Repro. Nutri. Develop. 24(6): 807-833.
29. Dawood, M.A.O., Eweedah, N.M., Khalafalla, M.M., Khalid, A., Astley, A.E., Fadl, S.E., Amin, A.A., Paray, B.A., Ahmed, H.A. (2020a). *Saccharomyces cerevisiae* increases the acceptability of Nile tilapia (*Oreochromis niloticus*) to date palm seed meal. Aquac. Rep. 17, 100314.
30. Dawood, M.A.O., Koshio, S., Ishikawa, M., Yokoyama, S. (2015). Interaction effects of dietary supplementation of heat-killed *Lactobacillus plantarum* and β-glucan on growth performance, digestibility and immune response of juvenile red sea bream, *Pagrus major*. Fish Shellfish Immunol. 45, 33–42
31. Dawood, M.A.O. (2021a). Nutritional immunity of fish intestines: Important insights for sustainable aquaculture. Rev. Aquac. 13, 642–663.
32. Dawood, M.A.O., Ali, M.F., Amer, A.A., Gewaily, M.S., Mahmoud, M.M., Alkafafy, M., Assar, D.H., Soliman, A.A., Van Doan, H. (2021b). The influence of coconut oil on the growth, immune, and antioxidative responses and the intestinal digestive enzymes and histomorphometry features of Nile tilapia (*Oreochromis niloticus*). Fish Physiol. Biochem., 1–12.
33. Dawood, M.A.O., Noreldin, A.E., Ali, M.A.M., Sewilam, H. (2021c). Menthol essential oil is a practical choice for intensifying the production of Nile tilapia

(*Oreochromis niloticus*): Effects on the growth and health performances. Aquaculture. 543, 737027.

34. Dawood, M.A.O., Koshio, S., Ishikawa, M., Yokoyama, S., El Basuini, M.F., Hossain, M.S., Nhu, T.H., Moss, A.S., Dossou, S., Wei, H., (2017). Dietary supplementation of β- glucan improves growth performance, the innate immune response and stress resistance of red sea bream, *Pagrus major*. Aquac. Nutr. 23 (1), 148–159.
35. Dawood, M.A.O., Koshio, S., Ishikawa, M., Yokoyama, S. (2016a). Effects of dietary inactivated *Pediococcus pentosaceus* on growth performance, feed utilization and blood characteristics of red sea bream, *Pagrus major* juvenile. Aquac. Nutr. 22, 923–932.
36. Dawood, M.A.O., Koshio, S., Ishikawa, M., Yokoyama, S. (2016b). Immune responses and stress resistance in red sea bream, *Pagrus major*, after oral administration of heat-killed *Lactobacillus plantarum* and vitamin C. Fish Shellfish Immunol. 54, 266–275.
37. Dawood, M.A.O., Noreldin, A.E., Sewilam, H. (2021d). Long term salinity disrupts the hepatic function, intestinal health, and gills antioxidative status in Nile tilapia stressed with hypoxia. Ecotoxicol. Environ. Saf. 220, 112412.
38. Dawood, M.A.O., Koshio, S. (2020b). Application of fermentation strategy in aquafeed for sustainable aquaculture. Rev. Aquac. 12, 987–1002
39. Dawood, M.A.O., Koshio, S., Fadl, S.E., Ahmed, H.A., El Astley, A., Abdel-Daim, M.M.; Alkahtani, S. (2020c). The modulatory effect of mannan oligosaccharide on oxidative status, selected immune parameters and tolerance against low salinity stress in red sea bream (*Pagrus major*). Aquac. Rep., 16, 100278.

40. De Silva, S., Anderson, T. (1995). Metabolism. In: Fish Nutrition in Aquaculture. Chapman and Hall. London, UK. pp 41-10.
41. Dossou, S., Dawood, M.A.O., Zaineldin, A.I., Abouelsaad, I.A., Mzengereza, K., Shadrack, R.S., Zhang, Y., El-Sharnouby, M., Ahmed, H.A., El Basuini, M.F. (2021). Dynamical hybrid system for optimizing and controlling efficacy of plant-based protein in aquafeeds. Complexity. 2021, 9957723
42. Dossou, S., Koshio, S., Ishikawa, M., Yokoyama, S., Dawood, M.A.O., El Basuini, M.F., Olivier, A., Zaineldin, A.I. (2018a). Growth performance, blood health, antioxidant status and immune response in red seabream (*Pagrus major*) fed *Aspergillus oryzae* fermented rapeseed meal (rm-koji). Fish Shellfish Immunol. 75, 253–262.
43. Dossou, S., Koshio, S., Ishikawa, M., Yokoyama, S., Dawood, M.A.O., El Basuini, M.F., El-Hais, A.M., Olivier, A. (2018b).Effect of partial replacement of fish meal by fermented rapeseed meal on growth, immune response and oxidative condition of red sea bream juvenile, *Pagrus major*. Aquaculture. 490, 228–235.
44. Duan, C. (1998). Nutritional and developmental regulation of insulin-like growth factors in fish. J. Nutr. 128, 306S–314S.
45. El Basuini, M.F., El-Hais, A.M., Dawood, M.A.O., Abou-Zeid, A.E.-S., El-Damrawy, S.Z., Khalafalla, M.M.E.L.S., Koshio, S., Ishikawa, M., Dossou, S. (2016). Effect of different levels of dietary copper nanoparticles and copper sulfate on growth performance, blood biochemical profiles, antioxidant status and immune response of red seabream (*Pagrus major*). Aquaculture, *455*, 32–40

46. Emery, J.A., Norambuena, F.,Trushenski, J., Turchini, G.M. (2016). Uncoupling EPA and DHA in fish nutrition: Dietary demand is limited in Atlantic salmon and effectively met by DHA alone. Lipids. 51, 399–412.
47. El Asely, A.M., Reda, R.M., Salah, A.S., Mahmoud, M.A., Dawood, M.A.O. (2020). Overall performances of Nile tilapia (*Oreochromis niloticus*) associated with using vegetable oil sources under suboptimal temperature. Aquaculture Nutrition. 26, 1154–1163.
48. Eslamloo, K., Falahatkar, B., Yokoyama, S. (2012). Effects of dietary bovine lactoferrin on growth, physiological performance, iron metabolism and non-specific immune responses of Siberian sturgeon *Acipenser baeri*. Fish Shellfish Immunol. 32, 976–985.
49. Espe, M., Lemme, A., Petri, A., El-Mowafi, A.(2006). Can Atlantic salmon (*Salmo salar*) grow on diets devoid of fish meal? Aquaculture. 255, 255–262.
50. Eppler, E., Berishvili, G., Mazel, P., Callers, A., Hwang, G., Maclean, N., Reinecke, M.(2010). Distinct organ-specific up-and down-regulation of igf-i and igf-ii mRNA in various organs of a gh-overexpressing transgenic Nile tilapia. Transgenic Res. 19, 231–240.
51. FAO, Fisheries and Aquaculture Department. (2012). The state of world fisheries and aquaculture Rome, http://www.fao.org/docrep/016/i2727e/i2727e.pdf; 2012.
52. FAO (Food and Agriculture Organization). (2009). World review of fisheries and aquaculture. In, State of the world fisheries and aquaculture 2008. FAO Fish Tech. Paper. Rome, Italy

53. FAO (Food and Agriculture Organization of the United Nations). (2018) State of the world's fisheries and aquaculture - Meeting the sustainable development goals. Rome, Italy. Licence: CC BY-NC-SA 3.0 IGO**.**
54. Fenton, J.I., Hord, N.G., Ghosh, S., Gurzell, E.A. (2013). Immunomodulation by dietary long chain omega-3 fatty acids and the potential for adverse health outcomes. Prostaglandins Leukot. Essent. Fatty Acids. 89, 379–390.
55. Furukawa, A. (1966) On the acid digestion method for the determination of chromic oxide as an index substance in the study of digestibility of fish feed. Nippon. Suisan Gakkaishi. 32, 502–506.
56. Gao, J. S. Koshio, M. Ishikawa, S. Yokoyama, R. E. P. Mamauag, and Y. Han (2012). "Effects of dietary oxidized fish oil with vitamin E supplementation on growth performance and reduction of lipid peroxidation in tissues and blood of red sea bream (*Pagrus major*) Aquaculture, vol. 356-357, pp. 73–79
57. Glencross, B.D., Baily, J., Berntsen, M.H.G., Hardy, R., MacKenzie, S., Tocher, D.R. (2020). Risk assessment of the use of alternative animal and plant raw material resources in aquaculture feeds. Rev. Aquac. *12*, 703–758.
58. Gómez-Requeni, P., Calduch-Giner, J., Vega-Rubín de Celis, S., Médale, F., Kaushik, S.J., Pérez-Sánchez, J. (2005). Regulation of the somatotropic axis by dietary factors in rainbow trout (oncorhynchus mykiss). Br. J. Nutr. 94, 353–361
59. Hevrøy, E.M., Ezpeleta, C., Shimizu, M., Lanzén, A., Kaiya, H., Espe, M., Olsvik, P.A. (2011). Effects of short-term starvation on ghrelin, gh-igf system, and IGF-binding proteins in Atlantic salmon. *Fish Physiol. Biochem.*, *37*, 217–232.
60. Hammer, Ø., Harper, D.A., Ryan, P. (2001). Paleontological statistics software package for education and data analysis. Paleontol. Electron. 4, 9.

61. Higgs, D., Dong, F. (2000) Lipids and fatty acids. In: Stickney R (ed) Encyclopedia of aquaculture. Wiley, New York, pp 476–496**.**

62. Hassaan, M.S., Goda, A.M.A.S., Kumar, V. (2017). Evaluation of nutritive value of fermented de-oiled physic nut, jatropha curcas, seed meal for Nile tilapia *Oreochromis niloticus* fingerlings. Aquac. Nutr. 23, 571–584.

63. Hossain, M.S., Koshio, S., Ishikawa, M., Yokoyama, S., Sony, N.M., Dawood, M.A.O., Kader, M.A., Bulbul, M., Fujieda, T. (2016). Efficacy of nucleotide related products on growth, blood chemistry, oxidative stress and growth factor gene expression of juvenile red sea bream, *Pagrus major*. Aquaculture. 464, 8–16

64. Huyben, D., Rimoldi, S., Ceccotti, C., Montero, D., Betancor, M., Iannini, F., Terova, G. (2020). Effect of dietary oil from *Camelina sativa* on the growth performance, fillet fatty acid profile and gut microbiome of gilthead sea bream (*Sparus aurata*). PeerJ, 8, e10430.

65. Hixson, S.M.; Parrish, C.C.; Wells, J.S.; Winkowski, E.M.; Anderson, D.M.; Bullerwell, C.N. (2016). Inclusion of camelina meal as a protein source in diets for farmed salmonids. Aquac. Nutr. 22, 615–630.

66. Hixson, S.M., Parrish, C.C., Anderson, D.M. (2013). Effect of replacement of fish oil with camelina (*Camelina sativa*) oil on growth, lipid class and fatty acid composition of farmed juvenile Atlantic cod (*Gadus morhua*). Fish Physiol. Biochem., *39*, 1441–1456.

67. Hixson, S.M., Parrish, C.C., Anderson, D.M. (2014a). Changes in tissue lipid and fatty acid composition of farmed rainbow trout in response to dietary camelina oil as a replacement of fish oil. Lipids. 49, 97–111.

68. Hixson, S.M., Parrish, C.C., Anderson, D.M. (2014b) Full substitution of fish oil with camelina (*Camelina sativa*) oil, with partial substitution of fish meal with camelina meal, in diets for farmed Atlantic salmon (*Salmo salar*) and its effect on tissue lipids and sensory quality. Food Chem. 157, 51–61.
69. Hixson, S.M., Parrish, C.C. (2014).Substitution of fish oil with camelina oil and inclusion of camelina meal in diets fed to Atlantic cod (*Gadus morhua*) and their effects on growth, tissue lipid classes, and fatty acids1. J. Anim. Sci. 92, 1055–1067.
70. Huang, S.S.Y., OO, A.N., Higgs, D.A., Brauner, C.J., Satoh, S. (2007). Effect of dietary canola oil level on the growth performance and fatty acid composition of juvenile red sea bream, *Pagrus major*. Aquaculture. 271, 420–431.
71. Ido, A., Iwai, T., Ito, K., Ohta, T., Mizushima, T., Kishida, T., Miura, C., Miura. (2015). T. Dietary effects of housefly (*Musca Domestica*) (Diptera: Muscidae) pupae on the growth performance and the resistance against bacterial pathogen in red sea bream (*Pagrus major*) (Perciformes: Sparidae). Appl. Entomol. Zool. 50, 213–221.
72. Iheanacho, S. C., Ikwo, T. N., Igweze, N., Chukwuidha, C., Ogueji, E. O., Onyeneke, R. (2018). Effect of different dietary inclusion levels of melon seed (*Citrullus lanatus*) peel on growth, haematology and histology of *Oroechromis niloticus* juvenile. *Turkish Journal of Fisheries and Aquatic Science*, *18*(3), 377–384.
73. Iheanacho, S.C., Odo, G.E. (2020). Neurotoxicity, oxidative stress biomarkers and haematological responses in African catfish (*Clarias gariepinus*) exposed to

polyvinyl chloride microparticles. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 232, 108741.

74. Ishikawa, M., Teshima, S.i., Kanazawa, A., Koshio, S. (1997). Measurements of digestibilities of cholesterol and fatty acids using 5α-cholestane as an inert marker in the tilapia, *Oreochromis niloticus*, and the freshwater prawn, *Macrobrachium rosenbergii*. J. Appl. Ichthyol.13, 31–35.
75. Jabir, M. A. R., Razak, S., Vikineswary, S. (2012). Chemical composition and nutrient digestibility of super worm meal in red tilapia juvenile. Pakistan Veterinary Journal. 32, 489–493.
76. Jiang, D., Zheng, J., Dan, Z., Tang, Z., Ai, Q.; Mai, K. (2019). Effects of five compound attractants in high plant-based diets on feed intake and growth performance of juvenile turbot (*Scophthalmus Maximus* l.). Aquac. Res. 50, 2350–2358.
77. Jiménez-Amilburu, V., Salmerón, C., Codina, M., Navarro, I., Capilla, E., Gutiérrez, J. (2013). Insulin-like growth factors effects on the expression of myogenic regulatory factors in gilthead sea bream muscle cells. Gen. Comp. Endocrinol. 188, 151–158.
78. Jin, M., Yuan, Y., Lu, Y., Ma, H., Sun, P., Li, Y., Qiu, H., Ding, L., Zhou, Q. (2017). Regulation of growth, tissue fatty acid composition, biochemical parameters and lipid related genes expression by different dietary lipid sources in juvenile black seabream, *AcanthoPagrus schlegelii*. Aquaculture. 479, 25–37.
79. Jobling, M., Leknes O., Saether B., Bendiksen A. (2008). Lipid and fatty acid dynamics in Atlantic cod, *Gadus morhua*, tissues: influence of dietary lipid concentrations and feed oil sources. Aquaculture. 281:87–94

80. Juaneda, P., Rocquelin, G., (1985). Rapid and convenient separation of phospholipids and non-phosphorus lipids from rat heart using silica cartridges. Lipids 20, 40–41. https://doi.org/10.1007/BF02534360.
81. Kader, M.A., Koshio, S., Ishikawa, M., Yokoyama, S., Bulbul, M.(2010). Supplemental effects of some crude ingredients in improving nutritive values of low fishmeal diets for red sea bream, *Pagrus major*. Aquaculture 308, 136–144
82. Kokou, F., Fountoulaki, E. (2018). Aquaculture waste production associated with antinutrient presence in common fish feed plant ingredients. Aquaculture. 495, 295–310.
83. Komilus, C.F., Shichi, N., Koshio, S., Ishikawa, M., Yokoyama, S., Michael, F.R., Gao, J., Makita, C. (2008). Influences of palm oil blended with fish oil on growth performances and lipid profiles of red seabream *Pagrus major*. Aquac. Sci. 56, 317–326.
84. Kanazawa, A., Teshima, S., Inamori, S., and Matsubara H. (1983). Effects of dietary phospholipid on growth of the larval red sea bream and knife jaw., mem.fac. Fish. Kagoshima Unive,m 32. 115-120.
85. Kanazawa, A. (1991). Essential phospholipid of larval fish and crustanceans., VI International Symposium of fish nutrition and Feeding,Biarritis , France.
86. Kohen, R., Nyska, A. (2002). Invited review: Oxidation of biological systems: Oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. Toxicol. Pathol. 30, 620–650.
87. Kolkovski, S., Tandler, A., Kissil, G.W., Gertler, A. (1993) The effect of dietary exogenousdigestive enzymes on ingestion, assimilation, growth and survival of

gilthead seabream (*Sparus aurata*, Sparidae, Linnaeus) larvae. Fish Physiol. Biochem. 12:203-209.

88. Koshio, S. (2002). Red sea bream, *Pagrus major. Nutr. Requir. Feed. Finfish Aquac.* 51-63, doi:10.1079/9780851995199.0051.
89. Kumar, V., Makkar, H.P.S., Becker, K. (2011) .Detoxified *Jatropha curcas* kernel meal as a dietary protein source: Growth performance, nutrient utilization and digestive enzymes in common carp (*Cyprinus Carpio* l.) fingerlings. Aquac. Nutr. 17, 313–326.
90. Kubilay, A., Uluköy, G. (2002). The effects of acute stress on rainbow trout (*Oncorhynchus mykiss).* Turk. J. Zool. 26, 249–254.
91. Lall, S., Nanton, D. (2002). Nutrition of Atlantic cod. Bull. Aquac. Assoc. Can. 102: 23-26
92. Lall, S., Tibbetts, S. (2009). Nutrition, feeding and behaviour of fish. Vet. Clin. North Am. Exot. Anim. Pract. 12: 361-372.
93. Lauff, M., Hoffer, R. (1984). Proteolytic enzymes in fish development and the importance of dietary enzymes. Aquaculture 37:335-346.
94. Lin, S., Luo, L. (2011). Effects of different levels of soybean meal inclusion in replacement for fish meal on growth, digestive enzymes and transaminase activities in practical diets for juvenile tilapia, *Oreochromis niloticus* × *O. aureus*. Anim. Feed. Sci. Technol.168, 80–87.
95. Lygren, B., Sevier, H.; Hjeltness, B., Waagbø, R. (1999). Examination of the immunomodulatory properties and the effect on disease resistance of dietary bovine lactoferrin and vitamin c fed to Atlantic salmon (Salmo salar) for a short-term period. Fish Shellfish Immunol. 9, 95–107.

96. Long, X.,Wu, R., Wu, X., Hou, W., Pan, G., Zeng, C., Cheng, Y. (2019). Effects of dietary fish oil replacement with blended vegetable oils on growth, lipid metabolism and antioxidant capacity of subadult swimming crab *Portunus trituberculatus*. Aquac. Nutr. 25, 1218–1230

97. Lu, J., Tibbetts, S.M., Lall, S.P., Anderson, D.M. (2020). Use of dietary oil, solvent-extracted meal and protein concentrate from camelina sativa for rainbow trout, *Oncorhynchus mykiss*, at the early fry stage. Aquaculture. 524, 735252.

98. Luo, Y., Ai, Q., Mai, K., Zhang, W.,Xu, W., Zhang, Y. (2012).Effects of dietary rapeseed meal on growth performance, digestion and protein metabolism in relation to gene expression of juvenile cobia (Rachycentron canadum). Aquaculture, 368–369, 109–116.

99. Matthäs, B. (1997). Antinutritive compounds in different oilseeds.(1997). Lipid/Fett 99, 170–174.

100. Matthäus, B., Zubr, J. (2000). Variability of specific components in *camelina sativa* oilseed cakes. Ind. Crop. Prod. 12, 9–18.

101. Mazumder, A., Dwivedi, A.; Du Plessis, J. (2016). Sinigrin and its therapeutic benefits. Molecule*s*, 21, 416.

102. Mzengereza, K., Ishikawa, M., Koshio, S., Yokoyama, S., Yukun, Z., Shadrack, R.S., Seo, S., Duy Khoa, T.N., Moss, A., Dossou, S., et al. (2021). Effect of substituting fish oil with camelina oil on growth performance, fatty acid profile, digestibility, liver histology, and antioxidative status of red seabream (*Pagrus major*). Animals , 11, 1990.

103. Moe, Y.Y.; Koshio, S.; Teshima, S.I.; Ishikawa, M.; Matsunaga, Y.; Panganiban, A. (2004). Effect of vitamin c derivatives on the performance of larval kuruma shrimp, *marsupenaeus japonicus*. Aquaculture , *242*, 501–512.

104. Mourente, G.; Bell, J.G.; Tocher, D.R. (2007). Does dietary tocopherol level affect fatty acid metabolism in fish? Fish Physiol. Biochem. 33, 269–280

105. Morais, S., Edvardsen, R, Tocher, D, Bell, G. (2012). Transcriptomic analyses of intestinal gene expression of juvenile Atlantic cod (Gadus morhua) fed diets with Camelina oil asreplacement for fish oil. Comp Biochem Physiol B, 161:283–293.

106. Moss, A., S, Koshio, S., Ishikawa, M., Saichiro, Y., Truong, H. N., Mahmoud, A.O. D., Weilong, W. (2018). Replacement of squid and krill meal by snail meal (*Buccinum striatissimum*) in practical diets for juvenile of kuruma shrimp (*Marsupenaeus japonicus*). Aquaculture Research. 2018; 49:3097–3106.

107. Mudalkar, S., Golla, R., Ghatty, S., Reddy, A.R. (2014). De novo transcriptome analysis of an imminent biofuel crop, *camelina sativa* l. Using Illumina gaiix sequencing platform and identification of SSR markers. Plant Mol. Biol. 84, 159–171.

108. Mushirata, K., Matsunari, H., Furuta, H., Rønnestad, I.; Oku, H.; Yamamoto, T. (2018). Effects of dietary soybean meal on the digestive physiology of red sea bream *Pagrus major*. Aquaculture, 493, 219–228.

109. Nagel, F., Appel, T., Rohde, C., Kroeckel, S., Schulz, C. (2017) Blue mussel protein concentrate versus prime fish meal protein as a dietary attractant for turbot (*Psetta maxima* l.) given rapeseed protein-based diets. Aquac. Res. Dev. S 2, 2.

110. Natalia, Y., Hashim, R., Ali, A., Chong, A. (2004). Characterization of digestive enzymes in a carnivorous ornamental fish, the Asian bony tongue *Scleropages formosus* (osteoglossidae). Aquaculture. 233, 305–320

111. NRC. (2011).Nutrient requirements of fish and shrimp. *Animal Nutrition Series, National Research Council of the National Academies*; The National Academies Press: Washington, DC, USA, 2011; p. 376.

112. Ni Eidhin, D., Burke, J., O'Beirne, D. (2003). Oxidative stability of ω-3 rich Camelina oil and Camelina oil-based spread compared with plant and fish oils and sunflower spread. J. Food Sci. 68: 345-35.

113. Ogueji, E., Iheanacho, S. C., Mbah, C., Yaji, A. J., Ezemagu, U. (2020). Effect of partial and complete replacement of soybean with discarded cashew nut (*Anacardium occidentale* L) on liver and stomach histology of *Clarias gariepinus* (Burchell, 1822). Aquaculture and Fisheries, *5*(2), 86–91.

114. Oliva-Teles, A. (2012). Nutrition and health of aquaculture fish. J. Fish Dis. 35: 83-108.

115. Olivotto, I., Mosconi, G., Maradonna, F., Cardinali, M., Carnevali, O. (2002). *Diplodus sargus* interrenal–pituitary response: Chemical communication in stressed fish. Gen. Comp. Endocrinol.127, 66–70.

116. Olsen, R.E., Myklebust, R., Ringø, E., Mayhew, T.M. (2000). The influences of dietary linseed oil and saturated fatty acids on caecal enterocytes in arctic char (*Salvelinus alpinus* L.): A quantitative ultrastructural study. Fish Physiol. Biochem. *22*, 207–216.

117. Osmond, A.T.Y., Arts, M.T., Bazinet, R.P., Napier, J.A., Han, L., Colombo, S.M. (2021). Transgenic camelina oil is an effective source of eicosapentaenoic

acid and docosahexaenoic acid in diets for farmed rainbow trout, in terms of growth, tissue fatty acid content, and fillet sensory properties. J. World Aquac. Soc. doi:10.1111/jwas.12788.

118. Onyilagha, J., Bala, A., Hallett, R., Gruber, M.; Soroka, J., Westcott, N. (2003). Leaf flavonoids of the cruciferous species, *camelina sativa*, Crambe spp., Thlaspi arvense and several other genera of the family Brassicaceae. Biochem. Syst. Ecol. 31, 1309–1322.

119. Pan, X., Xie, W., Caldwel, C., Anderson, D. (2011). Growth performance and carcass composition of rainbow trout (*Oncorhynchus mykiss*) fed practical diets containing graded levels of high fat residue camelina meal. *Can. J. Anim. Sci*. 91, 484.

120. Panserat, S., Kaushik, S.J. (2010). Regulation of gene expression by nutritional factors in fish. Aquac. Res. 41, 751–762.

121. Picha, M.E., Turano, M.J., Beckman, B.R., Borski, R.J.(2008). Endocrine biomarkers of growth and applications to aquaculture: A minireview of growth hormone, insulin-like growth factor (IGF)-i, and IGF-binding proteins as potential growth indicators in fish. N. Am. J. Aquac. 70, 196–211.

122. Piccinetti, C.C., Grasso, L., Maradonna, F., Radaelli, G., Ballarin, C., Chemello, G.; Evjemo, J.O., Carnevali, O., Olivotto, I. (2017).Growth and stress factors in Ballan wrasse (*labrus bergylta*) larval development. *Aquac. Res. 48*, 2567–2580.

123. Pickova , J., Mørkøre, T. (2007). Alternate oils in fish feeds. Eur J Lipid Sci Technol 109:256–263.

124. Peres, H., Santos, S., Oliva-Teles, A., (2013). Selected plasma biochemistry parameters ingilthead seabream (*Sparus aurata*) juveniles. J. Appl. Ichthyol. 29, 630‑636.

125. Pham-Huy, L.A., He, H., Pham-Huy, C. (2008) Free radicals, antioxidants in disease and health. Int J. Biomed Sci, *4*, 89–96.

126. Putnam, D., Budin, J., Field, L., Breene, W. (1993). Camelina: a promising low output oilseed. In: New Crops. Janick, J., Simon, J. (eds.). John Wiley and Sons. New York, USA. pp 314-32.

127. Qing, P.L., Xing,W.K. (1997), The experimental studies on activities of digestive enzyme in the larvae Penaeus chinensis. J. Fish. China. Available online: https://en.cnki.com.cn/Article_en/CJFDTotal-SCKX701.004.htm (accessed on 3 August 2021)

128. Radovanović, T.B., Borković-Mitić, S.S., Perendija, B.R., Despotović, S.G., Pavlović, S., Cakić, P., Saičić, Z.S. (2010). Superoxide dismutase and catalase activities in the liver and muscle of barbel (*Barbus barbus*) and its intestinal parasite (*Pomphoryinchus laevis*) from the danube river, serbia. Arch. Biol. Sci. 62, 97–105.

129. Ren, T., Koshio, S., Teshima, S., Ishikawa, M., Alam, M. S., Panganiban, A., Moe, Y. Y., Kojima, T., Tokumitsu, H. (2005) Optimum dietary level of L-ascorbic acid for Japanese eel, Anguilla japonica. J. World Aquacult. Soc., 36, 437–443.

130. Refstie, S., Storebakken, T., Roem, A.J. (1998) Feed consumption and conversion in Atlantic salmon (*Salmo salar*) fed diets with fish meal, extracted soybean meal or soybean meal with reduced content of oligosaccharides, trypsin inhibitors, lectins and soya antigens. Aquaculture, 162, 301–312.

131. Richard, D., Kefi, K., Barbe, U., Bausero, P., Visioli, F. (2008) Polyunsaturated fatty acids as antioxidants. Pharmacol. Res, 57, 451–455.

132. Roberts, I.M.(1985). Hydrolysis of 4-methylumbelliferyl butyrate: A convenient and sensitive fluorescent assay for lipase activity. Lipids, 20, 243–247.

133. Salinas, I., Abelli, L., Bertoni, F., Picchietti, S., Roque, A., Furones, D., Cuesta, A., Meseguer, J., Esteban, M.Á. (2008).Monospecies and multispecies probiotic formulations produce different systemic and local immunostimulatory effects in the gilthead seabream (*sparus aurata* l.). Fish Shellfish Immunolology, *25*, 114–123.

134. Seong, T., Matsutani, H., Haga, Y., Kitagima, R., Satoh, S. (2019) First step of non-fish meal, non-fish oil diet development for red seabream, (*Pagrus major*), with plant protein sources and microalgae *Schizochytrium* sp. Aquaculture research. 50, 2460–2468.

135. Seong, T., Kitagima, R., Haga, Y., Satoh, S. (2020) Non-fish meal, non-fish oil diet development for red sea bream, *Pagrus major*, with plant protein and graded levels of *Schizochytrium* sp: Effect on growth and fatty acid composition. Aquaulture Nutrition, *26*, 1173–1185.

136. Shen, J., Liu, H., Tan, B., Dong, X., Yang, Q., Chi, S., Zhang, S. (2020). Effects of replacement of fishmeal with cottonseed protein concentrate on the growth, intestinal microflora, haematological and antioxidant indices of juvenile golden pompano (*Trachinotus ovatus*). Aquaculture Nutrition. 26, 1119–1130.

137. Sigurgisladottir, S., Lall, S.P., Parrish, C.C., Ackman, R.G. (1992) Cholestane as a digestibility marker in the absorption of polyunsaturated fatty acid ethyl esters in atlantic salmon. Lipids. 27, 418–424.

138. Shourbela, R.M., El-Hawarry, W.N., Elfadadny, M.R.; Dawood, M.A.O. (2020). Oregano essential oil enhanced the growth performance, immunity, and antioxidative status of Nile tilapia (*Oreochromis niloticus*) reared under intensive systems. Aquaculture.542, 736868.
139. Siriwardhana, N., Kalupahana, N., Moustaid-Moussa, N. (2012). Health benefits of n-3 polyunsaturated fatty acids: eicosapentaenoic acid and docosahexaenoic acid. In: Advances in Food and Nutrition Research (Vol. 65): Marine Medicinal Foods: Implications and Applications: Animals and Microbes. Kim S (ed). Elsevier. Oxford, UK, pp 211-219.
140. Tacon, A., Metian, M. (2015). Feed matters: Satisfying the feed demand of aquaculture. Rev. Fish. Sci. Aquac. 23, 1–10.
141. Takeuchi, T., Toyota, M., Satoh, S., Watanabe, T., (1990). Requirement of juvenile red seabream (*Pagrus major*) for eicosapentaenoic and docosahexaenoic acids. Nippon Suisan Gakkaishi 56, 1263–1269.
142. Takeuchi, T., Shiina, Y.,Watanabe, T., (1991). Suitable protein and lipid levels in diet for fingerlings of red sea bream (*Pagrus major*). Nippon Suisan Gakkaishi. 57, 293–299.
143. Takeuchi, T., Shiina, Y., Watanabe, T., (1992). Suitable levels of n-3 highly unsaturated fatty acids in diet for fingerlings of red sea bream (*Pagrus major*). Nippon Suisan Gakkaishi 58, 509–514.
144. Tamaru, C. S., Ako, H., & Lee, C. S. (1992). Fatty acid and amino acid profiles from spawn eggs of the striped mullet, *Mugil cephalis L. Aquaculture*, 105, 83– 94.

145. Taufek, N. M., Aspani, F., Muin, H., Raji, A. A., Razak, S. A.,Alias, Z. (2016). The effect of dietary cricket meal (*Gryllus bimaculatus*) on growth performance, antioxidant enzyme activities, and haematological response of African catfish (*Clarias gariepinus*). *Fish Physiology and Biochemistry*,
146. Teshima, S., Kanazawa, A. (1971). Biosynthesis of sterols in the lobster, Panulirus japonica, the prawn, *Penaeus japonicus*, and the crab *Portunus trituberculatus*. Comparative Biochemistry and Physiology, 38B, 597–602.
147. Teshima, S.I., Kanazawa, A., Yamashita, M.(1986) Dietary value of several proteins and supplemental amino acids for larvae of the prawn *Penaeus japonicus*. Aquaculture. 51, 225–235.
148. Tovar-Ramírez, D., Mazurais, D.; Gatesoupe, J.F., Quazuguel, P., Cahu, C.L., Zambonino-Infante, J.L.(2010). Dietary probiotic live yeast modulates antioxidant enzyme activities and gene expression of sea bass (*dicentrarchus labrax*) larvae. Aquaculture. 300, 142–147
149. Toyes-Vargas, E.A., Parrish, C.C., Viana, M.T., Carreón-Palau, L.; Magallón-Servín, P., Magallón-Barajas, F.J. (2020). Replacement of fish oil with camelina (*Camelina sativa*) oil in diets for juvenile tilapia (var. GIFT *Oreochromis niloticus*) and its effect on growth, feed utilization and muscle lipid composition. (Aquaculture). *523*, 735177.
150. Turchini, G.M., Mentasti, T., Caprino, F., Giani, I., Panseri, S., Bellagamba, F., Moretti, V.M., Valfré, F. (2005). The relative absorption of fatty acids in brown trout (*Salmo trutta*) fed a commercial extruded pellet coated with different lipid sources. Ital. J. Anim. Sci., 4, 241–252

151. Turchini, G., Torstensen, B., Ng, W. (2009). Fish oil replacement in finfish nutrition. Reviews in Aquaculture, 1, 10–57.

152. Turchini, G.M., Mentasti, T., Caprino, F., Giani, I., Panseri, S., Bellagamba, F., Moretti, V.M., Valfré, F. (2012). The relative absorption of fatty acids in brown trout.

153. Watanabe, T. (2002). Strategies for further development of aquatic feeds. Fish. Sci. 68: 242-252.

154. Waraich, E.A., Ahmed, Z., Ahmad, R., Ashraf, M.Y., Saifullah, Naeem, M.S., Rengel, Z., (2013). Camelina sativa, a climate proof crop, has high nutritive value and multiple-uses: a review. AJCS 7 (10), 1551–1559.

155. Wei, B., Yang, Z., Cheng, Y., Wang, J., Zhou, J. (2018). Effects of the complete replacement of fish oil with linseed oil on growth, fatty acid composition, and protein expression in the Chinese mitten crab (*Eriocheir sinensis*). Proteome Sci. 16, 1–11.

156. Wei, M., Anderson, D.M., Zhang, Z., Colombo, S.M.(2020). High-oil residue camelina meal, a viable source of protein at low levels in diets for juvenile salmonids. Aquac. Nutr. 26, 558–567.

157. Wilson, R. (2002). Amino acids and proteins. In: Fish Nutrition. Halver, J., Hardy, R. (eds). Academic Press. San Francisco, USA. pp. 144-175.

158. Wijekoon, M. (2012). Effect of water temperature and diet on cell membrane fluidity and fatty acid composition of muscle, liver, gill and intestine mucosa of adult and juvenile steelhead trout, *(Oncorhynchus mykiss)*. Dissertation. Memorial University of Newfoundland. St. John’s, Newfoundland, Canada.

159. Worm, B. Barbier, E., Beaumont, N., Duffy, J., Folke, C., Halpern, B., et al. (2006). Impacts of biodiversity loss on ocean ecosystem services. Science. 314: 787-790.

160. World Health Organization, (2008). Fats and Fatty Acids in Human Nutrition: Report of an Expert Consultation. FAO Food and Nutrition Paper, Geneva, Switzerland, pp. 3.

161. Ye, C.L., Anderson, D.M., Lall, S.P. (2016). The effects of camelina oil and solvent extracted camelina meal on the growth, carcass composition and hindgut histology of Atlantic salmon (*Salmo salar*) parr in freshwater. Aquaculture. 450, 397–404.

162. Yu, J., Shuguo, L., Huaxin, N., Jie, C., Zongfu, H., Ying H. (2019). Influence of dietary linseed oil as substitution of fish oil on whole fish fatty acid composition, lipid metabolism and oxidative status of juvenile Manchurian trout, *Brachymystax lenok.* Nature Scientific Reports 9:13846

163. Yu, H., Gao, Q., Dong, S., Lan, Y., Ye, Z., Wen, B. (2016). Regulation of dietary glutamine on the growth, intestinal function, immunity and antioxidant capacity of sea cucumber *Apostichopus japonicus* (selenka). Fish Shellfish Immunol. 50. 56–65.

164. Yoshida, K. (1992). Studies on nutritional requirements of tiger puffer,Takifugu rubripes(in japanese)MSc. Thesis, Kagoshima University, Japn,85-135.

165. Zuo, R., Ai, Q., Mai, K., Xu, W. (2013). Effects of conjugated linoleic acid on growth, non-specific immunity, antioxidant capacity, lipid deposition and related gene expression in juvenile large yellow croaker (*Larmichthys crocea*) fed soyabean oil-based diets. Br. J. Nutr. 110, 1220–1232.

166. Zubr, J. (1997). Oil-seed crop: *Camelina sativa*. Ind. Crop. Prod. 6: 113-119.

Appendices.

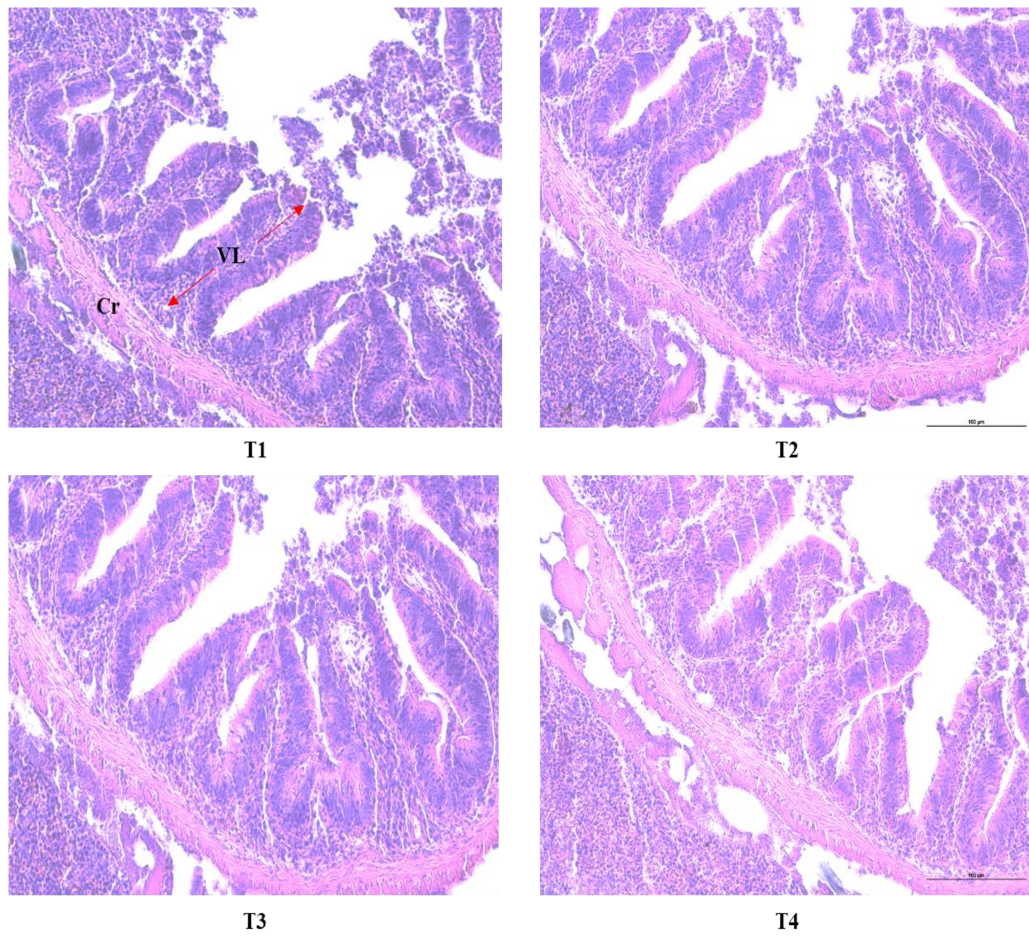


Figure 13: Intestine histomorphology of red sea bream fed experimental diets for 45 days. VL = Villus length, Cr = Crypt depth. Where T1=CM0, T2=SBM20.5, T3=CM20.5, T4=CM33.

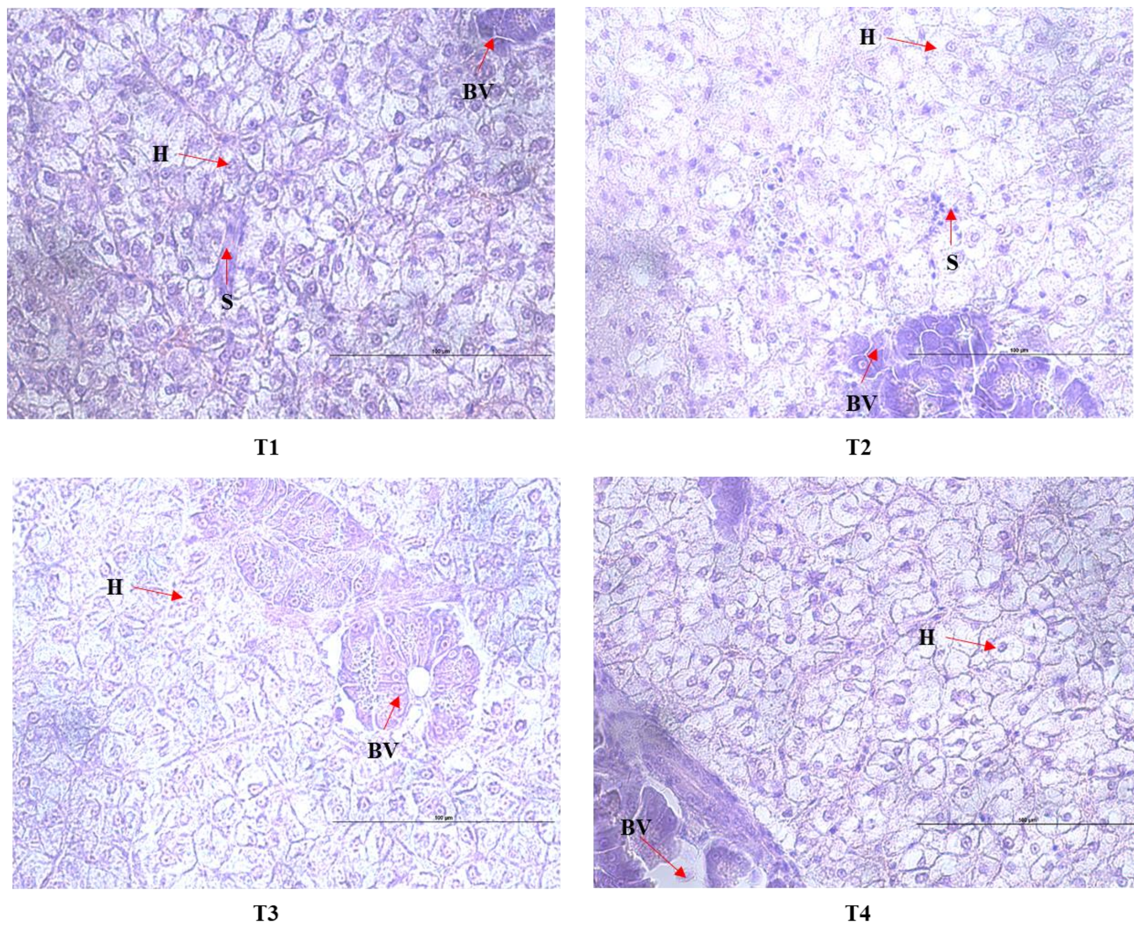


Figure 14: Liver histomorphology of red sea bream fed experimental diets for 45 days. BV = Blood vessel, H = Hepatocytes, S = Sinusoids. Where T1=CM0, T2=SBM20.5, T3=CM20.5, T4=CM33.

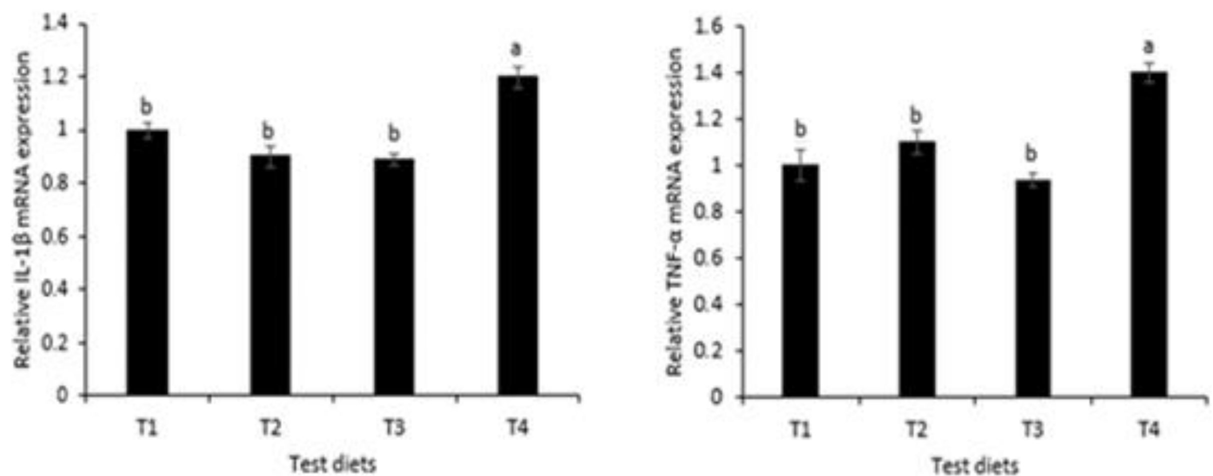


Figure 15: Immune-related gene expression (IL-1b and TNF-a) of red sea bream fed experimental diets for 45 days. Bars (mean ± SEM, n =3) with various superscripts are different (P < 0.05). Where T1=CM0, T2=SBM20.5, T3=CM20.5, T4=CM33.

Table 28: Forward and Reverse primers for immune-related genes

Gene	**Primer Sequence (5′–3′)** **[F] = Forward – [R] = Reverse**	**Accession number**
β-actin	TCTGTCTGGATCGGAGGTC [F] AAGCATTTGCGGTGGACG [R]	JN226150.1
TNF-α	CCAAACAGAAGCACTAACCAAGA [F] CTAAATGGATGGCTGCCTTG [R]	AY314010.1
IL-1β	CGAGTACCAAACAGCATGGA [F] GTGTAGGGGGCAGGTAGGTC [R]	AY257219.1

Table 29: Intestinal morphometric in red sea bream fed experimental diets for 45 days.

Items	Experimental diets			
	T1	T2	T3	T4
Villous length (Vl), µm	214.37±17.70 [b]	294.32±22.10 [a]	279.30±27.40 [a]	288.08±23.40 [a]
Cryptal depth (Cd), µm	135.24±12.22	118.28±25.31	124.34±14.39	112.89±27.30
Goblet cell count per villi	12.99±1.32 [b]	14.22±1.90 [ab]	18.17±2.21[a]	17.62±1.27 [a]

Values (mean ± SEM, *n* =3) within a row with various superscripts are different ($P < 0.05$). Where T1=CM0, T2=SBM20.5, T3=CM20.5, T4=CM33.

Table 30: Plasma biochemical constituents in juvenile red sea bream fed test diets

Items	Experimental diets			
	T1	T2	T3	T4
Hematocrit (%)	34±0.3[a]	33±0.5[b]	34±0.2[c]	28±2.1[d]
Glucose (mg/dl)	76.6±5.8	67.5±5.7	76.3±4.3	79.3±8.1
Total bilirubin (mg/dl)	5.3±0.3	7.3±0.6	5.6±0.3	6.6±0.8
Total protein (mg/dl)	3.6±0.1	3.5±0.3	3.5±0.1	3.7±0.2
Total cholesterol g/dl)	188.3±5.4	180.3±12.0	181.3±4.3	190.0±2.8
Triglyceride (g/dl)	129±14.6	134.3±38.8	112.6±20.1	143.6±20.2
GOT (UI/I)	72.3±20.3	100.0±45	110.6±46.3	116.6±30.7
GPT (UI/I)	17.3±7.3	26±16	48.6±34.2	26.6±5.7

Values are displayed as mean ± SEM (n=3). Data with common alphabets are not significantly different ($P < 0.05$). GOT = Glutamyl oxaloacetic transaminaseGPT =

Glutamic pyruvate transaminase Where T1=CM0, T2=SBM20.5, T3=CM20.5, T4=CM33.