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Use of mixtures of algal and vegetable oils as fish oil replacers in Asian seabass (*Lates calcarifer*) feeds and their effects on growth, digestive enzymes, immune biomarkers, fatty acid profiles, and expression of genes involved in fatty acid biosynthesis

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Abstract
An experiment was conducted to assess the potential use of algal oil (AO) and vegetable (soya and palm oil; 1:1) oil (VO) mixtures as replacers for fish oil (FO) in Asian seabass (*Lates calcarifer*) diets. Six iso-nitrogenous (45% crude protein) and iso-lipidic (10% crude lipid) diets were formulated incorporating FO, VOs, and AO at 100:0:0 (T1), 0:0:100 (T2), 50:25:25 (T3), 25:50:25 (T4), 0:50:50 (T5), and 0:75:25 (T6), respectively. Each diet was given to five tanks (100 L with 10 fish in each) of juvenile fish (3.89±0.23 g initial weight) fed to satiation for 8 weeks. At termination, five replicate groups of fish were used for survival rate (SR), final weight, weight gain, consumed feed, feed conversion ratio (FCR), and specific growth rate (SGR) determination. Proximate composition, nutrient utilization, digestive enzyme activities of the pyloric caeca and intestine, and immune parameters were assessed using fish from three replications. A pooled sample consisting of three replicates was used for the determination of fatty acid profile and lipid quality indices. The liver samples from four replicate groups of fish were pooled into two samples and analyzed for Δ6-desaturase and Elovl5 gene expressions. The SR, growth performance, and feed conversion ratio were not significantly different ($p>0.05$) among treatments; however, feed consumption was significantly different ($p<0.05$) among treatments. The highest feed consumption (36.45±2.01 g/fish), final weight (32.41±2.78 g fish^{-1}), and weight gain (28.53±2.78 g fish^{-1}) were observed in T4 feeding group. Moreover, substituting FO with AO and VOs significantly affected ($p<0.05$) viscerosomatic index, intraperitoneal fat, carcass lipids, and lipid retention efficiency (LRE). The VSI (5.45±0.42) and IPF (1.53±0.03) were the lowest in the T4 feeding group ($p<0.05$). The 100% AO feeding group (T2) showed significantly lower carcass lipid and LRE ($p<0.05$). Specific lipase activity in pyloric caeca was significantly higher in T4 feeding group ($p<0.05$). Docosahexaenoic acid (22:6n-3) and eicosapentaenoic acid (20:5n-3) contents of fish carcasses were higher in the 100% AO-fed group. There was a significant ($p<0.05$) impact on the expression of Δ6-desaturase and Elovl5 genes in liver tissue. There were no significant

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differences ($p>0.05$) in innate immune parameters among the treatments. The findings of this study indicate that 75% of FO can be replaced by a combination of 25% AO and 50% VOs in Asian seabass (*L. calcarifer*) diets without impairing growth performance, nutrient utilization, and health status. These results can be used to develop more sustainable feeds that are less reliant on marine resources.

Keywords Algal oil · *Lates calcarifer* · Growth · Fatty acid profile · Gene expression

Introduction

Fish meal (FM) and fish oil (FO) have been widely used as ingredients in fish feeds because of their desirable nutritional properties. Fish meal provides a balanced amino acid profile, while FO provides beneficial levels of n-3 long-chain polyunsaturated fatty acid profile (n-3 LC-PUFA) (Tacon and Metian 2008). The continued global growth of aquaculture production has increased demand for FO, while the production of this commodity has not increased in recent years resulting in increased prices (FAO 2022). Because FO supply is directly related to the sustainable development of the aquaculture industry, alternatives to FO for use in aquafeed have received increasing attention from researchers. Terrestrial vegetable oils (VOs) such as linseed oil, rapeseed oil, soybean oil, palm oil, and sunflower oil have received the most attention for their availability and price stability (Nasopoulou and Zabetakis 2012). These VOs are deficient in n-3 LC-PUFA such as eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), but some are rich in C18-polyunsaturated fatty acids (C18-PUFA) including linoleic acid (LA, 18:2n-6) and α-linolenic acid (ALA, 18:3n-3) (Turchini et al. 2009). EPA and DHA play crucial roles in the physiological development of marine fish, but because these cannot be produced endogenously, they must be supplied in diets to meet the requirements (Tocher 2015). Studies with a variety of fish species have shown that complete substitution of dietary FO with VOs results in decreased growth performance due to the deficiency of n-3 LC-PUFA, although partial replacement can support good growth (Turchini et al. 2011; Mu et al. 2020; Peng et al. 2008; Torrecillas et al. 2017). The use of VOs as dietary lipid sources has resulted in decreased n-3 LC-PUFA content in fish tissues, reducing the potential health benefits for consumers (Nasopoulou and Zabetakis 2012). Furthermore, n-3 LC-PUFA deficiency due to VO incorporation in fish diets has led to immunological disorders in some fishes (Jiang et al. 2013; Montero et al. 2008). Recently, microorganisms have received attention as sources of alternative ingredients for aquaculture diets due to their good nutritional properties, particularly high EPA and DHA content (Shah et al. 2018). Several nutritional studies have been conducted with microalgae-derived lipid sources in diets for marine fish without impacting growth performance or n-3 LC-PUFA concentration in tissue. In marine Atlantic salmon (*Salmo salar*) diets, supplementation with different levels of the thraustochytrid DHA-producing *Schizochytrium* sp. (up to 150 g kg^{-1} of diet) resulted in similar growth performance compared to the FO control group (Kousoulaki et al. 2015). In another study with smaller fish of the same species held in fresh water, replacing FO with *Schizochytrium* sp. oil or a 4:1 combination of palm oil and algal oil (AO) revealed no significant differences in feed intake and growth performance, although there were differences in the fatty acid profiles of the fish carcass (Miller et al. 2007). Carvalho et al. (2020) reported that microalgal oil combined with poultry oil and rapeseed oil could completely substitute FO in gilthead sea bream (*Sparus aurata*) diet. Algal meal from *S. limacinum* can be used to

replace FO in the diet of red drum (*Sciaenops ocellatus*) (Perez-Velazquez et al. 2018) and giant grouper (*Epinephelus lanceolatus*) (García-Ortega et al. 2016). The high market price and low availability of algal oil have limited its widespread usage in aquaculture (Sarker et al. 2016). However, combining VOs and AO may keep feed costs low, while enhancing the dietary n-3 LC-PUFA, resulting in high-quality products for consumers.

Asian seabass (*Lates calcarifer*) is an economically important cultured species in the Indo-West Pacific region due to its rapid growth rate and high tolerability to salinity fluctuation (Glencross 2006). Since Asian seabass is a highly carnivorous and voracious feeder (Davis 1985), its culture mostly relies on high protein and energy-containing commercial diets that source costly raw materials, including fish meal and FO (Glencross 2006). The feasibility of substituting FO by alternative lipid sources, including VOs (Rahman et al. 2021, 2022), poultry fat (Salini et al. 2015), non-marine phospholipid-rich oils (Salini et al. 2016), and rice bran oil (Glencross et al. 2016), has been investigated in Asian seabass diets. However, AO in combination with VOs has not been studied in Asian seabass diets. Hence, the current study was designed to assess the effects of replacing FO with AO combined with VOs on growth performance, fatty acid profiles, nutrient utilization, digestive enzyme activities, immunological parameters, and expression of related genes for fatty acid bioconversion in juvenile Asian seabass.

Materials and methods

Experimental design and diet preparation

AO (Veramaris, Netherlands, produced from *Schizochytrium* sp. and which contains more than 50% EPA and DHA combined) and a plant-based vegetable oil (VO) mixture (1:1) of food-grade soybean oil (Thai Vegetable Oil Public Company Limited) and palm oil (P.K. Trading, Thailand, Company Limited) were used for FO substitution. The control (T1) diet contained FO alone. Five experimental diets were prepared using the same ingredients as the control, but with FO substituted by either AO alone or with VO in the following proportions: T2 (100% AO), T3 (50 FO:25 VOs:25 AO), T4 (25 FO:50 VOs:25 AO), T5 (0 FO:50 VOs:50 AO), and T6 (0 FO:75 VOs:25 AO), respectively. The ingredients were mixed and made into a dough by addition of 30% water and extruded (Hobart Legacy Mixer, USA). The pellets were manually cut to 2-mm and 3-mm lengths and dried in a hot air drier at 50 °C for 4 h and kept at −4 °C until use. The formulations, ingredients with proximate composition, calculated price of the diets, and fatty acid profile are shown in Tables 1 and 2, respectively. The proximate composition (moisture, protein, lipid, and ash) of prepared diet was determined according to the standard of AOAC (1995). All samples were analyzed in triplicate. The moisture content of the diets was determined using thermal drying process. Approximately 1 g of sample was placed into a pre-weighed crucible and dried in an oven at 105 °C to achieve a consistent weight. The moisture content of the samples was calculated using the following formula:

$$Moisture\ (\%)\ :\ \{(W-D)/D\} \times 100;\ here, W = wet\ weight\ and\ D = dry\ weight$$

The crude protein content of diets was determined following the Kjeldahl method using the Kjeldahl apparatus (Gerhardt, Germany). Initially, nitrogen content was determined. Then, the following formula was used to calculate crude protein (%).

Table 1 Ingredients and proximate composition (% as fed-basis) of the experimental diets for Asian seabass

	Diets (fish oil to vegetable oils to algal oil)					
	1 (100:0:0)	2 (0:0:100)	3 (50:25:25)	4 (25:50:25)	5 (0:50:50)	6 (0:75:25)
Ingredients (% of diet)						
Poultry by-product meal (protein 60.01% and lipid 13.05%)	37.00	37.00	37.00	37.00	37.00	37.00
Soybean meal (protein 48.50% and lipid 2.70%)	18.00	18.00	18.00	18.00	18.00	18.00
Soy protein concentrate (protein 58.29% and lipid 1.16%)	18.50	18.50	18.50	18.50	18.50	18.50
Wheat flour	5.00	5.00	5.00	5.00	5.00	5.00
Ground rice husk	7.29	7.29	7.29	7.29	7.29	7.29
Fish oil	6.00	-	3.00	1.50	-	-
Soybean oil	-	-	0.75	1.50	1.50	2.25
Palm oil	-	-	0.75	1.50	1.50	2.25
Algal oil[1]	-	6.00	1.50	1.50	3.00	1.50
Vitamin and mineral premix[2]	1.00	1.00	1.00	1.00	1.00	1.00
Carboxymethyl cellulose (CMC)	2.00	2.00	2.00	2.00	2.00	2.00
DL-methionine	0.21	0.21	0.21	0.21	0.21	0.21
Dried tuna viscera[3] (protein 46.13% and lipid 10.05%)	5.00	5.00	5.00	5.00	5.00	5.00
Proximate composition[4] (%)						
Moisture	2.20	2.30	2.14	2.40	2.42	2.39
Ash	11.94	11.87	11.72	11.81	11.82	11.60
Crude protein	45.25	45.77	45.41	45.49	45.50	45.36
Crude lipid	9.76	9.15	9.24	9.20	9.74	9.42
Price of the diet (USD/kg)[5]	1.11	1.44	1.16	1.12	1.20	1.09

[1]Obtained from Veramaris (DSM, Thailand)

[2]Obtained from Thai Union Group PCL, Thailand

[3]Tuna viscera powder was made freshly at the laboratory

[4]By analysis (AOAC, 1995)

[5]Based on the gathered price of the ingredients from a local supplier (34.18 Bath/USD)

Table 2 Fatty acid profile (% of total fatty acids) of Asian seabass diets containing different levels of vegetable oils and algal oil replacing fish oil

Fatty acids (% of total fatty acids)	Diets (fish oil to vegetable oils to algal oil)					
	T1 (100:0:0)	T2 (0:0:100)	T3 (50:25:25)	T4 (25:50:25)	T5 (0:50:50)	T6 (0:75:25)
Butyric acid (4:0)	0.09	0.09	0.09	0.08	0.08	0.08
Lauric acid (12:0)	0.60	0.66	0.62	0.61	0.62	0.61
Myristic acid (14:0)	2.52	2.25	2.01	1.60	1.53	1.19
Pentadecylic acid (15:0)	0.37	0.38	0.30	0.23	0.23	0.16
Palmitic acid (16:0)	20.55	28.00	23.37	24.18	26.02	24.91
Margaric acid (17:0)	0.29	0.25	0.25	0.22	0.21	0.19
Stearic acid (18:0)	5.06	4.54	5.14	5.18	5.05	5.36
Arachidic acid (20:0)	0.21	0.32	0.26	0.28	0.31	0.31
Behenic acid (22:0)	0.16	0.17	0.18	0.18	0.20	0.20
Total SFA[1]	**29.85**	**36.66**	**32.22**	**32.56**	**34.25**	**33.01**
Myristoleic acid (14:1n-5)	0.26	0.20	0.23	0.20	0.19	0.19
Palmitoleic acid (16:1n-7)	4.87	2.10	3.38	2.69	2.04	2.01
Heptadecenoic acid (17:1)	0.39	0.08	0.22	0.15	0.08	0.09
Vaccenic acid (18:1n-7)	3.27	0.00	2.47	2.18	1.79	1.97
Oleic acid (18-1n-9)	27.59	15.77	25.25	26.66	23.66	27.67
Gondoic acid (20:1n-9)	3.20	1.16	1.90	2.07	1.83	2.20
Erucic acid (22:1n-9)	0.68	0.16	0.37	0.22	0.10	0.07
Nervonic acid (24:1n-9)	0.62	0.12	0.41	0.31	0.15	0.18
Total MUFA[2]	**40.88**	**19.59**	**34.23**	**34.48**	**29.84**	**34.38**
Linoleic acid (18:2n-6)	14.82	13.32	17.77	20.81	20.27	23.78
Gamma-linolenic acid (18:3n-6)	0.10	0.13	0.09	0.08	0.08	0.07
Eicosadienoic acid (20:2n-6)	0.39	0.10	0.25	0.18	0.11	0.12
Dihomo-gamma-linolenic acid (20:3n-6)	0.15	0.16	0.14	0.12	0.11	0.11
Arachidonic acid (20:4n-6)	0.99	1.51	1.02	0.89	1.03	0.82
Adrenic acid (22:4n-6)	0.23	0.22	0.19	0.17	0.14	0.15

Table 2 (continued)

Fatty acids (% of total fatty acids)	Diets (fish oil to vegetable oils to algal oil)					
	T1 (100:0:0)	T2 (0:0:100)	T3 (50:25:25)	T4 (25:50:25)	T5 (0:50:50)	T6 (0:75:25)
n-6 Docosapentaenoic acid (22:5n-6)	0.23	1.32	0.47	0.43	0.67	0.38
Total n-6 PUFA[3]	**16.91**	**16.76**	**19.93**	**22.68**	**22.41**	**25.43**
α-Linolenic acid (18:3n-3)	1.77	1.17	1.86	2.03	1.83	2.17
Stearidonic acid (18:4n-3)	0.38	0.18	0.24	0.14	0.10	0.06
Eicosapentaenoic acid (20:5n-3)	2.33	5.76	2.68	2.09	2.92	1.50
n-3 Docosapentaenoic acid (22:5n-3)	0.75	0.63	0.53	0.37	0.34	0.21
Docosahexaenoic acid (22:6n-3)	4.88	19.24	7.30	6.03	9.44	4.90
Total n-3 PUFA[4]	**10.11**	**26.98**	**12.61**	**10.66**	**14.63**	**8.84**
n-3/n-6	0.60	1.61	0.63	0.47	0.65	0.35

[1]SFA: (4:0), (12:0), (14::0), (15:0), (16:0), (17:0), (18:0), (20:0), (22:0), and (24:0).

[2]MUFA: (14:1n-5), (16:1n-7), (18:1n-7), (18-1n-9), (20:1n-9), (22:1n-9), and (24:1n-9)

[3]n-6 PUFA: (18:2n-6), (20:2n-6), (20:3n-6), (20:4n-6), (22:4n-6), and (22:5n-6)

[4]n-3 PUFA: (18:4n-3), (20:5n-3), (22:5n-3), and (22:6n-3)

$$Crude\ protein\ (\%) : Nitrogen\ (\%) \times 6.25, where\ 6.25\ is\ protein\ conversion\ factor.$$

Crude lipid was assessed by exhaustive Soxhlet extraction using petroleum ether on a Soxtec System (Soxtec System HT6, FOSS Tecator, Sweden). Approximately 1 g of the sample was used to extract crude lipid using 80 mL of petroleum ether (40–60 °C, boiling point) as the solvent. The crude lipid was collected in a pre-weighed Soxhlet extraction cup. The cup was then placed in the oven to allow the solvent to evaporate and finally allowed to cool down to room temperature in a desiccator and reweighed. The following formula was used:

$$Crude\ lipid\ (\%) : \left[(W3 - W2)/W1\right] \times 100$$

where $W1$ is the sample weight, $W2$ is the extraction cup weight, and $W3$ is the extraction cup and oil residue.

The ash content was determined by an ash combustion method. A pre-weighed crucible containing approximately 1 g of sample was placed in a muffle furnace at 550 °C for 6 h. Then, the samples were taken out from the furnace, cooled to room temperature in a desiccator, and reweighed. The percentage of ash content was calculated as below:

$$Ash\ (\%) : \{Ash\ weight\ (g)/Sample\ weight\ (g)\} \times 100$$

For fatty acid profile determination of the diets, a single solvent, hexane, and a catalyst, 14% boron trifluoride (BF_3), in methanol were used to prepare the fatty acid methyl esters (FAMEs) following one-step procedure as mentioned by Abdulkadir and Tsuchiya (2008). Nonadecanoic acid (19:0) obtained from Nu-Chek Prep, Inc., located in Elysian, Minnesota, USA, was used as the internal standard. The FAMEs were quantified using a Varian 3900 gas chromatograph at facilities of Fisheries and Oceans Canada, West Vancouver, British Columbia, Canada, as described by Arney et al. (2015).

Fish and feeding trial

An 8-week feeding trial was performed at the facilities of the Songkhla Inland Aquaculture Research and Development Center, Khlong Hoi Khong, Thailand. Three-week-old juvenile Asian seabass were obtained from the Coastal Aquaculture Technology and Innovation Research and Development Center, Songkhla, Thailand, reared in rectangular cement tanks for 4 weeks and salinity acclimatized by gradual adjustment from 20 to 0 g L^{-1} (5 g L^{-1} day^{-1}) for freshwater environment.

The fingerlings (3.89±0.23 g; mean±SD) were individually weighed and assigned in groups of 10 fish to 30 glass aquaria (100 L, 60 cm×40 cm×50 cm). Each diet was assigned to 5 aquaria according to a completely random design. Fish were fed by hand twice daily to apparent satiation at 8:30 and 17:00 for 8 weeks. The apparent satiation of the fish was assessed by not moving to the feed. Feed intake was recorded daily. Fifty fish from a pool common to all tanks were taken at the beginning of the trial for proximate composition and fatty acid analysis. The rearing water was changed at a rate of 80% daily and supplied with continuous aeration using an air blower. The water quality parameters such as temperature, pH, and dissolved oxygen were monitored throughout the trial period.

Growth performance and feed utilization measurement

At the termination of the trial, diets were withheld for two meals to evacuate the stomach of the fish. The fish of each aquarium were then euthanized with clove oil (0.10 mL L^{-1} water) and individually weighed. Survival rate (SR, %), weight gain (WG, g $fish^{-1}$), feed consumption (g $fish^{-1}$), FCR, and specific growth rate (SGR, % day^{-1}) were assessed. Two fish from three aquaria (6 fish $treatment^{-1}$) were dissected for viscera, liver, and intraperitoneal fat to measure and calculate for viscerosomatic index (VSI), hepatosomatic index (HSI), and intraperitoneal fat (IPF), respectively. The following formulas were used for the calculations.

$$Survival\ rate\ (\%) = 100 \times (final\ fish\ number / initial\ number)$$

$$Weight\ gain\ \left(g\ fish^{-1}\right) = final\ weight\ (g) - initial\ weight\ (g)$$

$$Food\ conversion\ ratio\ (FCR) = feed\ intake\ \left(g\ fish^{-1}\right) / weight\ gain\ \left(g\ fish^{-1}\right)$$

$$\begin{aligned} Specific\ growth\ rate\ \left(SGR, \%day^{-1}\right) &= (lnW2 - lnW1/T2 - T1) \times 100; W1 \\ &= initial\ weight, W2 = final\ weight, T2 - T1 \\ &= cultured\ period\ (days) \end{aligned}$$

$$Viscerosomatic\ index\ (VSI, \%) = 100 \times (viscera\ weight / body\ weight)$$

$$Hepatosomatic\ index\ (HSI, \%) = 100 \times (liver\ weight / final\ body\ weight)$$

$$Intraperitoneal\ fat\ ratio\ (IPF, \%) = 100 \times (intraperitoneal\ fat\ weight / final\ body\ weight)$$

Proximate composition and fatty acid profile analysis of final fish

Two fish from three replications (6 fish $treatment^{-1}$) were sampled at the termination for proximate composition and fatty acid profile analysis. Moisture, ash, crude protein, and crude lipid of fish were assessed according to the methods of AOAC (1995) as mentioned in the "Experimental design and diet preparation" section. For fatty acid profile determination, fish samples were freeze-dried (Labconco, FreeZone 6), ground (Philips HR2115), pooled, and vacuum packed according to feeding group for analysis at facilities of Fisheries and Oceans Canada, West Vancouver, British Columbia, Canada. The lipid quality indices, such as index of atherogenicity (IA), index of thrombogenicity (IT), and fish lipid quality (FLQ), were calculated from the obtained fatty acid profile data as mentioned by Chen and Liu (2020). The following equations were used for the calculations:

$$Index\ of\ atherogenicity\ (IA) : [C12 : 0 + (4 \times C14 : 0) + C16 : 0] / \Sigma UFA$$

$$Index\ of\ thrombogenicity\ (IT)\ :\ (C14:0+C16:0+C18:0)$$
$$/\left[(0.5\times\Sigma MUFA)+(0.5\times\Sigma n-6\ PUFA)+(3\times\Sigma n-3\ PUFA)+(n-3/n-6)\right]$$

$$Fish\ lipid\ quality\ (FLQ)\ :\ 100\times(C22:6n-3+C20:5n-3)/\Sigma FA$$

Protein efficiency ratio (PER), protein retention efficiency (PRE, %), lipid efficiency ratio (LER), and lipid retention efficiency (LRE, %) were calculated according to Martino et al. 2005. Three replications per treatment were used for the calculations by following these formulas.

$$Protein\ efficiency\ ratio\ (PER) = weight\ gain/total\ protein\ intake$$

$$Lipid\ efficiency\ ratio\ (LER) = weight\ gain/total\ lipid\ intake$$

$$Protein\ retention\ efficiency\ (PRE,\%) = 100\times(body\ retained\ protein/protein\ intake)$$

$$Lipid\ retention\ efficiency\ (LRE,\%) = 100\times(body\ retained\ lipid/lipid\ intake)$$

Digestive enzyme activity determination

At the termination of the trial, the intestines and pyloric caeca from two fish from three replicates (6 fish treatment^{-1}) were collected and preserved in liquid nitrogen for determination of digestive enzyme activity. Specific trypsin, lipase, and α-amylase activities were determined as described in Srichanun et al. (2013). Briefly, the crude enzyme of each organ was extracted by homogenizing the samples in 5.0 volumes (v/w) ice-cold distilled water and centrifuged at 4 °C, 12,000*g* for 30 min. The collected supernatant was used as crude enzyme extract for further reactions. The activity of trypsin (EC 3.4.21.4) was determined using N-benzoyl-DL-arginine-p-nitroanilide (BAPNA, Sigma B4875) as a substrate. BAPNA was mixed in 43.5 mg:1 mL dimethyl sulfoxide (DMSO) and diluted to 100 mL with 0.05 M Tris–HCl buffer containing 0.02 M $CaCl_2.H_2O$. The reactions were performed at 25 °C and 8.5 pH. An optical reader (Multiskan GO Microplate Spectrophotometer, Thermo Scientific) was used to check absorbance at 410-nm optical density in a 96-well plate every 20 s for 5 min. To calculate the enzymatic activity, the initial rate of the reaction was calculated. The specific trypsin activity was reported in units of μmol of p-nitroaniline liberated per mg protein per min.

Lipase activity (EC 3.1.1.3) was assessed using the Winkler and Stuckmann (1979) technique, which used para-nitrophenyl palmitate (p-NPP, Sigma N2752) as a substrate. Two solutions were mixed until completely dissolved to get the substrate solution: solution A (30-mg p-NPP in 10-mL isopropanol) and solution B (0.1-g gum arabic and 1-mL Triton-X100 in 90 mL of previously prepared 50 mM Tris–HCl buffer of pH 8.5). The absorption was measured every 20 s for 5 min at 410-nm optical density. The μmol of p-nitrophenol released per mg protein per min was used as the unit of specific lipase activity.

The α-amylase activity was measured using 1% starch solution in 50-mM Tris–HCl buffer with 10-mM NaCl (pH 8.0) as a substrate. Extracted enzyme (0.05 mL) was mixed with 0.5-mL substrate solution and 0.5-mL buffer. Then, this mixture was incubated at room temperature (25 °C) for 10 min. Production of reducing sugars was measured by following the dinitrosalicylic acid method (Somogyi, 1945). Then, samples were taken with 0.5-mL 1% dinitrosalicylic acid (DNS) and incubated in a water bath (boiling) for 5 min. Finally,

absorbance values were recorded at 540-nm optical density. The maltose standard curve was used to determine the amount of maltose released from this assay. The quantity (μmol) of maltose generated mg^{-1} protein min^{-1} was expressed as units of specific amylase activity.

Hemato-immunological parameter determination

To assess innate-immune parameters, blood was drawn from the caudal vein of two fish from three replicates (6 fish $treatment^{-1}$) using a 1-mL syringe with a 25-G needle. Collected blood was instantly processed for total blood cell count (red blood cell (RBC) and white blood cell (WBC)), hemoglobin (Hb), hematocrit (Ht), respiratory burst activity, and total serum protein determination as described by Suwannasang et al. (2014). Briefly 0.5 μL of blood was taken into a diluting pipette and mixed up to 1 mL of Yokoyama solution to count the blood cells under a microscope using hemocytometer.

Hb and Ht were determined using the methodology of Blaxhall and Daisley (1973) and Larsen and Snieszko (1961), respectively. For Hb, a micropipette was used to put 20-μL blood sample into a test tube filled with 5-mL Drabkin's solution. These were then mixed in a vortex mixer and incubated for 20 min at room temperature. Finally, 300 μL sample was placed in a 96-well plate and measured at 540 nm optical density. In the case of Ht, blood was drawn into a capillary tube and sealed with clay. The capillary tube was then centrifuged at 10,000*g* for 5 min using a hematocrit centrifuge unit, and the percentage of hematocrit was calculated using the formula below.

$$Hematocrit\ (Ht, \%) = (Packed\ cell\ volume / Total\ blood\ volume) \times 100$$

Leucocyte respiratory burst activity was assessed using the reduction of nitroblue tetrazolium to formozan as an indicator of superoxide anion (O_2^-) generation. Microplate readers were used to measure the absorbance at 640 nm where DMSO/KOH was employed as a blank (Stasiak and Baumann, 1996). Total serum protein was assessed colorimetrically by the method of Lowry et al. (1951). The blood sample was collected in a centrifuge tube and placed at room temperature for 1 h to facilitate blood clotting. To separate out the serum, it was centrifuged for 15 min at 5000 *g*. Then, 5 μL of serum was applied to 995 μL of double distilled water (DDW) in a test tube and mixed in a vortex mixer. After that, 2-mL alkaline copper solution (mixed in the vortex) was added to the test tube and incubated for 10 min at room temperature. Then, 3 mL of Folin reagent was also added to the test tube (also mixed in the vortex) and incubated for another 10 min. Finally, 300-μL sample was placed in a 96-well plate and measured in a microplate reader at 640-nm optical density.

Expression of Δ6 fatty acyl desaturase (Δ6 Fad) and elongase (Elovl5) genes through real-time quantitative reverse transcription-PCR (qRT-PCR)

Total RNA extraction and cDNA preparation

The liver was collected from two fish from four tanks per treatment (8 fish $treatment^{-1}$) and pooled to two replications and stored until use in RNAlater Solution (Invitrogen, USA) according to the company's protocol. The extraction of total RNA was conducted using Total RNA Mini Kit (Tissue) (Geneaid, Taiwan) following the manufacturer's

protocol with slight modification after Chirapongsatonkul et al. (2019), and the genomic DNA was removed by treating with DNase 1, RNase free (Thermo Fisher Scientific, USA). The quality and quantity of the extracted RNA were evaluated using a NanoDrop Spectrophotometer (BioDrop, UK). Then, 1 µg of total RNA was used for first-strand cDNA synthesis using High-Capacity cDNA Reverse Transcription Kits (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. The reverse transcription was executed in a thermal cycler (Bio-Rad T100™ Thermal Cycler, USA). After diluting, each reaction product with DNase/RNase-free water (Promega, USA) was stored at −20 °C until used for qRT-PCR analysis.

Quantitative real-time PCR (qRT-PCR)

Quantitative real-time PCR (qRT-PCR) technique with β-actin as a reference gene was used to assess the expression of Δ6 fatty acyl desaturase (Δ6-Fad) and elongase of very long-chain fatty acids (Elovl5) in the liver tissue of Asian seabass. The primers of each gene used in this study were designed based on the nucleotide sequence submitted to the GeneBank (Table 3). The qRT-PCR reaction (total volume 20 µL) composed of 1-µL cDNA, 4-µL 5× HOT FIREPol® EvaGreen® qPCR Mix Plus (no ROX), 2-µL primer cocktail (10 pmol/ µL), and 13 µL of DEPC-treated water. The reactions were duplicated with a negative control containing no cDNA. The reaction was initiated at 95 °C for 12 min, followed by 40 cycles of 95 °C for 15 s, 60 °Cfor 20 s, and 72 °C for 20 s, as suggested by the manufacturers. The fold change of the relative expression of the genes previously mentioned was assessed by following the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Statistical analysis

All data except for fatty acid profiles were checked for normality and homogeneity of variance by the Shapiro–Wilk test and Levene's test, respectively. The data were then analyzed using one-way ANOVA in the statistical package SPSS 22 (SPSS, Inc., Chicago, IL, USA) for Windows. Tukey's HSD test was performed to differentiate the treatment means at 95% confidence level ($p<0.05$). The data were expressed as mean±SD.

Table 3 Real-time qPCR primers for relative mRNA expression of Δ6 fatty acyl desaturase (Δ6 Fad) and elongase of very long-chain fatty acid (Elovl5) genes in liver tissue of Asian seabass

Gene	Primer ID	Sequence (5′-3′)
Δ6 Fad	qLcDesF	TTAATTCCCTTTGCCGTATTTAAA
	qLcDesR	AAGAAATCCTGCACAGAATCTGAA
Elovl5	qLcEloF	ATGGTCACGCTCATTATCCTTTT
	qLcEloR	AGCATTGGGTGGCGGTTTC
B-actin	Lcal-qActF	CTTCACCACCACAGCCGAGA
	Lcal-qActR	TGCCGATGGTGATGACCTGT

Results

Acceptance of diets

At the completion of the feeding trial, there was a significant difference ($p<0.05$) in the voluntary consumption by fish (Table 4). The highest feed consumption was observed in the T4 (25:50:25) group, which was significantly higher than in the control group ($p<0.05$) and T6 (0:75:25), while the consumption of the control and other diets were in a similar range ($p>0.05$).

Survival and growth performance

No fish died (SR 100%) during the trial period. There were no significant differences ($p>0.05$) in final weight, growth, and FCR among the treatments (Table 4). The lipid sources had no effect on HSI ($p>0.05$) but did significantly affect VSI and IPF ($p<0.05$), with the lowest values in the fish fed the T4 (25:50:25) diet.

Proximate composition of fish carcass and nutrient efficiency

The whole-body carcass composition and nutrient utilization data are presented in Table 5. There was no effect ($p>0.05$) of treatment on the whole-body moisture, ash, or crude protein content. Lipid content, however, differed significantly ($p<0.05$) among the dietary groups. The lipid contents of fish in groups T3 (50:25:25), T4 (25:50:25), T5 (0:50:50), and T6 (0:75:25) were significantly ($p<0.05$) lower than of fish in the control group but significantly higher than the 100% AO diet group. In terms of nutrient utilization, no significant differences related to treatment were found in PER, LER, and PRE ($p>0.05$), but LRE saw a significant impact ($p<0.05$). The fish fed the diet containing 100% AO had the lowest LRE, which was significantly lower ($p<0.05$) than that of the control and other treatment groups except T5.

Fatty acid profile of diets and fish carcass

The fatty acid profiles of the fish sampled at the end of the trial (Table 6) generally reflected those of the diets they had been fed (Table 2). Fish fed the control diet and the 100% AO diet exhibiting the highest and lowest average MUFA, respectively. As predicted, LA and total n-6 PUFA levels were higher in fish that received the diet consisting of 75% VOs and 25% AO (T6, 0:75:25). Fish that were fed the 100% AO diet had the highest ARA, EPA, DHA, and n-3/n-6 ratio, while fish in the T6 group (0:75:25) had the lowest. Furthermore, the concentrations of EPA decreased, and DHA increased, in fish in the experimental groups in comparison with those in the control group. The EPA concentration in the diets and carcass ranged from 1.50 to 5.76% and 1.28 to 4.57% of total fatty acids, respectively. The observed correlation coefficient between dietary EPA (Y) and carcass EPA content (X) was $y = 0.7798x + 0.0961$, where R^2 is 0.9959 (Fig. 1). On the other hand, the DHA concentration in the diets and carcasses ranged between 4.88 and 19.24% and 6.10 and 21.68% of total fatty acids, respectively. The correlation coefficient between dietary and carcass DHA was $y = 1.0641x + 1.5535$ where R^2 is 0.99 (Fig. 1). The slops indicated that EPA

Table 4 Final weight, weight gain, survival rate, consumed feed, food conversion ratio (FCR), specific growth rate (SGR), viscerosomatic index (VSI), hepatosomatic index (HSI), and intraperitoneal fat (IPF) of Asian seabass after 8-week feeding with diets containing different levels of vegetable oils and algal oil replacing fish oil

Growth parameters	Diets (fish oil to vegetable oils to algal oil)						*p*-value
	T1 (100:0:0)	T2 (0:0:100)	T3 (50:25:25)	T4 (25:50:25)	T5 (0:50:50)	T6 (0:75:25)	
Initial weight (g fish^{-1})	3.89±0.23	3.89±0.23	3.88±0.24	3.89±0.23	3.89±0.22	3.89±0.24	
Final weight (g fish^{-1})	27.57±3.04	29.36±3.24	27.17±2.77	32.41±2.78	30.11±2.83	26.75±3.33	0.052
Weight gain (g fish^{-1})	23.69±3.04	25.47±3.23	23.29±2.77	28.53±2.78	26.22±2.83	22.86±3.32	0.052
Survival rate (%)	100	100	100	100	100	100	
Consumed feed (g fish^{-1})	31.39±2.53^{b}	32.44±3.45^{ab}	31.78±2.85^{ab}	36.45±2.01^{a}	35.30±0.89^{ab}	31.21±2.96^{b}	0.013
FCR	1.33±0.10	1.28±0.09	1.37±0.07	1.28±0.07	1.36±0.12	1.38±0.12	0.456
SGR (% day^{-1})	3.49±0.19	3.60±0.19	3.47±0.18	3.78±0.16	3.65±0.17	3.43±0.22	0.054
VSI (%)	6.27±0.51^{a}	5.78±0.57^{ab}	5.55±0.83^{ab}	5.45±0.42^{b}	5.66±0.25^{ab}	6.23±0.36^{ab}	0.014
HSI (%)	1.70±0.15	1.43±0.27	1.44±0.14	1.57±0.21	1.57±0.49	1.60±0.25	0.469
IPF (%)	1.75±0.23^{ab}	1.56±0.33^{ab}	1.65±0.21^{ab}	1.53±0.09^{b}	1.79±0.42^{ab}	2.12±0.49^{a}	0.033

Values are mean ± SD of five replicates except VSI, HSI, and IPF where n=6. Means of main effects in the same column with different superscripts (a, b, and c) are significantly different (p <0.05)

Weight gain (g fish^{-1}) = final weight (g) − initial weight (g)

Survival rate (%) = 100 × (final fish number/initial number)

Food conversion ratio (FCR) = feed intake/weight gain (g/fish)

Specific growth rate (SGR, % day^{-1}) = ($\ln W2 - \ln W1/T2-T1$) ×100; $W1$ = initial weight, $W2$ = final weight, $T2-T1$ = cultured period (days)

Viscerosomatic index (VSI, %) = 100 × (viscera weight/body weight)

Hepatosomatic index (HSI, %) = 100 × (liver weight/final body weight)

Intraperitoneal fat ratio (IPF, %) = 100 × (intraperitoneal fat weight/final body weight)

and DHA showed different slops. The slope value greater than unity means the concentration of the fatty acid in carcass lipid was higher than in dietary lipid. The indices of atherogenicity and thrombogenicity were similar in all feeding groups, while incorporation of AO in the diets improved fish lipid quality (FLQ) over the control group. The highest FLQ value was observed in fish fed on the 100% AO diet.

Digestive enzyme activities

Specific digestive enzyme activities in the pyloric caeca and intestine are reported in Table 7. Dietary FO replacement by AO or a combination of VOs and AO did not ($p^{>}0.05$) affect the specific trypsin and amylase activities in the pyloric caeca. Specific lipase activities, however, were influenced by dietary lipid source ($p<0.05$). Fish in the T4 (25:50:25) and T6 (0:75:25) groups exhibited the highest and lowest specific lipase activity in the intestine, respectively, while the fish fed the FO replacement diets were not impacted ($p>0.05$).

Hemato-immunological parameters

Innate immune parameters of Asian seabass fed diets containing alternate lipid sources are documented in Table 8. After 8 weeks of the feeding trial, there were no significant effects of either AO only, or the VOs and AO in combination, on RBC and WBC counts, Hb, serum protein, or respiratory burst activity ($p>0.05$).

Expression of Δ6 fatty acyl desaturase (Δ6 Fad) and elongase (Elovl5) genes

The expressions of the target genes are shown in Fig. 2. Asian seabass fed FO replacement diets in freshwater exhibited a significant difference in mRNA expression of Δ6-Fad and Elovl5 ($p<0.05$), with Elovl5 exhibiting a higher degree of mRNA expression than Δ6-Fad. Fish in the T5 (0:50:50) group had significantly higher Δ6-Fad and Elovl5 mRNA expression ($p<0.05$) than the control group, which was statistically similar to the fish in the T2 (0:0:100) and T6 (0:75:25) groups. The expression of Δ6-Fad and Elovl5 mRNA in fish in the T3 (50:25:25) group was statistically similar ($p>0.05$) to fish in the control group, but significantly lower ($p<0.05$) than fish in the other groups.

Discussion

The goal of this study was to determine the feasibility of replacing FO in diets for juvenile Asian seabass with a combination of AO and VOs (soybean and palm oil). Fish that were fed diets containing either AO as the sole lipid source or in combination with VOs demonstrated good growth and 100% survival rate. The substitution of AO for FO in diets increased the availability of n-3 PUFA, especially EPA and DHA, which may have been responsible for the improved growth factors. Similar findings in terms of growth performance were observed in Atlantic salmon fed diets that sprayed with dried microalgae as compared to the control group fed with 100% FO (Kousoulaki et al. 2015, 2016). The growth performance of Asian seabass has been reported to be sensitive to vegetable oil

Table 5 Proximate composition, protein efficiency ratio (PER), lipid efficiency ratio (LER), protein retention efficiency (PRE), and lipid retention efficiency (LRE) of Asian seabass after 8-week feeding with diets containing different levels of vegetable oils and algal oil replacing fish oil

Parameters	Initial fish	Diets (fish oil to vegetable oils to algal oil)						*p*-value
		T1 (100:0:0)	T2 (0:0:100)	T3 (50:25:25)	T4 (25:50:25)	T5 (0:50:50)	T6 (0:75:25)	
Moisture (%)	76.50	72.87±0.25	72.72±0.68	73.23±0.92	72.59±0.86	72.46±0.65	72.55±0.52	0.636
Ash (%)	3.55	3.95±0.07	4.14±0.09	4.18±0.20	4.32±0.27	4.21±0.20	4.37±0.03	0.105
Protein (%)	15.50	17.13±0.55	17.28±0.28	17.04±0.63	17.62±0.40	17.58±0.14	17.39±0.36	0.138
Lipid (%)	3.66	4.66±0.24^{a}	2.87±0.06^{c}	4.23±0.26^{b}	4.10±0.15^{b}	4.18±0.23^{b}	4.19±0.19^{b}	0.000
PER	-	1.73±0.14	1.71±0.17	1.66±0.08	1.73±0.05	1.59±0.14	1.75±0.19	0.706
LER	-	8.03±0.67	8.56±0.83	8.16±0.38	8.56±0.26	7.42±0.67	8.43±0.90	0.329
PRE (%)	-	30.06±1.37	30.02±2.64	28.71±0.99	31.03±1.24	28.48±3.18	30.96±3.61	0.693
LRE (%)	-	38.81±5.28^{a}	23.42±2.88^{b}	35.27±1.94^{a}	36.39±3.06^{a}	31.70±4.76^{ab}	36.12±6.22^{a}	0.008

Values are mean ± SD of three replicates. Means of main effects in the same column with different superscripts (a, b, c) are significantly different ($p < 0.05$)

Protein efficiency ratio (PER) = weight gain/total protein intake

Lipid efficiency ratio (LER) = weight gain/total lipid intake

Protein retention efficiency (PRE, %) = 100 × [{(final body weight × final body protein) − (initial body weight × initial body protein)}/total protein intake]

Lipid retention efficiency (LRE, %) = 100 × [{(final body weight × final body lipid) − (initial body weight × initial body lipid)}/total lipid intake]

Table 6 Fatty acid profile (% of total fatty acids) of Asian seabass carcass after 8-week feeding with diets containing different levels of vegetable oils and algal oil replacing fish oil

Fatty acids (% of total fatty acids)	Initial fish	Diets (fish oil to vegetable oils to algal oil)					
		T1 (100:0:0)	T2 (0:0:100)	T3 (50:25:25)	T4 (25:50:25)	T5 (0:50:50)	T6 (0:75:25)
Lauric acid (12:0)	0.04	0.25	0.34	0.26	0.31	0.33	0.32
Myristic acid (14:0)	3.34	2.75	2.21	2.10	1.82	1.61	1.47
Pentadecylic acid (15:0)	0.77	0.43	0.40	0.37	0.28	0.28	0.23
Palmitic acid (16:0)	20.53	20.08	21.95	20.21	21.07	20.61	21.26
Margaric acid (17:0)	0.68	0.31	0.24	0.27	0.23	0.21	0.20
Stearic acid (18:0)	5.98	4.97	4.78	4.87	5.46	5.21	5.63
Arachidic acid (20:0)	0.32	0.21	0.28	0.25	0.24	0.26	0.26
Behenic acid (22:0)	0.15	0.05	0.18	0.15	0.15	0.19	0.19
Total SFA[1]	**31.81**	**29.05**	**30.38**	**28.48**	**29.56**	**28.70**	**29.56**
Myristoleic acid (14:1n-5)	0.06	0.13	0.10	0.11	0.11	0.10	0.09
Palmitoleic acid (16:1n-7)	5.02	5.36	2.72	3.82	3.44	2.57	2.71
Heptadecenoic acid (17:1)	0.86	0.14	0.10	0.14	0.13	0.15	0.17
Vaccenic acid (18:1n-7)	3.87	3.40	1.71	2.74	2.55	2.00	2.31
Oleic acid (18-1n-9)	22.35	29.32	17.38	26.80	24.89	23.05	25.36
Gondoic acid (20:1n-9)	5.72	0.23	0.49	2.47	1.45	0.45	0.60
Erucic acid (22:1n-9)	0.77	0.61	0.22	0.43	0.29	0.18	0.19
Nervonic acid (24:1n-9)	1.04	0.62	0.32	0.50	0.45	0.37	0.34
Total MUFA[2]	**39.69**	**39.81**	**23.04**	**37.01**	**33.31**	**28.87**	**31.77**
Linoleic acid (18:2n-6)	5.53	12.91	12.37	15.87	20.71	20.26	24.04
Gamma-linolenic acid (18:3n-6)	0.30	0.67	0.52	0.72	1.02	0.84	1.11
Eicosadienoic acid (20:2n-6)	0.43	0.41	0.17	0.32	0.31	0.22	0.27
Dihomo-gamma-linolenic acid (20:3n-6)	0.20	0.34	0.25	0.30	0.45	0.33	0.40
Arachidonic acid (20:4n-6)	1.44	1.04	1.61	1.15	1.04	1.28	1.04
Adrenic acid (22:4n-6)	0.21	0.26	0.28	0.26	0.26	0.24	0.22

Table 6 (continued)

Fatty acids (% of total fatty acids)	Initial fish	Diets (fish oil to vegetable oils to algal oil)					
		T1 (100:0:0)	T2 (0:0:100)	T3 (50:25:25)	T4 (25:50:25)	T5 (0:50:50)	T6 (0:75:25)
n-6 Docosapentaenoic acid (22:5n-6)	0.86	0.32	1.44	0.62	0.55	0.89	0.52
Total n-6 PUFA[3]	**8.97**	**15.95**	**16.64**	**19.24**	**24.34**	**24.06**	**27.60**
α-Linolenic acid (18:3n-3)	0.31	4.77	1.07	1.56	1.89	1.73	2.01
Stearidonic acid (18:4n-3)	0.59	0.44	0.28	0.31	0.26	0.21	0.19
Eicosapentaenoic acid (20:5n-3)	2.85	1.81	4.57	2.17	1.72	2.51	1.28
n-3 Docosapentaenoic acid (22:5n-3)	1.64	1.28	1.44	1.09	0.94	0.99	0.68
Docosahexaenoic acid (22:6n-3)	12.88	6.10	21.68	9.60	7.70	12.61	6.74
Total n-3 PUFA[4]	**18.27**	**14.40**	**29.04**	**14.73**	**12.51**	**18.05**	**10.90**
n-3/n-6	2.03	0.90	1.74	0.76	0.51	0.74	0.39
IA[5]	0.51	0.45	0.45	0.41	0.41	0.39	0.39
IT[6]	0.37	0.39	0.27	0.37	0.42	0.34	0.45
FLQ[7]	15.93	7.97	26.49	11.83	9.45	15.17	8.03

[1]SFA: (4:0), (12:0), (14::0), (15:0), (16:0), (17:0), (18:0), (20:0), and (22:0)

[2]MUFA: (14:1n-5), (16:1n-7), (18:1n-7), (18-1n-9), (20:1n-9), (22:1n-9), and (24:1n-9)

[3]n-6 PUFA: (18:2n-6), (18:3n-6), (20:2n-6), (20:3n-6), (20:4n-6), (22:4n-6), and (22:5n-6)

[4]n-3 PUFA: (18:3n-3), (18:4n-3), (20:5n-3), (22:5n-3), and (22:6n-3)

[5]IA (index of atherogenicity): [C12:0 + (4 × C14:0) + C16:0]/ΣUFA

[6]IT (index of thrombogenicity): (C14:0 + C16:0 + C18:0)/[(0.5 × ΣMUFA) + (0.5 × Σn-6 PUFA) + (3 × Σn-3 PUFA) + (n-3/n-6)]

[7]FLQ (fish lipid quality): 100 × (C22:6n-3 + C20:5n-3)/ΣFA

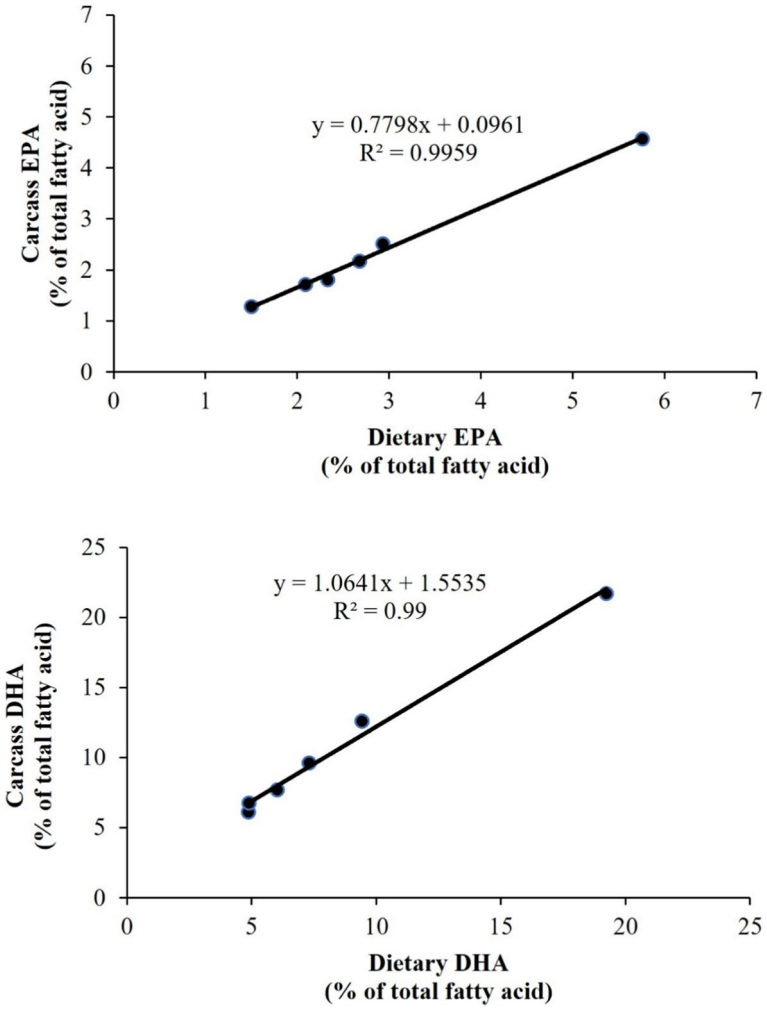

Fig. 1 Relationship between dietary fatty acid and carcass fatty acid concentrations for EPA and DHA in the total lipids of Asian seabass after 8-week feeding with diets containing different levels of vegetable oils and algal oil replacing fish oil

levels when used to replace fish oil. For example, Rahman et al. (2021) found that when FO was substituted by more than 25% soybean oil (SO), palm oil (PO), or sunflower oil (SFO), fish growth decreased, whereas an equal proportion of SO and PO at 37.5% substitution allowed growth improvement (Rahman et al. 2022). The IPF in this study was higher in the T6 fed fish (0 FO:75 VOs:25 AO) than the T4 (25 FO:50 VOs:25 AO) fish group. This finding is consistent with the study by Qiao et al. (2014) in olive flounder (*Paralichthys olivaceus*), who found that high VO levels in the diet led to increased visceral fat but that there was no effect when raw microalgae were substituted for FO. The combination of AO and VOs used in the current study may have supplied a better balanced dietary fatty acid profile for various important metabolic processes, resulting in decreased visceral fat deposition in Asian seabass. Our findings indicate that the combination of AO and VOs

Table 7 Digestive enzyme activity in pyloric caeca and intestine of Asian seabass after 8-week feeding with diets containing different levels of vegetable oils and algal oil replacing fish oil

Specific digestive enzyme (unit mg^{-1} protein min^{-1})	Diets (fish oil to vegetable oils to algal oil)						*p*-value
	T1 (100:0:0)	T2 (0:0:100)	T3 (50:25:25)	T4 (25:50:25)	T5 (0:50:50)	T6 (0:75:25)	
Pyloric caeca							
Specific trypsin activity	1.37±0.24	1.30±0.36	1.23±0.55	0.90±0.06	0.79±0.10	1.03±0.34	0.052
Specific lipase activity	2.73±0.18^{ab}	2.78±0.60^{ab}	2.23±0.45^{ab}	2.95±0.82^{a}	2.11±0.33^{ab}	1.96±0.29^{b}	0.011
Specific amylase activity	0.79±0.10	0.78±0.13	0.85±0.08	0.79±0.07	0.83±0.10	0.83±0.11	0.817
Intestine							
Specific trypsin activity	0.51±0.25	0.52±0.09	0.59±0.11	0.61±0.22	0.62±0.13	0.64±0.04	0.781
Specific lipase activity	1.77±0.36	1.60±0.24	1.64±0.43	1.49±0.36	1.65±0.63	1.32±0.16	0.657
Specific amylase activity	0.72±0.09	0.73±0.13	0.72±0.13	0.71±0.05	0.72±0.06	0.66±0.05	0.870

Values are mean ± SD where n=6. Means of main effects in the same column without superscripts are not significantly different ($p > 0.05$)

Table 8 Red blood cell (RBC), white blood cell (WBC), hemoglobin (Hb), serum protein, and respiratory burst activity in Asian seabass after 8-week feeding with diets containing different levels of vegetable oils and algal oil replacing fish oil

Specific digestive enzyme (unit mg^{-1} protein min^{-1})	Diets (fish oil to vegetable oils to algal oil)						*p*-value
	T1 (100:0:0)	T2 (0:0:100)	T3 (50:25:25)	T4 (25:50:25)	T5 (0:50:50)	T6 (0:75:25)	
RBC cells/mL ($\times 10^9$)	4.35±0.50	3.91±0.98	3.80±0.79	4.44±0.67	3.95±0.41	3.61±0.43	0.119
WBC cells/mL ($\times 10^7$)	4.71±1.23	5.54±1.35	3.88±1.59	4.53±0.91	4.58±1.00	4.27±0.90	0.228
Hb (g dL^{-1})	6.85±0.68	7.17±0.73	6.79±0.59	7.32±0.72	7.10±0.64	6.85±0.74	0.602
Serum protein (mg mL^{-1})	100.40±6.46	104.17±5.61	102.05±3.89	102.49±5.60	103.78±6.50	100.11±6.06	0.640
Respiratory burst activity (OD 640)	0.029±0.003	0.038±0.014	0.035±0.010	0.039±0.014	0.042±0.011	0.037±0.005	0.352

Values are mean ± SD where n=8. Means of main effects in the same column without superscripts are not significantly different ($p > 0.05$)

was a suitable substitute for FO in Asian seabass diets in that they did not impair growth performance or major somatic parameters.

As expected, complete substitution of FO with AO had an effect on lipid metabolism and deposition. The decreased lipid content and IPF in fish fed diets containing AO suggests that the higher n-3 LC-PUFA content could have been an important factor. It has been documented that dietary DHA has an anti-obesity effect in rodents by inhibiting adipocyte differentiation, inducing apoptosis in postconfluent adipocytes, and increasing lipolysis, all of which resulted in a decrease in fat accumulation (Kim et al. 2006).

The FA composition of dietary lipid sources has a noticeable effect on the FA profile of fish carcasses and tissues (Miller et al. 2007; Qiao et al. 2014; Sarker et al. 2016). Since

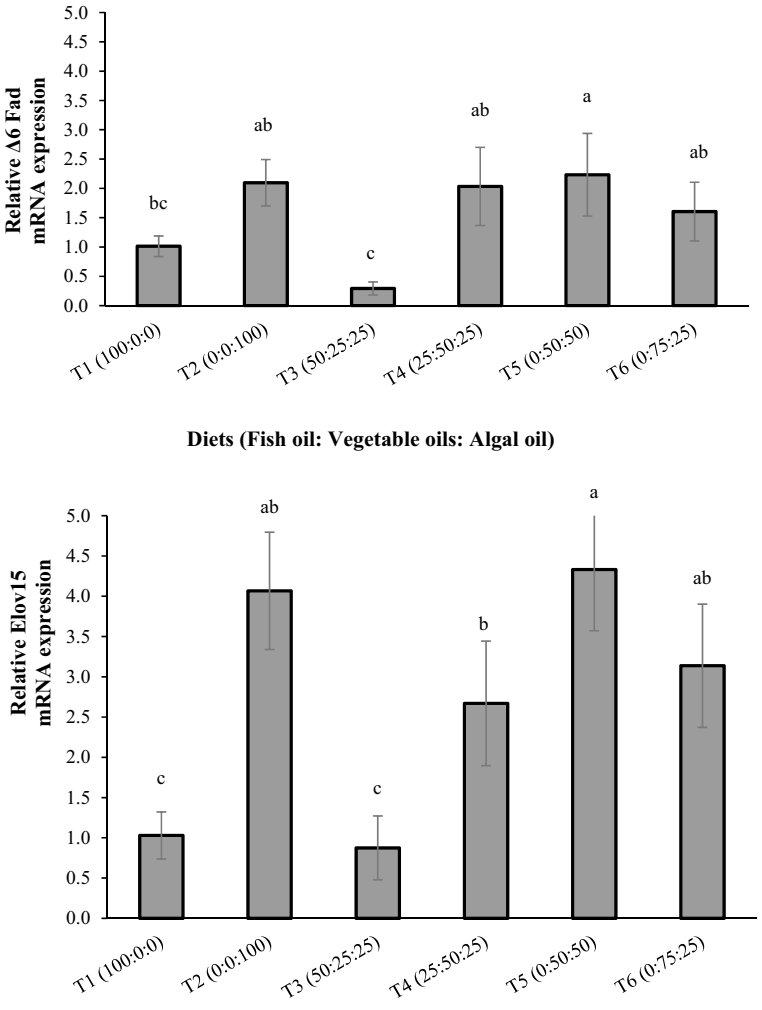

Fig. 2 Relative mRNA expression of Δ6 fatty acyl desaturase (Δ6 Fad) and elongase of very long-chain fatty acid (Elovl5) genes in liver tissue of Asian seabass after 8-week feeding with diets containing different levels of vegetable oils and algal oil replacing fish oil

the AO is a rich source of n-3 LC-PUFA (ARA, EPA, and DHA) (Carvalho et al. 2020), the increased AO content in the diets resulted in an increased level of ARA, EPA, DHA, and n-3/n-6 ratio, which was also reflected in the final fish carcass. The carcass FA composition of the experimental fish groups indicates that the DHA was selectively retained in fish body. Since the fish exhibited limited capacity to desaturate and elongate C18-PUFA to EPA and then DHA (Mohd-Yusofet al. 2010), it was expected that these FAs would be associated with selective retention of DHA rather than with synthesis. These findings are in line with other species such as trout (Turchini and Francis 2009) and common carp (*Cyprinus carpio*) (Ren et al. 2013). Fatty acid retention or catabolism largely depends on the dietary fatty acid, its level, and the tissue β-oxidation (Torstensen, Frøyland, and Lie 2004). Probable mechanisms of selective retention of DHA in this study may be related to high specificity of fatty acyltransferases for DHA, or reduced catabolism of DHA due to the complex peroxisomal β-oxidation (Bell et al. 2002). Substrate preferences for SFA and MUFA over PUFA were observed in β-oxidation of fish (Henderson 1996). Most fatty acids, including oleic acid (18:1n-9) and erucic acid (22:1n-11) as well as ALA, LA, and EPA, are readily oxidized in fish, whereas DHA is selectively retained in fish tissue due to preferential and selective catabolism of the FAs (Torstensen et al. 2004). In addition, the decreased EPA content may be attributed to the concomitant appearance of n-3 docosapentaenoic acid (n-3 DPA) via elongation of C20 to C22. Asian seabass probably respond to synthesis of DHA from dietary precursors through the PUFA conversion pathway. The enzyme Δ5 desaturase is required to synthesize n-3 DPA but is unlikely to exist in the species (Mohd-Yusof et al. 2010). Another possible explanation for the presences of n-3 DPA is as an intermediate product of peroxisomal β-oxidation of DHA to EPA for eicosanoid production (Brossard et al. 1996).

To further understand the impact of FO replacement on fatty acid metabolism, we examined the relative mRNA expression in the liver of the Δ6-Fad and Elovl5 genes which are involved in fatty acid synthesis and metabolism. Δ6-Fad is active in both ALA and LA, while Elovl5 is active in elongation of C18-C20, C20-C22, and C22-C24 (Mohd-Yusof et al. 2010). The action of Δ4-desaturase has been detected in some other species, suggesting that it may induce independent DHA synthesis from EPA (Monroig, Tocher, and Castro 2018). Fish bioconversion capability of C18-PUFA to n-3 LC-PUFA varies by species, with most marine fish species having lower bioconversion capacity than freshwater fish species (Turchini et al. 2009). Generally, PUFA bioconversion is mediated by a series of fatty acyl desaturase (Fad) and elongase of very long-chain fatty acids (Elovl) enzymes. ALA is first transformed into EPA by Δ6 desaturation, then elongation, and finally Δ5 desaturation (Cook and McMaster 2002). Another pathway requires converting EPA to DHA through two elongation processes: Δ6 desaturation and peroxisomal chain shortening (Sprecher 2000). In this analysis, the mRNA expression of Elovl5 gene was considerably higher than that of the Δ6-Fad gene in all dietary treatments. Although the expression of Δ6-Fad was significantly different in liver tissue in response to FO replacement in the Asian seabass diet, the expression was minor. Conversely, Elovl5 gene expression was nearly fourfold higher in T2 and T5 feeding groups containing 100% and 50% AO, respectively, than in the control group, except in T3 (50 FO:25 VOs:25 AO), indicating that EPA to DHA conversion may occur in liver tissue. Thus, the activity of the Δ4-desaturase gene is still needed to be explored in order to understand the conversion of EPA to DHA. Recent experiments on Asian seabass have demonstrated the possibility of DHA synthesis from EPA when fish were fed lower DHA levels, though the mechanism of DHA retention was unclear (Glencross and Rutherford 2011; Morton et al. 2014). In our study, however, DHA was abundant due to the use of AO for FO replacement in the diets, indicating that the fish had limited ability to desaturate and elongate LC-PUFA from their precursors C18-PUFA.

The quantity of n-3 LC-PUFA, particularly EPA and DHA, increased in the fish carcass due to AO substitution in the diets, which improved the nutritional value of the fish for human consumption. Fish fed the AO diet exhibited a good index of atherogenicity (IA) and an index of thrombogenicity (IT) relative to the control fish group. Furthermore, the high concentration of EPA and DHA in carcasses of AO fed fish improved fish lipid quality (FLQ) by 3.32 times in the 100% AO treatment relative to control and was also greater in other feeding groups compared to the control group. These findings are in line with the studies in the gilthead seabream (Carvalho et al. 2020) and rainbow trout (Teimouri et al. 2016), where algal sources were used to supply EPA and DHA in the diets. The IA, IT, and FLQ lipid quality indices are prominent indications of the possible nutritional value of fish for the cardiovascular health of the final consumer (Ulbricht and Southgate 1991). The IA considers the ratio of proatherogenic fatty acids, such as SFA, to antiatherogenic fatty acids, such as PUFA, to reflect the risk of lipid accumulation in blood vessels (Carvalho et al. 2020). In contrast, the IT represents the possibility of blood clotting in the vessels, which is supported by prothrombogenic SFA and opposed by antithrombogenic MUFA or PUFA. In this study, these indices were estimated to determine the possible effects of dietary AO and VOs on consumer health, even though our experimental fish did not reach a plate size. Thus, the use of AO and VO combinations in Asian seabass diets would allow delivery of quality products to the consumer.

The activity of digestive enzymes is closely related to the dietary intake of organisms (Eusebio and Coloso 2002). In our study, fish that were fed the FO replaced diet by 75% VOs and 25% AO had significantly lower specific lipase activity in the pyloric caeca. We previously observed alteration in specific lipase activity when FO was substituted by higher amounts of VO (Rahman et al. 2021, 2022). Similarly, Bowyer et al. (2012) reported that yellowtail kingfish exhibited lower lipase activity when fed a canola oil-containing diet than the control group. It is well established that LC-PUFA is normally highly digestible in most fish species, meaning that it is also efficiently absorbed (Turchini et al. 2009). Thus, high levels of VOs in the diet decrease dietary content of LC-PUFA, which modifies the function of lipase activity in fish.

Hematological parameters are important in determining the health of the fish (Peres et al. 2013). There were no significant differences in RBC and WBC counts, Hb, serum protein, or respiratory burst activity in fish feeding FO replacement diets in our study. FO substitution with alternative lipid sources in the diets may affect fish immunity by modifying the membrane fluidity of immune cells (Montero et al. 2008). Previously, we found that Asian seabass fed FO replacement diets with VOs had lower WBC and serum protein levels than the FO control group (Rahman et al. 2021, 2022). The findings of the current study, however, showed that replacing FO with a combination of AO and VOs had no detrimental impact on immune parameters. The results indicated that the fish obtained the necessary LC-PUFA from the supplied AO for its immune system to function properly.

Conclusion

Replacing FO with a combination of AO and VOs had no detrimental impact on immune parameters.

Fish oil can be effectively substituted in diets for juvenile Asian seabass (*L. calcarifer*) at 75% by combining 50% vegetable oils (soybean and palm oil) and 25% algal oil without compromising growth, nutrient utilization, fatty acid profile, digestive enzyme activities, and immunity.

Asian seabass demonstrated elongation capability of DHA from EPA that resulted in higher levels of DHA in the body than the dietary levels.

The current price of AO ($8.98 kg^{-1}) is almost 3 time higher than FO, and this may limit its use in aquaculture grow out feeds, but with increased efforts to contain production costs and the increasing body of information about its efficacy as a feed supplement, including the finding of the current study, it can be expected that AO will become an attractive replacement for fish oil in the near future.

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Author contributions All authors equally participated.

Data availability Data of this article will be provided on request.

Declarations

Ethical approval This research followed the National Research Council of Thailand's "Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes."

Conflict of interest The authors declare no competing interests.

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