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Vegetable Oil Blends as Fish Oil Alternatives in the Diets of *Heterobranchus longifilis* Fingerlings

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

This study was undertaken to determine the suitability of vegetable oil blends as a major source of lipid in place of fish oil in the diet of *Heterobranchus longifilis* fingerlings. The fish were fed one of the six experimental diets twice daily to satiation for twelve weeks. The test diets were identical in composition, except for the source of supplemental lipid which was either fish oil (FO), palm oil (PO), soybean oil (SO), equal blend of FO and PO (FOPO), equal blend of PO and SO (POSO) or equal blend of FO, PO and SO (FOPOSO). The growth parameters were significantly different (P<0.05) among dietary treatments. However, the apparent digestibilities were not significantly different (P>0.05). Malic enzyme activities in the liver were not significantly different among dietary groups. On the other hand, the activities of glucose-6-phosphate dehydrogenase were significantly different and over six-fold higher malic enzyme. Results indicated that FO may be replaced with either PO, SO and their blends in *H. longifilis* diets.

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1. INTRODUCTION

Wild marine fish populations are used to produce fish meals and oils that provide EFA and other nutrients in aquafeeds, although the production from these stocks are fully exploited, and is not expected to increase beyond the present level [1]. This has provided the impetus to investigate alternatives to marine lipids in aqua feeds. Alternative sources of lipids can be of vegetable origin, as they are often cheaper and more available than marine oils [2].

Endogenous lipids, synthesized via lipogenesis, and dietary lipids, not used for supply of energy, leads to lipid depots in tissues. Formation of tissue lipid depots involves transport of both absorbed and de novo synthesized lipids in peripheral tissues as lipoproteins, and release of fatty acids from triacylglycerol-rich core of circulating lipoproteins by lipoprotein lipase (LPL) for uptake by tissues. All these pathways (lipogenesis, transport of lipids by lipoproteins and uptake of fatty acids by tissues via LPL action) involved in lipid deposition and mobilization in fish tissues are similar to that of mammals [3-5]. They are affected by dietary fatty acid composition in mammals [6-8].

The objective of this study was to investigate the effects of dietary lipid blends on the growth performance of *H. longifilis*fed diets in which FO was replaced by vegetable oils and mixtures composed of fish oil (FO), Soyabean oil (SO) and palm oil (PO). The effects of this replacement were studied on fat synthesis by measuring the activities of lipogenic enzymes in liver.

2. MATERIALS AND METHODS

2.1 Source of Fish

Heterobranchus longifilis fingerlings, used in this study were obtained from National Institute for Freshwater Fisheries Research (NIFFR) hatchery and acclimatised for two weeks in the laboratory in a flow through system of circular tanks (62 l), and fed a commercial pelleted feed (NIFFR).

2.2 Oil Types

The rationale for the selection of the three oil types used in the study was based on the previous experiments in which the fillet *n*-3 fatty acid composition of fish fed 100% PO was reduced [9]. To improve the concentration of these fatty acids in fish fillet an oil rich in *n*-3 acids (FO) and one rich in *n*-6 acids (PO) fatty acids (FA) and Soybean oil (SO) having an appreciable amount of *n*-3 and *n*-6 acids amongst oils of plant origin, were chosen to evaluate their effects singly or in combinations on growth performance, digestibility, and lipogenic enzyme activities.

2.3 Diets Preparation

The composition of the experimental diet is given in Table 1. The diets were formulated to be isonitrogenous and isolipidic. The six experimental diets were prepared by substituting one of the following oils as the lipid source; fish oil (FO), palm oil (PO), Soyabean oil (SO), equal blend of FO and PO (FOPO), equal blend of PO and SO (POSO) or equal blend of FO, PO and SO (FOPOSO) at 60 g kg^{-1}. The proximate composition of the experimental diets and the dietary fatty acid compositions are shown in Tables 1 and 2 respectively. The experimental diets were prepared by mixing the dry ingredients with oil and pregelatinized starch and the resulting moist dough was pelleted using a locally assembled meat mincer through a 2-mm die. The moist pellets were then sun dried and stored under refrigeration in 200 g batches, until used.

2.4 Experimental Set-up

The digestibility experiments were carried out in a flow through system of circular tanks, (62 l). For this experiment, *Heterobranchus longifilis* were randomly selected from the laboratory stock, and eighteen groups of twenty fish (initial weight 4.65±0.23 g) were stocked in the experimental tanks. Fish were maintained under 12:12 light: dark regime, with constant aeration and a flow rate of 0.5 l min^{-1}. Water temperature and pH were maintained at 26±2.1ºC and 6.9±0.2, respectively.

Each group was fed one of the six experimental diets, assigned randomly, each diet being assigned to three groups. The *Heterobranchu slongifilis* were acclimatised to the experimental diets for 2 weeks, when the fish were fed to satiation between 0830 to 1030 h and 1630 to

1830 h. During the acclimatisation period, tanks were cleaned of feed particles after each feeding and faecal matter siphoned out prior to each feeding, thus simulating the conditions that would be in operation when the faecal material would be collected for the study.

2.5 Experimental Protocol

The eighteen groups of *Heterobranchu slongifilis* were fed the assigned diets (triplicated for each diet) for a period of 12 weeks, after acclimatisation. The faecal material egested between the two feeds (day-time collection) was negligible and irregular. As such, only the faecal material collected overnight, over a 4 day period, was used for the study. Although there would have been a certain degree of leaching of nutrient material from the faeces, and as such would affect the digestibility estimations, but such effects are believed to be minimal [10]. Furthermore, as the same procedure was adopted for all the diets this should not influence the results on the comparison of the different oil types. Pellets of faecal matter were siphoned out, taking care not to break, and sundried until used for chemical analysis. As the amount of material collected each day was insufficient to perform all the chemical analyses, faecal matter collected over four consecutive days was pooled, for each diet.

2.6 Sample Collection

At the end of the feeding trial, all fish were weighed and six fish were randomly sampled from each tank. The fish were killed by blow on the head, dissected and the livers removed, weighed and stored at 4°C for estimation of lipogenic enzymes activities. The fish was then skinned and muscle tissues removed, pooled, and stored frozen for subsequent analysis.

2.7 Chemical Analyses

Samples of the feeds were taken for analyses and the proximate composition of the feeds was estimated using standard procedures [11]; moisture by heating at 80°C to constant weight, protein by estimating the Kjeldahl nitrogen (=6.25) in an automated Kjeltech (Tecator, Sweden, Model 2300), lipid by chloroform methanol extraction [12], ash by incinerating in a muffle furnace at 550°C for 18 h. Chromic oxide in the diets and faecal matter were estimated by the wet digestion method of Furukawa and Tsukahara [13]. All analyses were performed in triplicate.

Fatty acid analysis was conducted on the six experimental diets samples. The sample preparation for fatty acid was done by direct methylation method [14] with methanolic HCl. Fatty acid methyl esters were resolved and analysed by a gas-liquid chromatography (Shimadzu GC-17A). Three aliquots of each of the esterified samples (fatty acid methyl esters) were analysed in a Shimadzu GC 17A, equipped with an Omegawax 250 capillary column (30mL x 0.32mm internal diameter), a FID detector and a split injection system (split ratio 50:1). The carrier gas was helium and injector port and detector temperatures were 240°C and 250°C, respectively.

The temperature program was 190ºC for 5 min, 190–240ºC at 2ºC min^{-1}, and held at 240ºC for 10 min. Fatty acids methyl esters were identified in comparison to an external standard (SupelcoTM 37 component FAME Mix).

2.8 Digestibility Estimations

Apparent percent digestibility estimations were based on pooled faecal samples, from each replicate, for each of the diets, collected over four days. A total of three faecal samples for each of the diets was analysed for protein, total lipid, ash, energy and fatty acids, in triplicate.

The percent apparent dry matter (%ADM) and protein (%PD), lipid (%LD) and energy (%ED) digestibility of the diets were determined using standard formulae [15,16]. The formulae used were:

% ADM = 100 – [100 (Cr_2O_3 in diet) ÷ (Cr_2O_3 in faeces)].

% Nutrient = 100 – 100 [(% Cr_2O_3 in diet) ÷ (% Cr_2O_3 in faeces)] x [(% nutrient in faeces) ÷ % nutrient in feed)].

2.9 Lipogenic Enzymes Assay

For the enzyme assays, liver samples were homogenized in three volumes of ice-cold buffer (0.02 M Tris-HCl, 0.25 M sucrose, 2 mm EDTA, 0.1M sodium fluoride, 0.5 mm phenylmethylsulfonyl fluoride, 0.01 M β-mercaptoethanol, pH 7.4), and homogenates were centrifuged with Eppendorf 5417R refrigerated centrifuge at 15,000 × g at 4°C for 20

min. Selected lipogenic enzyme activities of the supernatant were quantified with spectrophotometric procedures: glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49) according to Bautista et al. [17] and malic enzyme (ME, EC 1.1.1.40) according to Ochoa [18]. The enzymatic activity units (IU), defined as micromoles of substrate converted to product per minute at the assay temperature (30°C) were expressed per gram of liver tissue (wet weight).

2.10 Statistical Analysis

Data were subjected to one-way ANOVA followed by Duncan's multiple range tests for comparisons of the means among different dietary treatments. Analyses were conducted using the SPSS 13.0 software package.

3. RESULTS

3.1 Growth Performance

The diets were well accepted by *H. longifilis* and thus no significant differences were found in the average feed intake during the experimental period (Table 3). The weight gain of fish fed diets A(FO), C(SO), D(FOPO), E(POSO) and F(FOPOSO) were not significantly ($P>0.05$) different. Highest weight gain was observed in fish fed diet B(PO) and was not significantly higher than those of fish fed diets A and F. Feed conversion ratio (FCR) and specific growth rates (SGR) followed similar trends as weight gain. However, fish fed diet D (2.12 *n*-3/*n*-6 ratio) had the highest FCR and lowest SGR which were significantly ($P<0.001$) different from that of fish in other dietary groups.

3.2 Nutrient Digestibility

Digestibility values are shown in Table 4. The apparent digestibility for dry matter, crude protein and crude lipid were not significantly different ($P>0.05$) among dietary groups and ranged from 70.80% to 72.70%, 87.60% to 89.30% and 81.60% to 92.50% respectively for dry matter, protein and lipid.

3.3 Lipogenic Enzyme Activities

Hepatic glucose-6-phosphate dehydrogenase (G6PD) activities (IUg^{-1} liver) were higher in fish fed with diet C (0.57 *n*-3/*n*-6 fatty acid ratio) than the fish fed with diets A (1.97 *n*-3/*n*-6 fatty acid ratio) and D (2.12 *n*-3/*n*-6 fatty acid ratio) ($P<0.001$) (Fig. 1). The G6PD of fish fed diet C was similar to those of fish fed diets B, E and F. Moreover, the activity of G6PD was over six-fold higher than that of malic enzyme (ME) in fish fed with B, C, E and F diets. The activity of malic enzyme in *H. longifilis* liver was not significantly different among the dietary groups.

4. DISCUSSION

The results of this study and the earlier trials hold promise that total or partial replacement of FO by vegetable oils as sources oflipids in freshwater fish species is possible, resulting in good growth performance, feed conversion and growth rates [9,19-20]. Vegetable oils like PO and SO are rich in fatty acids with 18 carbon atoms especially linoleic and / or linolenic acids which are essential for freshwater species [20].

This study also demonstrated that feeding diets containing PO (0.70 *n*-3/*n*-6 fatty acid ratios) had improved and significant effects on growth rate and feed conversion ratio, compared with fish fed diet containing FO (1.97 *n*-3/*n*-6 fatty acid ratio). This finding is consistent with previous studies showing successful partial or total FO replacement in both freshwater and marine fish species without negative effects on growth [21-24]. Piedecausa et al. [23] indicated that FO can be completely replaced by Soyabean or linseed oil in sharp snout sea bream (*D. puntazzo*) diets for 92 days without reducing growth. The present study also showed that the substitution of palm oil for total dietary FO in *H. longifilis* had no negative influence on growth performance.

Apparent digestibility of dry matter, protein and lipid were high in all dietary treatments, this might be a contributing factor to the increased growth of fish in this study. Lipids are known to influence the rate of feed passage through the gut [25]. Indications exist that enhanced dietary lipid reduce passage rate, giving intestinal enzymes more time for hydrolyses [26]. Fat digestibility was highest (92.50%) in fish fed diet B (PO) and lowest (81.60%) in fish fed diet A. FO contains higher concentrations of long chain polyunsaturated fatty acid; the low lipid digestibility could be as a result of the possible resistance of DHA to lipolysis. This is in agreement with results from previous studies, which showed that digestibility and absorption of fatty acids in fish decreases with increasing chain length [27,28]. Additionally, dietary lipid influence bile salt production resulting in increased stability of some of the enzymes active in starch hydrolyses, like amylase [26]. However, lipids

influence on enzyme activities is found to vary according to fatty acid composition, highly unsaturated fatty acids do not promote the same positive effect on starch digestibility as saturated fatty acids [26].

Table 1. Composition of experimental diets (g kg $^{-1}$) for fingerling *Heterobranchus longifilis*

	Diets*					
	A	**B**	**C**	**D**	**E**	**F**
Ingredients						
Fish meal (Danish)	398.00	398.00	398.00	398.00	398.00	398.00
Soyabean meal	313.00	313.00	313.00	313.00	313.00	313.00
Corn flour (Maize)	172.00	172.00	172.00	172.00	172.00	172.00
Cassava starch	20.00	20.00	20.00	20.00	20.00	20.00
Methionine	10.00	10.00	10.00	10.00	10.00	10.00
Vit./Min. Premix	20.00	20.00	20.00	20.00	20.00	20.00
Salt (NaCl)	1.50	1.50	1.50	1.50	1.50	1.50
Vitamin C	0.50	0.50	0.50	0.50	0.50	0.50
Chromic Oxide	5.00	5.00	5.00	5.00	5.00	5.00
Fish oil (FO)	60.00	-	-	30.00	-	20.00
Palm oil (PO)	-	60.00	-	30.00	30.00	20.00
Soyabean oil (SO)	-	-	60.00	-	30.00	20.00
Proximate composition (n=3)						
Moisture (g/kg)	63.00	60.00	60.90	60.20	60.60	64.10
Crude protein (g/kg)	456.80	452.30	452.00	452.80	454.50	451.60
Lipid (g/kg)	105.00	106.70	105.80	105.90	105.70	106.40
Ash (g/kg)	83.00	82.90	83.20	82.40	82.90	82.10
Crude fibre	22.50	22.34	22.42	22.18	22.38	22.42
Carbohydrate (NFE)* *(g/kg)	269.70	275.66	273.58	276.52	273.92	273.38
Metabolizable energy (kJ/g)***	17.47	17.56	17.52	17.54	17.53	17.49

Diets: A = fish oil; B = palm oil; C = soyabean oil, D = palm oil and fish oil (1:1); E = palm oil and soyabean oil (1:1), F = fish oil, palm oil and soyabean oil (1:1:1), **NFE = nitrogen free extracts = 100 – (CP + lipid + ash + crude fibre); * calculated from the published compositions of the ingredients used (NRC, 1993)*

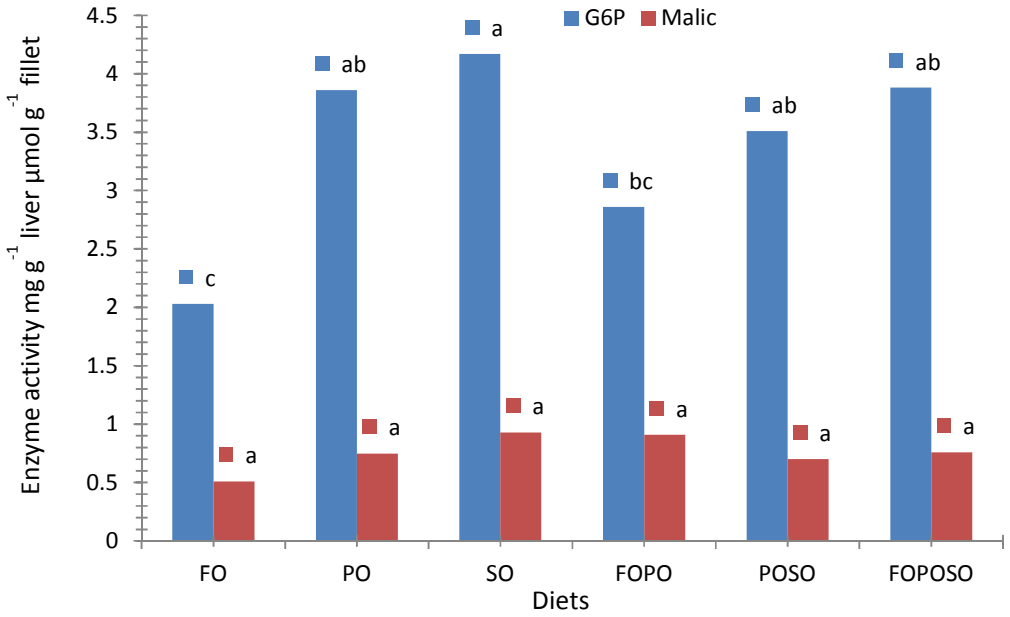

Fig. 1. Activities of hepatic glucose-6-phosphate dehydrogenase (G6PDH) and malic enzyme (ME) of *H. longifilis* fed diets containing fish oil, palm oil and Soyabean oil as sources of *n*-3 and *n*-6 for 12 weeks

^{abc}Bar with different letters are significantly different (P<0.001)

Table 2. Fatty acid composition of the experimental diets* (g/100 g of total FA)

Fatty acids	A	B	C	D	E	F
12:0	1.13	0.15	0.58	0.69	0.28	0.65
14:0	5.98	0.72	4.22	4.35	1.98	4.58
16:0	18.14	35.41	18.77	29.41	23.40	23.33
18:0	4.53	4.71	4.11	3.09	3.89	4.27
20:0	0.74	0.20	0.66	0.44	0.53	0.48
22:0	0.20	ND	0.22	0.09	0.14	0.14
24:0	0.19	ND	0.15	ND	0.10	0.10
∑ SAT	30.91	41.20	28.71	38.06	30.32	33.55
16:1*n*-7	5.79	3.97	3.70	3.45	4.17	3.60
18:1*n*-7	ND	ND	ND	1.61	ND	ND
∑ *n*-7	5.79	3.97	3.70	5.06	4.17	3.60
16:1*n*-9	8.61	0.12	6.49	ND	3.63	5.33
18:1*n*-9	9.61	31.34	13.95	25.38	23.53	23.49
20:1*n*-9	1.93	2.66	1.44	2.72	2.05	2.08
24:1*n*-9	0.63	0.01	0.28	0.55	0.18	0.43
∑ *n*-9	20.77	34.12	22.16	28.66	29.39	31.33
22:1*n*-11	2.72	1.07	1.32	3.59	1.31	2.18
∑ *n*-11	2.72	1.07	1.32	3.59	1.31	2.18
18:2*n*-6	7.55	10.93	26.33	6.86	19.09	12.66
18:3*n*-6	1.97	0.11	1.21	0.30	0.66	0.97
20:2*n*-6	2.83	0.19	0.09	0.17	0.18	0.17
20:3*n*-6	0.17	0.10	ND	ND	ND	ND
20:4*n*-6	0.91	0.20	0.43	0.38	0.36	0.43
∑ *n*-6	13.42	11.53	28.06	7.88	20.30	14.23
18:3*n*-3	1.47	0.11	3.19	1.33	1.99	2.32
18:4*n*-3	ND	ND	ND	2.07	ND	ND
20:4*n*-3	ND	ND	ND	0.38	ND	ND
20:5*n*-3	10.95	3.48	4.84	4.86	3.66	4.48
22:5*n*-3	1.36	0.50	0.56	0.61	0.70	0.62
22:6*n*-3	12.61	4.02	7.45	7.50	8.15	7.68
∑ *n*-3	26.39	8.11	16.04	16.74	14.50	15.10
∑ MUFA	29.28	39.16	27.18	37.31	34.87	37.11
∑ PUFA	39.81	19.64	44.10	24.63	34.80	29.33
∑ UNSAT	69.09	58.80	71.29	61.94	69.68	66.45
n-3/*n*-6	1.97	0.70	0.57	2.12	0.71	1.06
n-6/*n*-3	0.51	1.42	1.75	0.47	1.40	0.94

**Diets: A = fish oil; B = palm oil, C = soyabean oil; D = palm oil and fish oil (1:1); E = palm oil and soyabean oil (1:1), F = fish oil; palm oil and soyabean oil (1:1:1). ND = not detected*

Table 3. Growth performance of *H. longifilis* fed diets containing fish oil, palm oil and soyabean oil as sources of *n*-3 and *n*-6 for 12 weeks

	Initial weight (g/fish)	Final weight (g/fish)	weight gain (g/fish)	Av. Feed intake (g/fish)	FCR	SGR
Diets*						
A	4.57	35.27^{ab}	30.69^{ab}	45.05	1.29^{ab}	2.89^{ab}
B	4.78	42.29^{a}	37.51^{a}	46.90	1.12^{b}	3.09^{a}
C	4.67	27.00^{b}	22.34^{b}	36.72	1.40^{ab}	2.48^{bc}
D	4.67	23.41^{b}	18.74^{b}	35.63	1.52^{a}	2.31^{c}
E	4.59	26.29^{b}	21.70^{b}	36.96	1.41^{ab}	2.49^{bc}
F	4.60	34.95^{ab}	30.35^{ab}	45.14	1.31^{ab}	3.04^{a}
SEM	0.10	2.37	1.68	2.03	0.05	0.09

*FCR = feed conversion ratio (feed intake/weight gain; SGR = specific growth rate (100 x (ln [final body weight] – [initial body weight]/No. days); *Diets: A = fish oil, B = palm oil, C = soyabean oil, D = palm oil and fish oil (1:1); E = palm oil and soyabean oil (1:1); F = fish oil, palm oil and soyabean oil (1:1:1); values in the same column followed by the same letter are not significantly different at P<0.001*

Table 4.Apparent nutrient digestibility of *H. longifilis* fed diets containing fish oil, palm oil and soyabean oil as sources of *n*-3 and *n*-6 for 12 weeks

	Dry matter (%)	Protein (%)	Lipid (%)
Diets*			
A	70.70	87.60	81.60
B	71.40	89.30	92.50
C	70.80	89.00	91.20
D	71.90	86.70	88.60
E	72.70	87.90	91.80
F	72.20	88.20	90.70
SEM	1.10	1.97	1.28

**Diets: A = fish oil; B = palm oil; C = soyabean oil; D = palm oil and fish oil (1:1); E = palm oil and soyabean oil (1:1); F = fish oil; palm oil and soyabean oil (1:1:1)*

In fish, hepatic tissue is the preferential site of *de novo* fatty acid synthesis [29]. NADPH is essential for hepatic fatty acid biosynthesis; Malic enzyme, G6PD, P6GDH and IDH are involved in catalyzing production NADPH. The highest activities of hepatic malic enzyme and G6PD were found in fish fed with PO, SO, POSO and FOPOSO diets, and no significant difference was found between those fed with FOPO, PO, POSO and FOPOSO diets. With regard to the two key regulatory enzymes in the lipogenic pathway, the activity of G6PD was over 4 times higher than that of ME in fish fed with the experimental diets. This indicates that the cytoplasmic reducing equivalents NADPH are mainly provided by the pentose phosphate pathway. Similar results have been reported in juvenile cobia (*Rachycentron canadum*) by Wang et al. [30].

5. CONCLUSION

The results of this study clearly showed the possibility of feeding fish meal-based diets containing PO to *H. longifilis* fingerlings without any negative effects on growth performance or feed utilization. Further investigation is needed on the causes of reduced performance of *H. longifilis* fingerlings fed POSO diet.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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