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RESEARCH PAPER

Effects of Emulsifier Material and Vegetable Oils on Zebrafish (*Danio rerio*) Growth Performance

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Abstract

This study was conducted to investigate the effects of Polyglycerol polyricinoleate, (PGPR; E-476) as a dietary emulsifier supplement with sunflower and linseed oil on zebrafish (*Danio rerio*) growth performance and feed utilization. The control diet included only fish oil whereas other experimental diets included 6% sunflower oil and linseed oil with and without PGPR at %0.06. A total of 180 zebrafish larvae were used in the experiment. At the end of the experiment, the whole body samples were taken for proximate and fatty acid analysis. The fish were fed diets that contained PGPR, S0%L0% (PGPR), S6%L0% (PGPR) and S0%L6% (PGPR), had higher values ($p<0.05$) in final weight (557.1, 521.3 and 622 mg), weight gain (1982.83, 2129.5 and 2779.56%), specific growth rate (2.062, 2.099 and 2.272), digested lipids amount (127.23, 122.65 and 148.52 mg), feed conversion efficiency rate (1.683, 1.893 and 1.725), protein efficiency ratio (1.321, 1.181 and 1.31) and whole body lipid percentage (44.37, 37.9827 and 46.7063 %), respectively. The percentages of fatty acids in fish tissues that fed diet with PGPR diets, S0%L0% (PGPR), S6%L0% (PGPR) and S0%L6% (PGPR), were higher ($p<0.05$); ∑SFAs (16.695, 12.732 and 14.672 %), ∑MFAs (17.748, 16.014 and 17.922%), and ∑PUFAs (9.926, 9.235 and 14.111%), respectively, which reflected a positive influence of the emulsifier on zootechnical performance variables. Briefly, the results suggest that dietary use of linseed oil with particularly PGPR appeared to be a better alternative to fish oil.

Introduction

The increasing growth of aquaculture production globally has greatly raised the demand for protein and energy concentrates from fish meal and fish oil. Responsible attempts have been made to use alternative protein and energy sources, from animal or plant ingredients to decrease traditional sources in fish feeds (Watanabe, 2002). The percentage of lipids in fish diets has a direct relation with the gross energy of the whole body and daily protein and energy gains in fish (Martino *et al*., 2002). The body weight (BW), specific growth rate (SGR), feed utilization (FU) and other growth performance parameters of fish are influenced by dietary lipids levels (Chatzifotis *et al*., 2010). The percentages of n-3 fatty acids in Atlantic salmon (*Salmo salar*) diets affected directly growth rate (GR), hepato-somatic index (HSI) and fillet lipid components (Menoyo *et al*., 2003). There is a positive relationship between the fatty acid composition of fish fillets and liver, and dietary

fatty acids profile (Xue *et al*., 2006). Plant oils contain important fatty acids, including all spectrums of SFAs, MUFAs, and PUFAs, particularly a higher percentage of n-6 PUFAs (Orsavova *et al*., 2015). In juvenile Japanese sea bass (*Lateolabrax japonicas*), partial replacement of fish oil with linseed oil and soybean oil resulted in adequate growth performance (Xu *et al*., 2015).

Emulsifiers are amphiphilic molecules that contain polar and non-polar sides. They are considered to function as consolidators between the water and oil interface and work as decomposers to increment the active surface of lipids to improve the function of lipase, which disassemble triglyceride molecules into free fatty acids and monoglycerides and compose micelles from digested lipids (Hasenhuettl and Hartel, 2008, Roy *et al*., 2010 and Zhang *et al*., 2011). Bile salts as endogenous emulsifiers have the ability to convert lipid droplets to micrometric particles, and from hydrophobic to relatively hydrophilic phase, which allows more excess to lipase enzymes action, and increases the concentration of bile salts secretion (Sadeghpour *et al*., 2018). Previous research mentioned that larvae of Atlantic cod (*Gadus morhua*) have poor exploitation for neutral lipid classes due to insufficient levels of either bile acids or lipases or both (Olsen *et al*., 1991). Polyglycerol polyricinoleate, PGPR (E-476) is classified as an emulsifier produced from polymerized glycerol and polymerized ricinoleic acid and is widely used in food manufacture. The addition of polyethylene glycol ricinoleate supplemental exogenous emulsifier in broiler diets at concentrations above 1% of the added fat enhanced feed utilization and increased the live body weight by about 5% (Roy *et al*., 2010; Bastida, 2013). The use of lysophosphatidylcholine as an exogenous emulsifier in juvenile turbot (*Scophthalmus maximus L*.) diets enhanced the weight gain rate, specific growth rate, feed conversion ratio, protein efficiency ratio, and lipid utilization coefficient efficiency (Li *et al*., 2019). The use of 0.35 g/kg of commercial nutritional emulsifier NE (Excential Energy Plus, Orffa Additives BV, The Netherlands) with soybean oil in juvenile Nile tilapia (*Oreochromis niloticus*) diets improved feed utilization, growth performance, fatty acids profile in tissue, raise activation of lipase and reduce the cholesterol percentages (Wangkahart *et al*., 2022).

Zebrafish is considered an important experimental model as a teleost fish due to fast development, high offspring, and easy control of reproduction (Linbo, 2009). The digestion properties of zebrafish are very similar to many farmed fish species, including herbivores like common carp, and tilapia and carnivores like trout and salmon (Ulloa *et al*., 2011). Therefore, in this study, zebrafish was used as an experimental model to investigate the effects of two vegetable oils with or without exogenous emulsifier on the growth performance and tissue fatty acids profiles of fish.

Materials and Methods

The experiment place

This experiment was carried out in the Aquatic Vertebrate Living Experiment Unit, inside the Inland Fisheries Production Research and Application Station of the Faculty of Aquatic Sciences, Istanbul University, Sapanca, Sakarya, Türkiye.

Table 1. Composition of control and experimental diet groups with their proximate analysis

İngredients of control and experimental diets	Control (g/kg)	S6%L0% (g/kg)	S0%L6% (g/kg)	S0%L0% (PGPR)	S6%L0% (PGPR) (g/kg)	S0%L6% (PGPR) (g/kg)
Fish meal	650	650	650	650	650	650
Fish oil	90	30	30	90	30	30
Sunflower oil	0.0	60	0.0	0.0	60	0.0
Linseed oil	0.0	0.0	60	0.0	0.0	60
Gelatin	60	60	60	60	60	60
Corn starch	107	107	107	101	101	101
Dicalcium phosphate	15	15	15	15	15	15
Vitamin and mineral premix	3	3	3	3	3	3
Lysine	10	10	10	10	10	10
Methionine	5	5	5	5	5	5
Cellulose	60	60	60	60	60	60
PGPR	0.0	0.0	0.0	6	6	6
Total	1000	1000	1000	1000	1000	1000
Proximate composition						
Crude protein (%)	45.0416	44.9411	45.0458	44.9543	45.0085	44.9888
Crude lipid (%)	14.227	16.5180	15.7980	15.6613	14.0933	15.6177
NFE (%) *	19.7573	17.7189	18.3741	18.8222	20.006	18.5877
Cellulose (%)	5.9996	6.0030	6.0003	6.0099	5.9873	5.9883
Ash (%)	8.8763	9.0695	8.8570	8.5784	8.8282	8.8927
Gross energy(MJ/kg DM) **	19.618	20.13342	20.0005	19.9878	19.5848	19.9413

* Nitrogen free extract (soluble carbohydrate) calculated as 100 - (Crude protein % + Crude lipid + Cellulose %+ Ash % + moisture %)

** Calculated as total carbohydrate ·17.2 kJ/g; fat ·39.5 kJ/g; and protein ·23.5 kJ/g (Perera, 2016).

Zebrafish housing

In this experiment, we used a recirculation zebrafish system (Tecniplast Company – Italy). 180 zebrafish (*Danio rerio* Hamilton, 1822) larvae AB line were randomly distributed on eighteen plastic tanks (3.5L) (three tanks for each group diet). Each tank had baffles on the outlet side (300, 500 and 800 microns according to the fish size) to discharge the water. The effluent water from experimental tanks was treated via a fine mechanical filter, and carbon and UV filters. Water temperature, pH, dissolved oxygen, conductivity and salinity were automatically monitored and controlled with a computerized system.

Feed and feeding

All ingredients used in the experimental diets were obtained from the domestic markets. The control and five experimental diets were prepared in the Nutrition Laboratory, Faculty of Aquatic Sciences, Istanbul University. The ingredients were homogenously mixed through an electric blender following weighing with a precision balance according to the formulations (Table 1). The control diet contained only fish oil as the source of lipid without emulsifier material (PGPR). The S6%L0% diet group included 3% fish oil and 6% sunflower oil at a ratio of 1:2, S0%L6% diet group involved 3% fish oil and 6% linseed oil at a ratio of 1:2, S0%L0% (PGPR), S6%L0% (PGPR) and S0%L6% (PGPR) were the same as the control, second and third diet groups except they included 0.06% PGPR (Table1). All remaining ingredients in the control and experimental diets were kept at the same levels. During the adaptation period (the first three days after stocking), fish were fed the control diet. Fish in control and experimental groups were fed three times a day (at 09: 00, 13: 00 and 17: 00 o'clock). Every morning and during the day; the bottoms of the tanks were cleaned by a glass pipette to collect the faeces one hour before the first, second, and third feedings. The collected faeces were placed in falcon tubes and then kept at -20 C° until analysis. After the end of the experiment; the total collected faeces for every group were dried and then weighed to determine the faeces' dry matter weight for each group.

Samplings

All weightings were done after anesthetization with 164 mg/L of tricaine methane sulfonate (MS-222) (Acros Organics, Geel, Belgium) for 1-2 minutes to avoid stress and ensure the welfare of the fish (Matthews et al., 2018). At the end of the experiment, fish samples for analysis were taken after euthanization using an overdose of tricaine methane sulfonate (300 mg/L) for 10 minutes (Matthews and Varga, 2012).

Growth performance measurements

The growth and feed utilization performance of experimental fish were calculated using the following formulas (Ricker, 1979, Silva *et al*., 1995; Agbo, 2008):

Weight gain (WG):(Final live body weight (FBW) – Initial live body weight SBW)) × 100 (Final live body weight)

Specific growth rate (SGR):(lnFBW – lnIBW) × 100 (Experiment days (D))

Feed conversion ratio (FCR): Feed fed (g) (Live weight gain)

Protein efficiency ratio (PER): Weight gain (g) (Crude protein fed (g))

Percent digestibility: Nutrient intake – nutrient in faeces × 100 (Bob-Manuel, 2013) (Nutrient intake)

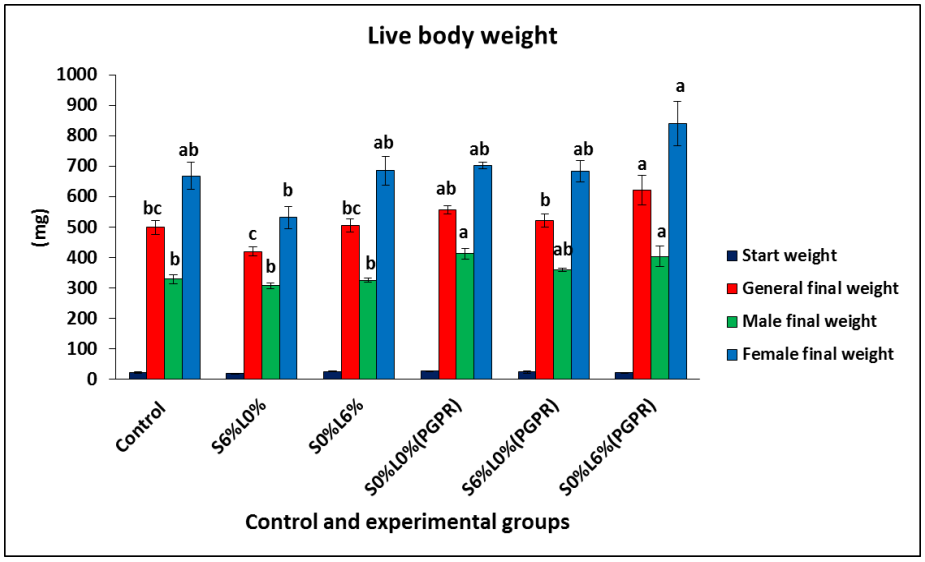

Figure 1. Illustrate the fish live body weight (mg) at the beginning and end of the experiment. All data are given as means ± SD with different superscript letters

Biochemical analysis

Feed, whole body and faeces samples were analyzed using the methods according to AOAC, (1990) in the Nutrition Laboratory, Faculty of Aquatic Sciences Istanbul University.

Fatty acids analysis

The experimental diets, tissues and faeces samples of control and experimental groups were analyzed for fatty acids analysis. In the first step, the lipids were extracted by homogenizing the sample with 2: 1 chloroform-methanol (v/v), and filtering the homogenate (Folch *et al*., 1952). Before analysis of fatty acids, 10 mL of high-purity hexane and 0.5 mL of 2 N methanolic potassium hydroxide solutions were added to 0.1 g oil, and the mixture was kept in the dark for 2 hours. For the esterification step; 2.0 ml of 14% BF methanolic solution was added to the fatty acids methanolic solution mixture and placed in a 70 C° water bath for 3 min. Then, 1.0 ml of deionized water was added followed by cooling to stop the reaction. The FAMEs were extracted from the aqueous methanol phase by adding 1 ml of methylene chloride and shaking the test tube for 1 min to favour mixing. Two layers formed, the methylene chloride layer was drawn off with a Pasteur pipette and transferred to another test tube while the aqueous methanol phase was extracted twice again with 1.0 ml of methylene chloride. The extracts were then mixed, dehydrated with anhydrous sodium sulfate, filtered and placed in a 10-ml volumetric flask, completed using methylene chloride (Casado *et al*., 1998). After pretreatment, fatty acid analysis was performed with GC/MS (Shimadzu GCMS QP 2010 ULTRA). During the analysis, RTX-5MS brand capillary column (30m; 0.25 mm; 0.25 μm) and helium as carrier gas were used in the device. The column furnace

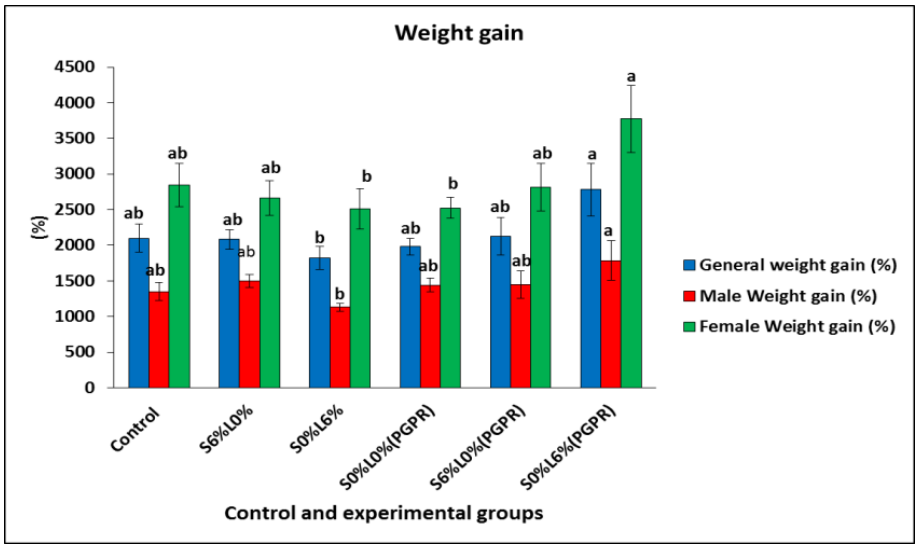

Figure 2. Illustrate the fish live body weight gain (%) at the end of the experiment. All data are given as means ± SD with different superscript letters**.**

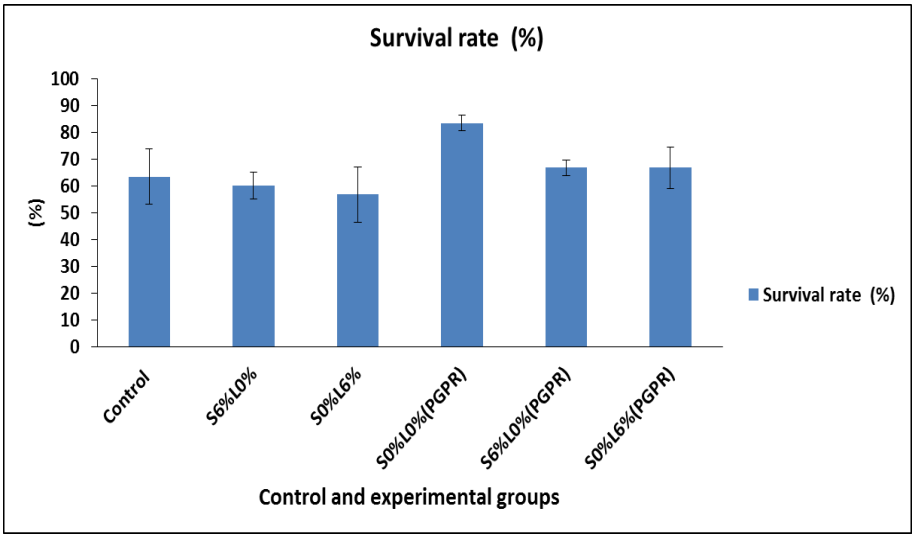

Figure 3. Show fish survival rate in control and experimental group until the end of the experiment. All data are given as means ± SD with different superscript letters.

temperature was set at 40°C, interface temperature at 250°C, ion source temperature at 200°C and injection temperature at 250°C. The injection volume is 1µL, and the split (1/5) method was used for injection. During the analysis, the oven program was applied as 3 min at 40°C, from 40°C to 240°C with 4°C/min increment, 240°C 10 min, from 240°C to 260°C with 4°C/min increment, 260 °C at 10 min, which lasted for a total of 78 minutes (Kesbiç, 2019).

Statistical Analysis

All the growth performance parameters and fatty acids findings were analyzed with the one-way analysis of variance (ANOVA) using SPSS statistical software (version 21.0). The Duncan's test was applied to distinguish the significantly different means among the experimental groups. The differences were regarded as significant at a significance level of 5 %. The data were presented as means ± SD.

Results

The change in mean live body weight and weight gain of zebrafish by sexes over the experimental period are shown in Figure1 and Figure2 respectively. Male and female zebrafish final weight and weight gain were significantly higher in S0% L6% (PGPR) than those in other groups ($p< 0.05$). There were no significant differences among the treatments in terms of survival rate ($p>0.05$) (Figure3). The SGR values for males and females (Figure4) were significantly higher in S0% L6% (PGPR) compared with the remaining treatment groups ($p< 0.05$). Fish in S0%L6% and S0%L6% (PGPR) groups had higher daily dry matter feed intake but without any significant differences ($p>0.05$). However, significantly higher crude and digestible lipid intakes were the case in fish fed S0%L6% and S0%L6% (PGPR) than those on other treatments (Figure5) ($p< 0.05$). There were no significant differences ($p>0.05$) among the treatments in terms of dry matter digestibility percentages (Figure6), but the apparent digestibility coefficients of lipid for fish in S0%L0% (PGPR) and S0%L6% (PGPR) groups were significantly different from those in the control and S0%L6% ($p<0.05$). The S0%L0% (PGPR) and S0%L6% (PGPR) groups were (Figure7) significantly better ($p<0.05$) than S6%L0% in terms of feed conversion efficiency and protein efficiency ratios. The whole body lipid levels of fish in S0%L0% (PGPR) and S0%L6% (PGPR) (Figure8) were significantly higher than those in the other groups ($p<0.05$). The S0%L0% (PGPR) group (Figure9) had significantly a higher percentage of total saturated fatty acids (∑SFAs) in both diet and fish tissue compared with other groups ($p<0.05$). The S0%L0% (PGPR) diet (Figure10) was higher total monounsaturated fatty acids (∑MFAs) percentage ($p<0.05$), while fish in S0%L6% (PGPR) diet was higher ∑MFAs ($p<0.05$). The total polyunsaturated fatty acids (∑PUFAs) percentage (Figure11) was significantly higher percentage in the diets of S6%L0%, S0%L6% and S0%L6% (PGPR) ($p<0.05$), whereas the whole body ∑PUFAs were significantly higher in S0%L6% (PGPR) group than the other groups ($p<0.05$).

Discussion

Most of the results in terms of growth, feed utilization and whole body components were significantly influenced by dietary treatments. This reflects the effects of dietary lipid types and whether they contain PGPR as an emulsifier or not. The fish in group S0%L6% (PGPR) recorded the highest final live

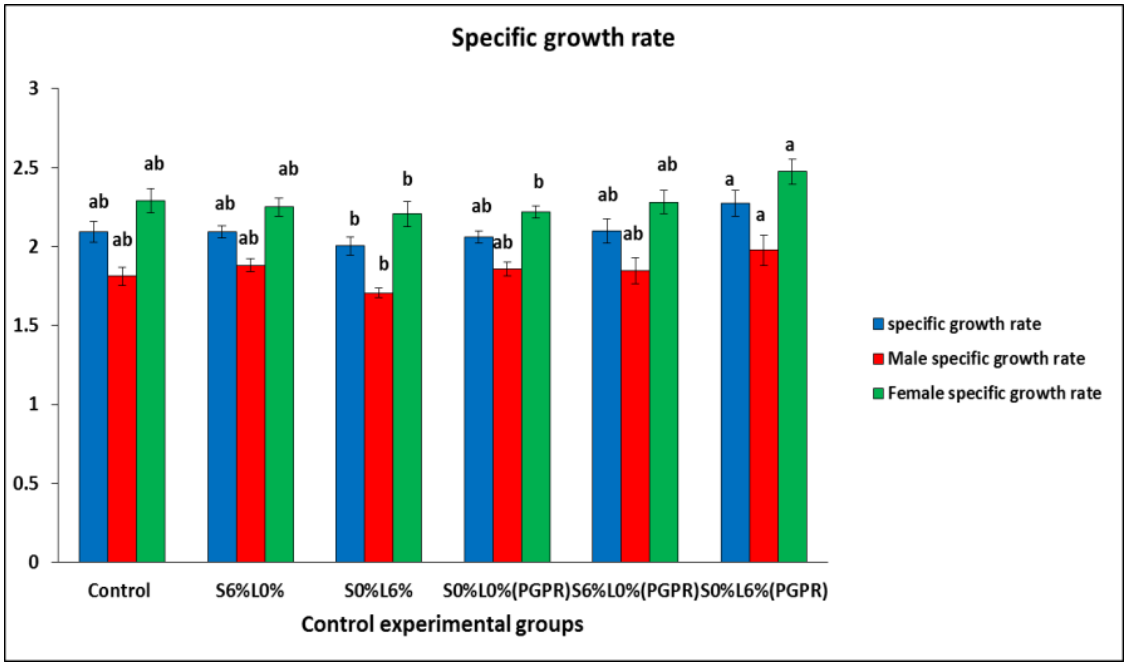

Figure 4. Illustrate the fish specific growth rate at the end of the experiment. All data are given as means ± SD with different superscript letters.

body weight and weight gain ($p<0.005$) among treatments (Figure1 and Figure2). This may demonstrate the beneficial effects of the dietary use of linseed oil as a partial substitute for fish oil with supplemental PGPR. Linseed oil includes over 85% unsaturated fatty acids, which were reported to have important roles in the growth of freshwater fish species such as rainbow trout (*Oncorhynchus mykiss*), coho salmon (*Onchorhynchus kisutch*) and chum salmon (*Onchorhynchus keta*) (Gruia *et al*., 2012, and Takeuchi and Watanabe, 1982). Dietary supplemental PGPR in the S0%L6% (PGPR) as an emulsifier had a significant role to enhance the apparent lipid and dry matter digestibility coefficients, which is consistent with the results of Roy et al (2010). When

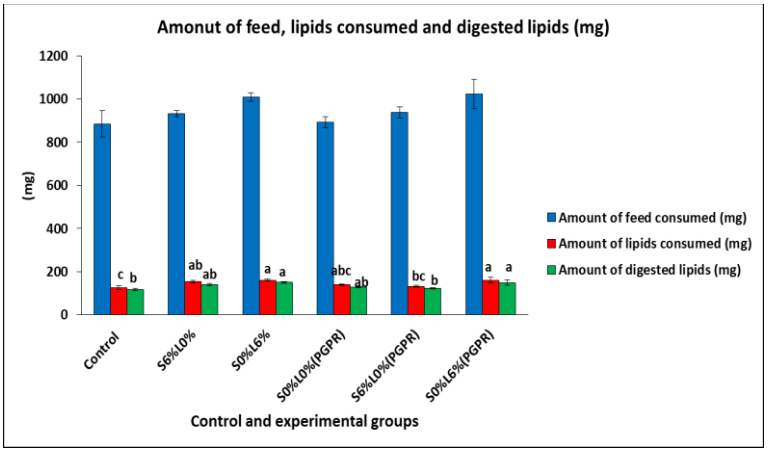

Figure 5. Illustrate amount of diet and dietary lipid (mg) that consumed by fish and digested lipids amount (mg) during experiment period. All data are given as means ± SD with different superscript letters.

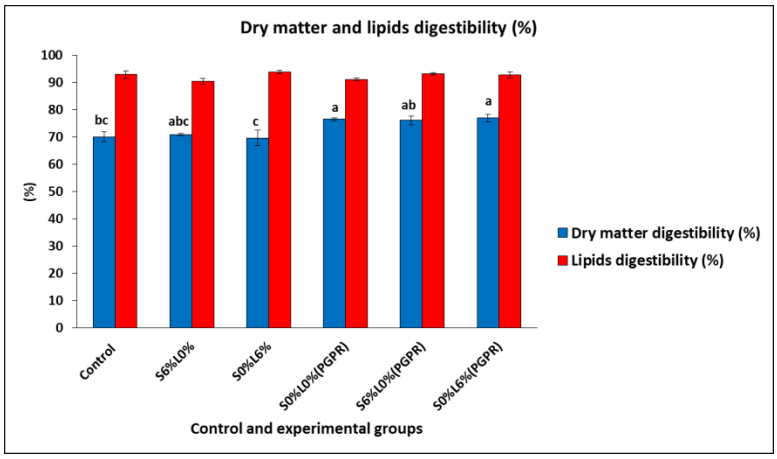

Figure 6. Illustrate dry matter and lipids digestibility (%) of fish during experiment period. All data are given as means ± SD with different superscript letters

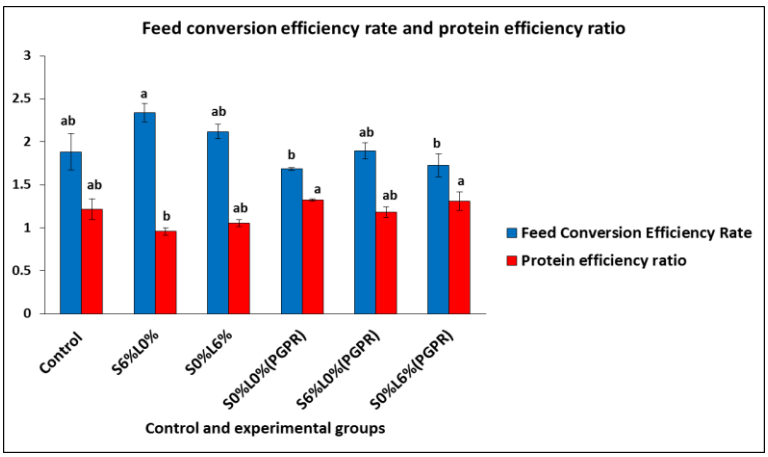

Figure 7. Illustrate the feed conversion efficiency rate protein efficiency ratio of fish during experiment period. All data are given as means ± SD with different superscript letters.

comparing two groups that contained sunflower oil (66.67%) as a partial replacement for fish oil with or without PGPR, the final live body weight and weight gain values were higher in S6%L0%(PGPR) than S6%L0% group, which could be a result of the effects of the emulsifier on lipid availability for fish.

There were no significant differences among the treatments ($p>0.05$) in survival rate (Figure3). When this finding was taken together with the no negative effects of dietary supplemental PGPR at 0.06% level on zebrafish health or growth performance, it can be claimed that (PGPR) is not toxic or carcinogenic for fish. This claim is consistent with the findings of a number of previous studies (Wilson and Smith, 1998, Wilson *et al*., 1998, Roy et al., 2010, Mortensen *et al*., 2017). S0%L6% (PGPR) diet resulted in a higher specific growth rate in zebrafish than S0%L6% ($p<0.05$) (Figure4), which may be due to a higher influence of dietary PGPR on the utilization of dietary linseed oil compared with fish and sunflower oils.

There were no significant effects of dietary PGPR on the feed intake of zebrafish in the present experiment ($p>0.05$). The amounts of lipid consumed and digested were significantly higher in groups containing linseed oil (6%) ($p<0.05$). In general, dietary emulsifiers play a positive role in the enhancement of lipid digestibility and feed efficiency (Drażbo *et al*., 2019). Dietary addition of PGPR in S0%L0% (PGPR) diet group (100% fish oil) numerically enhanced the amounts of digested lipid consumption in S0%L0% group. Bontempo *et al*. (2018) pointed out that exogenous emulsifiers are considered additives improving lipids utilization. The values of feed conversion and protein efficiency ratios (Figure7) for S0%L0% (PGPR) and S0%L6% (PGPR) groups were significantly better than those for S6%L0%. Although dietary supplemental PGPR in the experimental diets resulted in numerically better feed conversion and protein efficiency ratio, it could be speculated that the presence of dietary emulsifiers could increase the utilization of nutrients by fish to build tissues and promote the growth process. The whole body lipid levels of zebrafish in groups S0%L0% (PGPR) and S0%L6% (PGPR) were significantly higher than in other groups (Figure8). The ∑SFAs were significantly higher in fish fed S0%L0% (PGPR) and S0%L6% (PGPR) diets (Figure9) than those on other diets ($p<0.05$). In general, the fish fed experimental diets containing PGPR tended to have higher percentages of ∑SFAs in their tissues, meaning that the emulsifier increased the availability and deposition of this fatty acid group in the

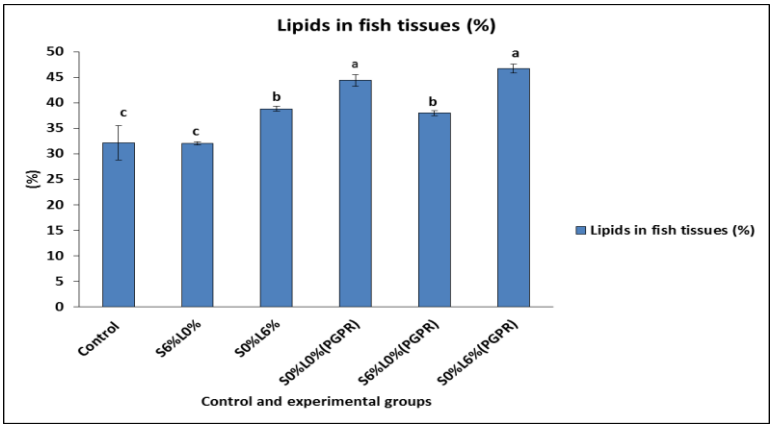

Figure 8. Illustrate the total lipids percentage (%) of fish tissue in control and experimental groups. All data are given as means ± SD with different superscript letters.

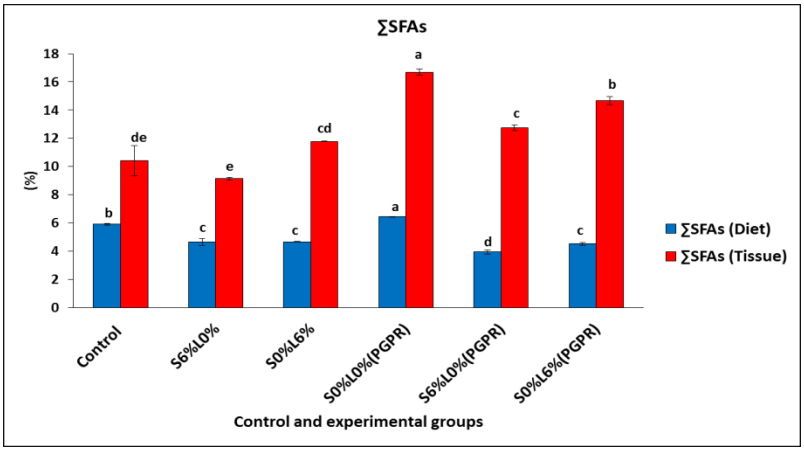

Figure 9. Illustrate the total saturated fatty acids (∑SFAs) percentage in control and experimental diets, and fish tissue. All data are given as means ± SD with different superscript letters.

fish body. A similar tendency was the case for the ∑MUFAs in fish tissues (Figure10), where dietary exogenous PGPR significantly increased the ∑MUFAs of the whole body ($p<0.05$) compared with their un-supplemented counterparts. Although the diet containing linseed oil had a significantly higher percentage of total polyunsaturated fatty acids (∑PUFAs) with ($p<0.05$) (Figure11), the fish fed S0%L6% (PGPR) showed a further significant increase in ∑PUFAs. Altogether; there is a close interaction between the dietary emulsifier PGPR and major fatty acid groups stored in the body of zebrafish.

Conclusion

In summary, the results of this study show that the dietary addition of PGPR as an emulsifier in zebrafish had a positive impact on the utilization of protein and lipids and growth performance. The close relationship between dietary PGPR and vegetable oils concerning promoting the exploitation of their fatty acids by fish clearly shows that dietary emulsifiers can increase lipid availability to fish. The presence of ∑PUFAs in the fish diet with suitable levels supports the growth process better than ∑SFAs and ∑PUFAs. It can be suggested the introduction of PGPR as an emulsifier feed additive into the diets of various commercial freshwater and marine fish species would be beneficial. Yet, this topic remains to be studied in future studies.

Ethical Statement

All this experiment steps were following the roles of Local Ethics Committee of Experimental Animals Istanbul University (Meeting Agenda No; 2021/03 IUHADYEK).

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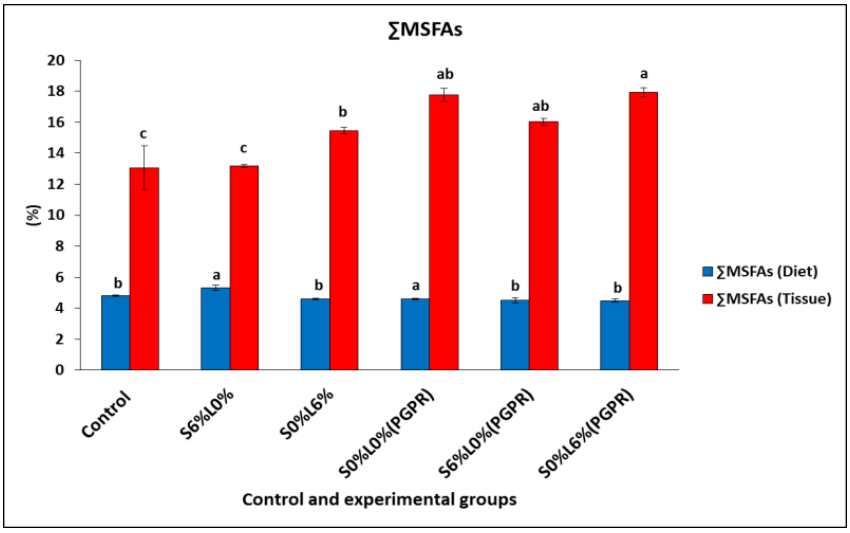

Figure 10. Illustrate the total mono-unsaturated fatty acids (∑MFAs) percentage in control and experimental diets, and fish tissue. All data are given as means ± SD with different superscript letters

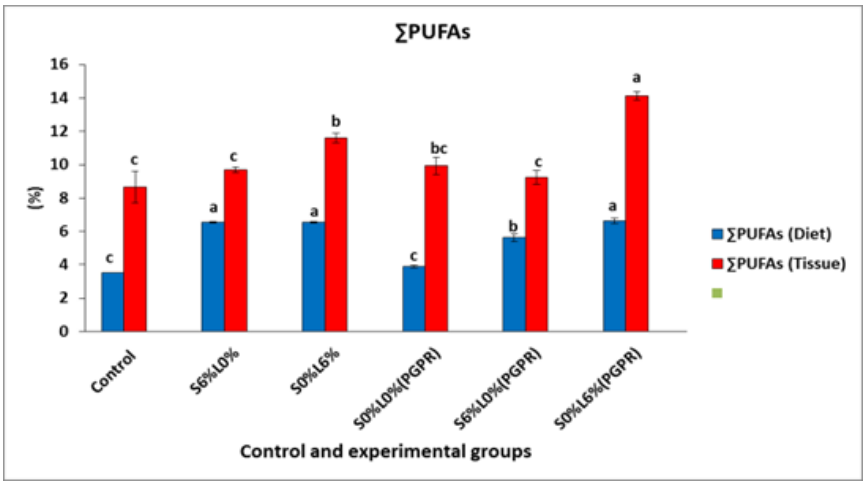

Figure 11. Illustrate the total poly mono-unsaturated fatty acids (∑PUFAs) percentage in control and experimental diets, and fish tissue. All data are given as means ± SD with different superscript letters

Author Contribution

First Author: designed the experiment, executed the practical steps of the experiment, laboratory analysis of the samples and the statistical analysis of results, and article writing. Second Author: full supervision of the stages of the experiment, coordination with legal and supportive scientific units to conduct the experiment, revisions and corrections of the article and decision to publish.

Conflict of Interest

The authors declare no conflict of intrest.

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