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The Effect of Fish Meal and Plant-Based Diets on the Growth and Nutritional Composition of White Worms (*Enchytraeus albidus* Henle, 1837) in Various Substrates

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Authors' Contribution
EE designed the study. MHI and SC performed the experiments and collected the data. EB performed the data analysis. EE and EB built the main structure of the manuscript.

Key words
***Enchytraeus albidus*, Growth performance, Nutritional value, Aquaculture, Fish feed, Recycling**

ABSTRACT

In this study, the growth and nutritional composition of *Enchytraeus albidus* (white worm) were investigated using different combinations of culture substrates and feeds. The aim was to determine the utilization of White worm for recycling the fish feeds in case of expiration. The white worms were either given a plant-based diet or a fish feed-based diet (commercial extruded Seabass feed) in four different culture substrates (rice husk, peat, cocopeat, garden soil). There were altogether eight experimental groups with triplicates. The initial stocking density of white worms was 150 worms/unit (2.2 Liters of cylindrical containers), and all the experiments were carried out in the dark at a constant temperature at 18°C. Worms were collected from the substrate by heat treatment and the counting was done manually, using dissection tools. Proximate composition of the produced white worms was measured with regard to given ratios of the protein, carbohydrate and lipid sources provided from the feed materials. The plant-based diet yielded the highest worm density of the study (2220 worms/unit) while the garden soil was used as substrate. In comparison, the fish feed-based diet fed white worms reached a significantly lower density (627 worms/unit) although the optimal nutritional value for the fish diet was ensured. These results showed that the carbohydrate content of the feed for white worm should be adjusted for optimal growth. Furthermore, the use of a combined plant- and fish feed-based diet can result in high growth performance and improved nutritional value during fish feed production.

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INTRODUCTION

Aquaculture is providing high-quality animal protein for human consumption, but the sustainability of the industry has been questioned. This is because feed has been produced using raw materials from less desirable fish species rather than directly as food for humans. Alternative energy sources (Buck and Krause, 2013), like plant-based feed (Arriaga-Hernandez *et al*., 2021; Rahimnejad *et al*., 2021) or insect-based feed (Makkar *et al*., 2014; Henry *et al*., 2015; Belghit *et al*., 2019), have been considered to replace fish meal-based feed or to decrease the fish meal requirement. The goal is to develop a more economically sustainable and eco-friendly aquaculture industry. In addition to plant- and insect meal, oligochaetes have also been investigated as a sustainable way to transform fish feed production (Walsh, 2012; Walsh *et al*., 2015; Holmstrup *et al*., 2020; Dai *et al*., 2021). The most promising and interesting candidate species of oligochaetes is *Enchytraeus albidus* Henle, 1837, belonging to class Clitellata in phylum Annelida (Henle, 1837), commonly known as white worms.

Propagation of white worms is easy and inexpensive, and they can be used for feeding both freshwater and marine fish species (Walsh, 2012; Fairchild *et al*., 2017). Historically, white worms were used as a live feed in sturgeon aquaculture (Ivleva, 1973). Providers of ornamental fish have also relied on white worms to be able to supply healthy and inexpensive fish feed for aquaria. Despite their successful but limited use as live feed, white worm meal has not been developed into an economically viable option as a base ingredient for formulated fish feed production. Unfortunately, due to the large demand for fish feed in a rapidly growing industry economic interests have outweighed ecological concerns.

The growth and health of farmed fish is directly related to the nutritional value of formulated fish feeds

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(Aksnes *et al*., 1997). Especially, successful fish larvae production cycles in hatcheries depend on live feeds and their nutritional value (Tocher, 2010). The quality of formulated fish feed is dependent on the nutritional value of the ingredients. The protein requirement of ornamental fishes has been reported as 30-50% (Lubzens *et al*., 1989; Sales and Janssens, 2003) and as 40-70% for marine fish juveniles (Cahu and Infante, 2001) in the study done by Fairchild *et al*. (2017). Several studies suggest that white worms can be a valuable source of ingredients for fish feed as evaluation has shown them to be rich in proteins (45-70% of dry weight) and lipid (15-20% of dry weight) content (Walsh, 2012; Walsh *et al*., 2015; Fairchild *et al*., 2017; Holmstrup *et al*., 2020; Dai *et al*., 2021). However, the nutritional value of white worms is also determined by interactions between feed, substrate and culture conditions (Fairchild *et al*., 2017). Therefore, it is important to develop appropriate feed materials for the white worm culture to provide an optimal nutritional value for fish feed production. According to literature, industrial scale fish feed production with white worms can be achieved by producing large quantities of white worms as it was produced for sturgeon culture in the former Soviet Union (Ivleva, 1973; Vedrasco *et al*., 2002; Fairchild *et al*., 2017).

In this study, the growth rate and nutritional composition of white worms were investigated after propagation on two different feeds (plant-based and fish feed-based) in the four different substrates (rice husk, peat, cocopeat, garden soil). Our primary purpose was to identify the best substrate and feed combination for optimal growth of white worms and to contribute to the further development of alternative ingredients that are nutritionally appropriate for fish feed production while considering white worms as biological recyclers.

MATERIALS AND METHODS

Origin of biological material and rearing conditions

White worms were obtained from the Aquaculture Research Facility of Muğla Sıtkı Koçman University (Muğla, Turkey) and maintained in laboratory cultures under controlled conditions in garden soil (moistened to 40-50%) before the experiment to obtain the required number of individuals for this study design. Rearing throughout the study took place at a constant temperature (18 ±1°C), a stable pH (6.2 ±0.2) in the soil (sterilized and dried before use) and in the dark. The start culture of *E. albidus* was fed twice a week with finely ground and autoclaved oats flakes (Amorim *et al*., 2005b). It took about one month to reach the required 5000 worms to start experiments.

Feed preparation

Commercial (Çamlı, İzmir, Turkey) extruded seabass feed (f, fish feed-based diet) and plant-based diet (p) were used in this experiment. Fish feeds were 8 months old which means that the shelf life expired 2 months ago (recommended shelf life was 6 months). Fish feeds were stored at room temperature in the fish farm. There was no air conditioning. These fish feed pellets were preserved with their physical features. All the feeds were immediately brought to laboratory then kept at +4 ^{o}C. Fish feeds were powdered with a grinder before the diet preparation. The plant-based feed (p) was provided as a dry powder from the local marketplace consisted of whey (10%), whey protein concentrate (1.5%), skimmed milk (9%), lactose (61%), galactooligosaccharide (2), fructooligosaccharides (2%), vegetable oil (10%) and fish oil (4.5%). Both of the diets were prepared as a paste with addition of water. Briefly, 15 ml of sterilized distilled water was added to 22.5 g of this dry powder in the sterilized glass petri dish and mixed until the paste was homogenized for all diets.

Experimental design

Cylindrical plastic containers (Ø: 25 cm) were used to culture *E. albidus.* All the equipment and materials were sterilized before use in an autoclave. Four substrate materials and two feed types (eight experimental groups in total) were tested in triplicate to evaluate their effect on the growth performance of *E. albidus*. Either peat (P), rice husk (R), cocopeat (C) or garden soil (G) were used as the substrate. All culture containers were filled with substrate material to a final depth of 4.5 cm (approx. 2.2 L). All the prepared diets were distributed equally (approx. 9.3 g/container) to each culture container by placing the paste on the top of the substrate and then covered with a sterilized glass-lid to reduce the risk of contamination. Feeding was done ad-libitum by checking absence of feed in the containers daily. Each culture was started with 150 individuals of *E. albidus,* equivalent to a density of 7 worms/100 cm^{3}. Mean weights of worms was measured as 0,011 ± 0,002 g. The feeding experiment started after a 10-day long adaptation period and continued for 50 days. Harvesting was done with the help of a heat source under the plastic container and white worms collected from top. Thereafter, the substrate was spread over drying paper so the remaining worms could be collected. Counting of white worms was done manually with dissection tools at the beginning and the end of the experiment.

Proximate composition analysis

The proximate composition analysis of produced white worms was done in the Seafood Processing Laboratories of Fisheries Engineering Faculty of Muğla Sıtkı Koçman University. The sampling pool consisted of 0.5 g organic material (white worms) from each replicate (totally 1.5 g from each group). Sampling and proximate composition analysis were done after 50 days of feeding trial. Initially all the worms came from same culture condition. Therefore, Initial proximate composition of the worms was not analyzed and discussion was made on final differences that was occurred between the experimental groups. Collected white worms from each group were homogenized in a glass beaker, then analyzed for the proximate proportion of protein (Kjeldahl method, AOAC 928.08, 2002), lipid (Bligh and Dyer; 1959), ash (AOAC 950.46, 1990), moisture (AOAC, 1995), and total carbohydrate (Merrill and Watt, 1974). The nutritional composition of the plant-based and fish feed-based diets were provided by the food producers (Table I). The content of vitamins, minerals and proximate composition of feeds were provided by the food producers.

Table I. Nutritional composition, vitamin, and mineral content of plant-based and fish feed-based diets which was used to feed *E. albidus*.

	Plant-based diet	Fish feed-based diet
Proximate composition (g/100g)		
Protein	4.70	50
Lipid	14.30	20
Carbohydrates	77.00	15
Moisture	3.00	12
Cellulose	1.00	3
Vitamins		
Vit A (IU/kg)	11200	12500
Vit D3 (IU/kg)	4000	2500
Vit E (IU/kg)	45	300
Vit C (mg/kg)	300	1000
Vit B1 (mg/kg)	5	-
Vit B2 (mg/kg)	8	30
Vit B12(mg/kg)	0.007	0.02
Inositol (mg/kg)	-	780
Choline (mg/kg)	-	3000
Minerals (mg/kg)		
Sodium	940	6
Calcium	4250	5
Phosphorus	3200	15

Statistical analysis

Normality of data was defined by using Shapiro-Wilk Test. The effect of feed and substrate types on the growth performance and the proximate composition of *E. albidus* were determined by two-way ANOVA. When ANOVA tests indicated significant effects of feed treatment, substrate type or combination of feed and substrate on worm numbers, Tukey's HSD/ Kramer tests were run to identify differences between the groups. Nutritional compositions of the produced worms were also compared with Tukey's HSD pairwise comparison tests. Replicate culture containers were considered experimental units (N = 3) for all statistical analysis. SPSS 22.0 software was used for the statistical analysis and the null hypothesis (no significant difference between experimental groups) was rejected when the calculated p-value was < 0.05. The error terms included with the symbol ± represents Standard Deviations.

RESULTS

The final population size and density of *E. albidus* were significantly affected by feed type and substrate type (one-way ANOVA, p < 0,001, Table II). All the feed and substrate combinations were also affected worm production and final density (two-way ANOVA p < 0.001, Table II, Fig. 1). The worm densities significantly increased in the Cp (cocopeat-plant-based diet), Cf (cocopeat-fish feed based diet), Pp (peat-plant based diet), Gp (garden soil-plant based diet), and Gf (garden soil-fish feed based diet) groups. Significantly decreased final worm numbers and densities were observed in the Rp (rice husk-plant based diet), Rf (rice husk-fish feed based diet), and Pf (peat-fish feed based diet) groups at the end. Best final worm density was observed at the Gp combination (101 ± 4.02 worm/100 cm^3), but Pp (89 ± 2.90 worm/100 cm^3) combination had similar final worm density although there was a significant difference between these two combinations (p < 0.001). Final numbers of worm counted as 2220 ± 88.36 worm/container in Gp combination and 1957 ± 63.75 worm/container in Pp combination. The lowest numbers of worms recorded as 57 ± 3.27 worm/container after fish feed treatment in rice husk substrate (Rf combination).

Table II. White worm population sizes after 50 days of feeding experiments. All the values are given as number of worms per culture box (2.2 Liter). Statistical differences and abbreviations were given in the related text. Abbreviation of feeding groups were created by using the cross section of rows and columns in the table (Rf, Rp, Cf, Cp, Pf, Pp, Gf, Gp). Standard deviation is given with (±).

	Fishfeed-based diet (f)	Plant-based diet (p)
Rice husk (R)	57 ± 3.26^{a} (Rf)	141 ± 6.53^{ab} (Rp)
Cocopeat (C)	271 ± 4.32^{b} (Cf)	528 ± 8.04^{c} (Cp)
Peat (P)	78 ± 2.16^{a} (Pf)	1957 ± 63.74^{d} (Pp)
Garden soil (G)	627 ± 28.08^{c} (Gf)	2220 ± 88.36^{e} (Gp)

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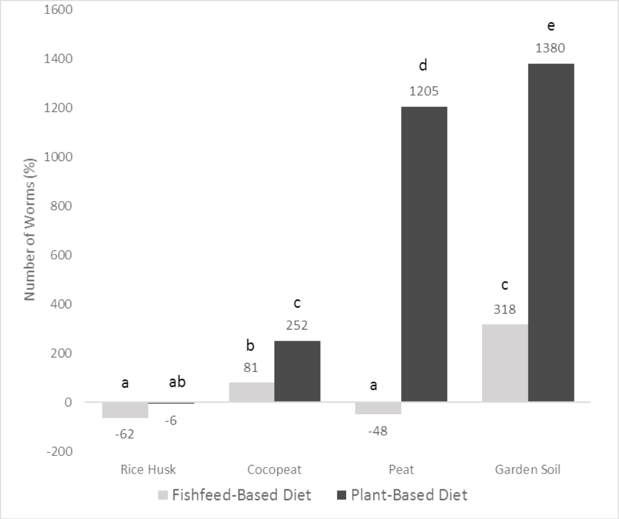

Fig. 1. Bars shows the worm numbers as percentage. Comparison done by substrate types and each bar belongs to different feed treatment. The zero point (-) of the graph is initial worm density (7 worms/100 cm^3). Lines on the bars shows errors and letters represents the significant differences between treatments (p-value given in the text as they are differentiated according to the experimental group).

If we evaluate the results on the basis of feeding treatment, the numbers of the worms obtained from the plant-based diet was greater than the fish feed-based diet in general. Fish feed fed groups were significantly different among all substrate groups and the best growth rate was observed in the garden soil substrate (Gf) with 627 white worms (Tukey's HSD, pairwise t-test, p<0.05). In contrast, even fewer worm numbers than the initial number of 150 worms/container were observed in the rice husk-fish feed (Rf) group and the peat - fish feed (Pf) group (57 worms and 78 worms, respectively). The cocopeat-fish feed (Cf) group sustained lower population growth; however, number of white worms (271 individuals) were significantly increased for this group compared to Rf and Pf groups (p<0.001). The number of worms were slightly decreased with the combination of Rice husk and plant-based feed (Rp), but it was not statistically significant from the initial numbers (p>0.05). The plant-based feed (p) yielded good results that could be observed with the cocopeat-plant based feed (Cp), peat-plant based feed (Pp), and garden soil-plant based feed (Gp) combinations. These combinations resulted in more than a 3-fold (24 worms/100 cm^3), a 13-fold (89 worms/100 cm^3), and a 14-fold (101 worms/100 cm^3) increase in worm numbers, respectively (p<0.001, Fig. 1).

The nutritional composition of white worms was analyzed only in the groups Pf, Pp, Gf, and Gp. The other experimental groups yielded with insufficient number of worms. The nutritional composition of analyzed groups was significantly altered during the feed treatment, except for the moisture content (p<0.001). However, all substrate types had little effect on protein, lipid, carbohydrate, and moisture levels, although a significant effect was observed on the ash content of the worms (p<0.001, Fig. 2). Fish feed-fed worms had significantly higher protein content (56.7-57.1% in dry weight), but lower lipid (22.5-24.2% in dry weight) and lower carbohydrate (2.6-2.7% in dry weight) levels compared to worms fed a plant-based diet (p<0.05, Table III, Fig. 2).

Table III. Nutritional values of white worms at the end of the experiments on dry weight basis. Statistical differences and significance were given in the text (Gp, garden soil-plant based diet; Gf, garden soil-fish feed based diet; Pp, peat-plant based diet; Pf, peat-fish feed base diet). Standard deviation is given with (±).

Dry (%)	Protein	Lipid	Carbohydrate	Ash
Gp	38.58±1.86^{a}	42.41±2.79^{a}	12.51±1.06^{a}	6.51±0.42^{a}
Gf	57.44±1.47^{b}	21.55±1.22^{b}	2.70±0.48^{b}	18.31±1.25^{b}
Pp	38.85±5.08^{a}	39.44±2.33^{a}	11.78±0.81^{a}	9.94±0.95^{c}
Pf	57.13±1.81^{b}	24.24±1.42^{b}	2.58±0.04^{b}	14.38±0.80^{d}

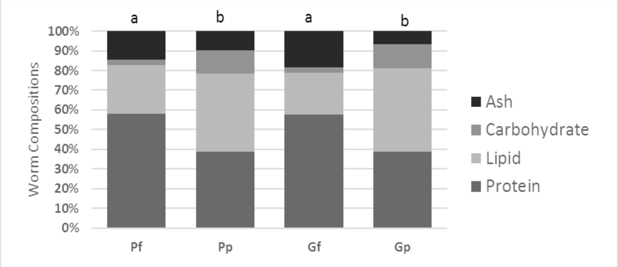

Fig. 2. Proximate composition of the four group in dry weight basis which were yielded enough to conduct composition analysis. The letters above the bars represents significance.

DISCUSSION

Most of the research articles were related on avoidance behavior (Amorim *et al.*, 2005a, 2008; Lobe *et al.*, 2018) and toxicity tests on survival, reproduction and growth (Arrate *et al.*, 2002; Amorim *et al.*, 2005b; Fernandes *et al.*, 2020) instead of commercial scale production of white worms. Only some growth and reproduction data were

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available in literature for the feeding potential of white worms (Memiş *et al*., 2004; Fairchild *et al*., 2017; Dai *et al*., 2021). Accordingly, the aim of the present study was to determine the optimal feed/substrate type combination for white worm culture that also allowed the best nutritional composition while using an expired (not degraded) fish feed for recycling purposes.

E. albidus productivity is largely dependent on finding the best combination of substrate and feed type, and notable effects can be observed even in short-term studies (Fairchild *et al*., 2017) because the white worm generation time is as short as 20 days (Ivleva,1973; Memiş *et al*., 2004). In the present study, feed and the substrate combinations had significant effects on propagation and nutritional composition of *E. albidus.* Highly significant differences were observed after 7 weeks and were most promising for the Pp (peat substrate/plant feed) and the Gp (garden soil substrate/plant feed) combination. At the start of the culture there were 7 worms/100 cm^3 that increased to 89 worms/100 cm^3 and 101 worms/100 cm^3 density per culture container over the course of the experiment, respectively. Fairchild *et al.* (2017) started with a higher density of 210 worms/100 cm^3 and reported a 6-fold population increase within 12 weeks; more specifically 1321 worms/100 cm^3 in the bread fed groups. In comparison, our result was a 13-fold population increase after only 6 weeks of culture. Furthermore, the plant-feed/natural garden soil combination sustained a similar worm population growth to the 12-fold increase of the mentioned study at University of New Hampshire (Fairchild *et al*., 2017) although their starting density was notable higher than in the present study. It is worth mentioning that the high population growth obtained in the present study could be related to the substrate (natural garden soil) that was used for the adaptation period as we used newly prepared substrate. It is supported by the findings of Ivleva (1973) who mentioned that the used substrates or combination of used and new substrates give the best growth results for new worm cultures (Fairchild *et al*., 2017). In this point of view, we can say that our results can be improved while the culture gets older by time and partial substrate replacement could be done for better culture conditions.

The inhibited growth of white worms cultured in rice husk (3-6 worms/100 cm^3) could be related to substrate type as worm numbers decreased below the initial numbers (7 worms/100 cm^3) in both feed groups. It is known that worms require a soft and more porous substrate (Amorim *et al*., 2005b) and the negative impact of rice husk was expected. Although the impact of the cocopeat on worm growth and final densities differed between the feed treatments, the results obtained from this groups were not sufficient for White worm culture. However, it was observed that the Fish feed diet (Cf) supported survival but it was not effective for white worm population growth in cocopeat substrate (12 worms/100 cm^3). In the case of Cocopeat and Plant-based feed combination (Cp), the white worms had a relatively increased density (24 worm/100 cm^3) which can be related to the high carbohydrate content of the Plant-based feed. But it is still more supportive for survival and relatively enough for population growth. The peat substrate had a negative impact on worm density in the Pf group (4 worms/100 cm^3) and it could be related to the high organic matter and clay content. Peat is organic soil that contains large amounts (>20%) of organic matter (OM) and that is rich in minerals (up to 60% clay) that support plant growth. Amorim *et al*. (2005b) recommended that suitable soils for the study of *E. albidus* should consist of 2.5-8.0% OM and 6-26% clay to achieve an acceptable worm reproduction. Thus, peat could not be a good substrate alone when fish feed was used for *E. albidus* production. Promising worm densities were obtained with the Pp (89 worms/100 cm^3) and Gp (101 worms/100 cm^3) combinations. Although the Pp and Gp combinations resulted in a similar final worm density, there was a significant difference between groups, and it appears to be related to substrate type. Worm densities are mostly determined by feed type and that would explain the highly significant changes of worm densities between experimental groups. Also, the plant feed results might suggest protective effect on white worm survival that counteract any negative effects of substrates. Dai *et al.* (2021) reported a highest worm density of 1300 worms/vial and it is approximately equal to 1625 worms/100 cm^3. In the same research, authors mentions that population density have negative impact on population growth in terms of biomass. Both of the studies (Fairchild *et al*., 2017; Dai *et al*., 2021) had higher densities then our study which means that the relation between density and biomass is not a concern for our research. Dai *et al.* (2021), fed the worms for 160 days to reach the maximum density and the biomass approximately 100 g live weight per liter of substrate. In our study highest population biomass was observed as 11.10 g live weight per liter of substrate. This means that the growth of the white worms with both fish-feed based and plant-based diet could not be hampered by crowding. One of our purposes was to determine the recycling potential of expired fish feeds by using white worms. The efficiency of recycling process can be increased by adjusting the environmental conditions according to the literature. Holmstrup *et al.* (2020) found that the white worms yield much higher if the substrate is moistened with saline water instead of freshwater.

The substrate type appears to have significant impact on *E. albidus* population growth (Fig. 1). It was, however,

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not possible to identify why, as the nutritional- and mineral composition of individual substrate types were not known.

The Rf and Rp groups were not included in this analysis as the total biomass and final population density is not promising for both feed type. However, our statistical analysis showed that the nutritional composition (protein, lipid, carbohydrates) of the propagated worms was highly dependent on feed type. The ash content was significantly affected by substrate type and it could be explained by the mineral contents of the substrate (Fig. 2). Therefore, we can conclude that the worm nutritional content is not affected by the substrate type alone but also that the combination of substrate and feed type notably altered the ash content of white worms.

The protein content (38.6-57.1%) of produced white worms in this study equals values reported for live feeds currently used in aquaculture. According to Radhakrishnan *et al.* (2020), the protein content of commonly used live feeds in aquaculture is reported to be 63.2% in Copepods, 53.8% in Artemia, 51.3% in Rotifers (Rocha *et al*., 2017), and 39.68% in Daphnia (Cheban *et al*., 2017). The said publication also reports that the lipid content of these live feeds is 8.8% for Copepods, 18.1% for Artemia, 12% for Rotifers (Rocha *et al*., 2017) and 24.99% for Daphnia (Cheban *et al*., 2017). It shows that the lipid content (22.5-42.4%) of white worms produced in present study is comparable to that of the mentioned live feeds. Worms from the fish feed-fed groups (Pf, Gf) had optimal protein and lipid values, but the final worm density was consistently lower than in the Plant-based feed groups (Pp and Gp). Taken together, our results suggest that expired fish feed can be recycled with white worms if the appropriate carbohydrate levels provided in ration and that these white worms have a sufficient protein and carbohydrate content to be made into a high-quality ingredient for fish feed production. This recycling strategy would be a step towards a more sustainable industry as fishmeal then could be replaced with white worm meal. White worm meal or similar meals (Belghit *et al*., 2020; Shekarabi *et al*., 2021) should be evaluated further as an alternative fish feed ingredient.

CONCLUSION

It is shown that expired fish feed can be recycled with the help of *E. albidus* and that white worms can be an alternative fish feed ingredient as it has been proven that their protein and lipid content is similar to the nutritional composition of live feeds and commercial extruded fish feeds.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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