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Recycling and repurposing food waste as feed for small-scale zebrafish (*Danio rerio*) aquaculture

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Abstract

Food waste is a globally abundant resource, but currently, it is primarily managed by disposal in landfills, wasting the valuable nutrients. At the same time, commercial fish feeds are expensive and contain unsustainably produced ingredients that are subject to price fluctuations and demand for other uses. Utilizing food waste to supplement or replace traditional feed ingredients could increase the profitability and sustainability of aquaculture, supporting fish species that provide economic, recreational, and ecological value. To explore the feasibility of using food waste in aquaculture, food waste was recycled into "converted fish flakes" (CFF) with a protein ratio appropriate for zebrafish (*Danio rerio*) in a small-scale aquaculture study. Juvenile zebrafish were fed diets with increasing proportions of food waste (25%, 50%, and 100% CFF) for 32 days and compared to a control group fed with commercial feed. Zebrafish fed CFF diets had high survival rates and growth that was comparable to the commercial feed. CFF diets did not significantly alter zebrafish fecundity or viscerosomatic index (VSI). Histology revealed an increased amount of goblet cells in the intestine and fat deposits in the liver associated with a 50% CFF diet. These results indicate that recycled food waste could be feasibly used in freshwater, small-scale aquaculture of omnivorous fish.

Keywords: aquaculture, food waste, sustainability, recycling, zebrafish

Introduction

Global aquaculture production was 110.2 million tons in 2016, including 80 million tons of food fish and 30.1 million metric tons of aquatic plants, with an estimated total worth of \$243.5 billion (FAO 2011) [6]. The profitability of aquaculture is dependent on the efficiency of feed intake and utilization, and it is desirable for fish farmers to control the feed quality to produce fish with maximum growth and minimal waste and environmental impact (da Silva et al. 2016) [3]. Currently, fish meal is a major source of high-quality protein for commercial fish feed because of its high protein content, amino acid balance, and digestibility; however, increasing demand for fishmeal as aquaculture feed and the decline of wild fish populations where fishmeal is sourced has led to price increases (Jiang et al. 2019) [11]. Capture fisheries have reached their maximum potential production and will, therefore, not be able to meet the increasing demand for fish products (Subasinghe et al. 2009) [20]. Overharvesting of wild fish populations has already resulted in dramatic losses in abundances that endanger local seafood industries (CNBC 2018). However, these ingredients will likely become expensive and unsustainable in the future, as currently, 60% of global fish stocks are maximally sustainably fished, and more than 30% of global fish stocks are fished at unsustainable levels (FAO 2020) [7]. The aquaculture industry is reported to consume more than 70% of the global supply of fish meal and 90% of the global supply of fish oil (Bostock et al. 2010) [1]. Expansion of aquaculture could further increase the pressure on a global fish meal or oil supply (Bostock et al. 2010) [1]. Fish meal and oil are popular ingredients in aquaculture feeds because of the high levels of easily digestible protein (Jiang et al. 2019) [11]. Most crustacean, marine, and other intensively cultured fishes require high quality feed, which usually contain fish meal or fish oil (Bostock et al. 2010) [1]. If the aquaculture industry is to grow, the supply of feed inputs will have to grow at a similar rate, although capture fishery production is projected to remain static (Tacon and Metian 2015) [21].

Unlike the production of capture fisheries, which is based on the natural productivity of the local ecosystem, the cultivation of farmed fish is dependent on external feed inputs (Tacon and Metian 2015) [21]. Utilizing nontraditional feeds such as food waste to supplement or replace commercial feed should decrease the cost and increase the sustainability and stability of aquaculture (Nasser *et al.* 2018) [15].

Several plant and animal-based protein alternatives are available and used in industrial aquaculture feeds, and with appropriate economic and regulatory incentives, transitioning to alternative feedstuffs could accelerate (Naylor et al. 2009) [17]. Food waste and food processing by-products are being explored as an alternative aquaculture feed in other countries, especially in China, which produces an estimated 195 million tons of food waste annually (Mo et al. 2018). Food waste is an abundant and valuable resource, and its disposal takes up limited and increasingly expensive space in landfills. Incineration of food waste is not a viable disposal option because the high moisture content reduces the energy produced (Mo et al. 2018). Due to its abundance and difficulty of disposal, there is an urgent need to recycle food waste into fuel or animal feed (Wong et al. 2016) [25]. However, ruminants like cows, sheep, and goats are vulnerable to mad cow disease spread by producing feed from animal blood, bone, and tissue; fish are not known to be vulnerable to this disease (Wong et al. 2016) [25]. Furthermore, fish are more efficient at converting feed into edible meat than warm-blooded animals (Robinson and Li 2015) [19]. Therefore, fish are an excellent candidate for food wastebased feed.

Zebrafish (*Danio rerio*) are a well characterized animal model which is closely related to other fishes (like carp and tilapia) used in aquaculture (Ulloa *et al.* 2011, Dahm and Geisler 2006) ^[23, 4]. The rapid development and small size of zebrafish allow for a fast and statistically powerful analysis of growth (Ulloa *et al.* 2014) ^[24]. Maximum weight gain and protein retention were found in juvenile zebrafish fed a diet with protein levels between 37.6% and 44.8%, respectively (Fernandes *et al.* 2016) ^[8]. The first objective of this study was to determine the feasibility of recycling university preconsumer food waste into "converted fish flakes" (CFF) for zebrafish. The second objective was to investigate the effects of food waste-based diets on zebrafish. Therefore, it was

hypothesized that food waste can be successfully processed into CFF with a protein level appropriate for zebrafish. It was further hypothesized that zebrafish survival, reproduction and growth will not be significantly different when fed CFF and zebrafish will not show adverse effects in the morphology of the intestine or liver.

Methods

Production of CFF

A target protein level of 38% was set for the CFF used in this study based on reported optimal levels for growth (Fernandes et al. 2016) [8]. Pre-consumer fruit, vegetable, and meat waste (created when preparing meals) was collected at a university campus (Fig. 1A, B) and frozen, which preserves the fresh food and is reported to increase the bioavailability of nutrients as ice crystals expand and puncture the cell walls of the fruits and vegetables (Jin and Chen 2018) [12]. This also helps to produce a smoother and more uniform texture in the CFF. After being frozen, food waste was thawed and then cooked thoroughly with a hot water bath. Treating food waste with heat is crucial when recycling food into animal feed because heat inactivates anti-nutritional factors such as tannins or alkaloids that may be present in peels, leaves, and root tubers (García et al. 2005) [10]. Cooking also increases the digestibility of carbohydrates by fish (National Research Council 1993) [16]. Further, heating food reduces undesirable microbial populations that may cause illness (García et al. 2005) [10]. After cooking, the food waste was processed with a food processor to produce a uniform mixture (Fig. 1C) with 38% by weight of meat waste to correspond with a protein level of 38%. The mixture was thinly spread onto silicone sheets and a food dehydrator was used to dry the mixture into thin sheets, which were broken into CFF (Fig. 1D). CFF was then mixed with commercially bought flakes to produce three treatment diets with different proportions of food waste: 25% CFF, 50% CFF, and 100% CFF (Fig. 1F, G, H). The Control group (0% CFF) was fed only with the commercial feed, Egg Yolk Flakes (Pentair, Minneapolis, MN, US; Fig. 1E). The control and treatment diets (Fig.1E, F, G & H) were sent to an independent laboratory (RL Food Testing Laboratory, Newbury Park, CA, US) for macronutrient analyses and the results of which can be seen in Table 1.



Fig 1: Collection and production of food waste-based feed for a small-scale aquaculture study using zebrafish. (A & B) Some parts of food, such as peels, rinds, and stems, are separated during food preparation as pre-consumer food waste and are collected in designated bins to reduce contamination with undesirable materials such as paper, plastic, and chemicals. (C) To produce palatable and consistent feed, only a few food items were selected and processed. After collection food was frozen, then cooked, and portioned to contain 38% protein by weight. The cooked portions were then processed with a food processor into a smooth paste, which was dried in a dehydrator (D) to produce Converted Fish Flakes (CFF). (E-F) Commercial egg yolk fish flakes from pentair (E), Mixtures of CFF with commercial egg yolk fish flakes at different ratios of 25% (F), 50% (G) and 100% (H).

Table 1: Macronutrient Analyses of Control and CFF diets.

Nutrient	Control	25% CFF	50% CFF	100% CFF
Minimum Crude Fat%)	9.0	18.6	26.8	39.8
Minimum Crude Protein (%)	50.5	48.4	46.2	39.7
Maximum Fiber (%)	1.8	3.1	3.1	2.8
Moisture (%)	6.3	3.0	3.0	5.1
Ash (%)	14.1	11.3	8.2	2.8

Animal husbandry

This study was reviewed and approved by Institutional Animal Care and Use Committee. Wild-type zebrafish were hatched and reared in standard conditions to the late juvenile stage (about 30 days). Zebrafish were weighed and measured for starting body weight, then randomly separated into 2.8-liter tanks in a recirculating tank system (Aquaneering, San

Diego, CA, US: ZTEDU235, Two Shelf for experimental groups and ZTT350, Three Shelf for the control group). Water quality parameters were tested regularly with API Freshwater Master Test Kit (Chalfont, PA, US). Water quality parameters can be seen in Table 2. During each trial fish were fed *ad libetum*, approximately 1 gram of flakes every morning and evening, for a total of 2 grams per day, for 8 weeks.

Table 2: Water quality of trials 1 & 2.

Temperature (C)	pН	High pH	Nitrites (ppm)	Nitrates (ppm)	Ammonia (ppm)
26.1 ± 0.69	7.4 ± 0.21	7.4 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.2 ± 0.17

Trial 1

Groups were monitored for individual mortality to determine the survival rate of each replicate tank. At the end of the study, zebrafish were again anesthetized with 0.01% tricaine before measurement for body weight. To assess reproductive success, at the conclusion of the trial, at least two pairs (2 males and 2 females) of zebrafish from each group were set up to breed. The offspring were collected and observed for 5 days post fertilization at standard conditions to measure percent viability in each group. Because reproduction requires high levels of energy, this experiment was performed after the measurement of final weight and length to avoid bias.

Trial 2

Mortality and start and final weights were measured as in Trial 1. Additionally, at the conclusion of the trial, five male and five female zebrafish from each group were euthanized and fixed in 37& formaldehyde, after which they were sent to an independent laboratory (College of Veterinary Medicine, University of Georgia, GA, US) for histological analysis of the intestine and liver. Fixed fish were embedded in paraffin, sectioned, and stained with Hemotoxylin & Eosin and Sudan IV dye. Liver sections were observed for excessive lipid accumulation or other abnormal morphology and intestine sections were observed for signs of inflammation. Finally, five male zebrafish from each group were euthanized and

dissected to obtain the viscerosomatic index (VSI).

Calculations and statistical analysis

The survival rate was calculated as ((final # / start #) * 100). Individual starting and final weights were averaged by replicate, and percent change in weight was calculated as ((average final – average start) / average start * 100). Fecundity was measured by manually counting the total number of embryos produced by each breeding pair. Embryo survival rates were calculated as ((# surviving at day 5 / # total) * 100). Individual VSI were calculated as ((viscera weight / final body weight) * 100). Data was input, visualized, and calculations were performed with Excel 2015 (Microsoft, Redmond, WA, US). Statistical analysis was performed using JMP (SAS, Cary, NC, US). Data for fecundity, embryo survival rates, and liver vacuole counts met the assumptions of normality and equal variance. Data for percent change in weight, survival rates, and intestine goblet counts were log transformed to meet the assumptions of normality with the Shapiro-Wilk test and equal variance with the Leven test. These data were analyzed with a one-way ANOVA for the difference by treatment (Control, 25% CFF, 50% CFF, or 100% CFF diet). Data for VSI did not meet the assumption of equal variance for ANOVA after log transformation, so a nonparametric Kruskal-Wallis test was used for these data. Statistical results can be seen in Table 3.

Table 3: Statistical outputs for one-way ANOVA and Kruskal-Wallis tests for differences between groups of zebrafish fed increasing proportions of food waste-based Converted Fish Flakes (CFF) for 32 days. Asterisks represent statistical significance.

	Trial	df (Effect, Total)	F	p>F
	Survival	3, 11	0.47	0.71
	Weight	3, 11	5.61	0.0228*
1	Length	3, 11	4.87	0.0326*
	Fecundity	3, 11	0.47	0.71
	Embryo Survival	3, 11	0.55	0.66
2	Survival	3, 11	0.55	0.66
2	Weight	2, 8	1.35	0.33
	Goblet Cells	2, 17	35.16	<0.0001*
	Vacuoles	2, 17	14.72	0.0003*
	Trial	df	ChiSquare	p>ChiSq
2	Length	2	2.22	0.33
2	VSI	2	0.38	0.83

Results Zebrafish survival was not affected by CFF

In this study, food waste-based CFF diets did not affect the survival rates of zebrafish compared to a commercially available diet (Fig. 2A). In the first trial, no mortality was observed in any replicate tank of the Control, 50% CFF, and

100% CFF groups. In the second trial, similarly high survival rates were observed, with the Control group again exhibiting no mortality (Fig. 2B). Survival rates were not significantly different between the Control and CFF diets in either trial (Table 3)

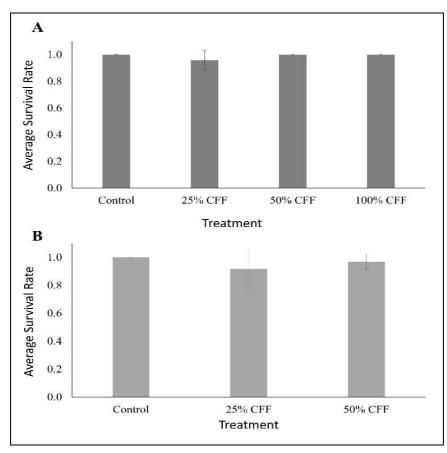


Fig 2: Survival of zebrafish that were fed with diets of increasing proportions of food waste-based CFF in Trial 1 (A) and Trial 2 (B).

Zebrafish fed 50% and 100% CFF had weight gain similar to commercial feed

In the first trial, a significant difference (p=0.0228, Table 3) was found between groups for in percent change in weight. The Control, 50% CFF, and 100% CFF groups were similar and shared a significance level, while the 25% CFF and 100% CFF group shared a different significance level (Fig. 3a). The 25% CFF group was observed to have the highest average percent change in weight in the first trial, with a weight gain

of nearly 100%, and it also had the highest level of variation (Fig. 3A). Similarly, the 25% CFF group had an approximately 100% weight gain in the second trial (Fig. 3B). However, in the second trial no significant differences were found between groups (Table 3). In the second trial, the groups also exhibited a higher average and higher variation in percent change in weight compared to the first trial (Fig. 3A, B).

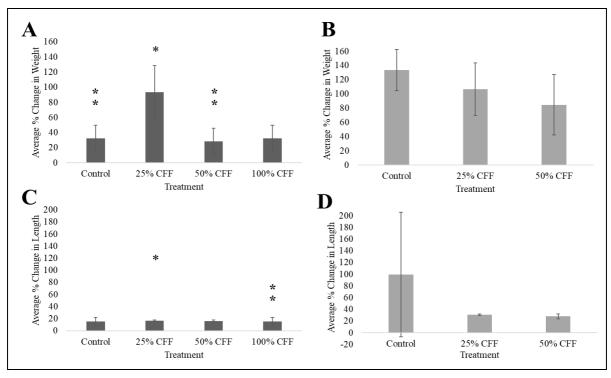


Fig 3: Growth in zebrafish-fed diets with increasing proportions of Converted Fish Flakes (CFF) for 32 days. Average percent change in weight in Trial 1 (A) and Trial 2 (B). Average percent change in length in Trial 1 (C) and Trial 2 (D). Error bars represent ± one standard deviation, n=3. Groups with the same asterisk (* or **) share the same level of statistical significance, groups without an asterisk share both levels.

In length for Trial 1, similar to the change in weight, the highest average percent change was also seen in the 25% CFF group (Figure 3C). The lowest average percent change was found in the 100% CFF group (Figure 3C). A significant difference (p=0.326, Table 4) was found between groups in percent change in length. The 25% CFF, 50% CFF, and Control groups shared a significance level, and the 50% CFF, 100% CFF, and Control groups shared a different significance level (Figure 3C). The 100% CFF and Control groups also had higher levels of variation than the other treatment groups. Interestingly, the 25% CFF and 100% CFF groups seem the most different in percent change in length, as these groups did not share a significance level.

There was no significant difference (p=0.33, Table 4) found between groups in percent change in length for Trial 2. In this trial, the Control group had the highest average percent change in length, and this group also had an extremely high level of variation compared to the other groups (Figure 3D). The lowest average percent change in length was in the 50% CFF group (Figure 3D). The 25% CFF and 50% CFF groups

had similar average percent changes in length, and these groups also has similar levels of variation, which were much smaller than the level seen in the Control group (Figure 3D). The 25% CFF and 50% CFF groups also had higher average percent changes in length compared to the first trial (Figure 3C, D).

CFF diets did not alter zebrafish fecundity or VSI compared to commercial feed

Zebrafish-fed commercial flakes and CFF produced on average, approximately 200 embryos in total (Fig. 4A). Although the 25% CFF group produced the highest average amount of embryos, no significant differences were found between groups in a total number of embryos (p=0.71, Table 3) and the groups exhibited similar variation (Fig. 4A). The average embryo survival rates were similarly consistent, at approximately 0.7 (Fig. 4B). Again, no significant differences were found between groups in embryo survival rates (p=0.66, Table 3) and the variation between groups was similar (Fig. 4B).

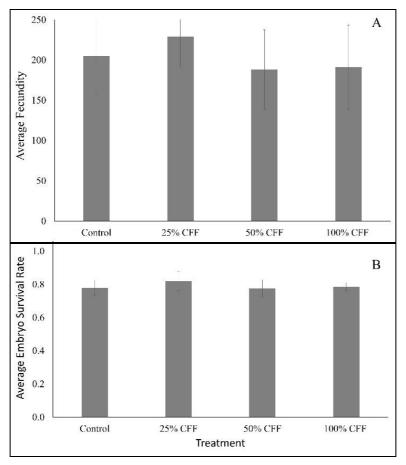


Fig 4: Average fecundity (A) and embryo survival rates (B) in zebrafish fed with diets containing increasing proportions of Converted Fish Flakes (CFF) for 32 days. n=3. Error bars represent ± one standard deviation. No significant difference was found between groups using a one-way ANOVA

Similar to the fecundity data, no significant differences (p=0.83, Table 3) were found between the Control and CFF groups in VSI. The Control, 25% CFF, and 50% CFF groups

had an average VSI of approximately 15 (Fig. 5). Interestingly, in this parameter, the Control group had the highest level of variation (Fig. 5).

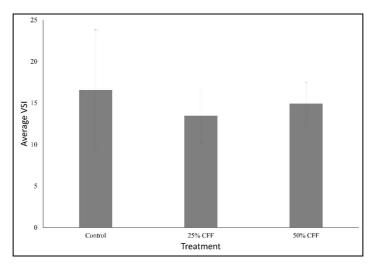


Figure 5. Average VSI in zebrafish-fed diets containing increasing proportions of Converted Fish Flakes (CFF) for 32 days. n=3. Error bars represent \pm one standard deviation. No significant difference was found between groups using a Kruskal-Wallis test

Alterations in intestine goblet cells and liver vacuoles of zebrafish fed 50% CFF

Histological analysis of zebrafish intestine and liver sections revealed changes in the 50% CFF group compared to the Control and 25% CFF groups. The 50% CFF group had significantly increased (p<0.0001, Table 3) numbers of goblet cells in the intestine (Fig. 6 and 7). This change seems to be more severe in female zebrafish, with the females of the 50%

CFF group having more than double the number of goblet cells in the male intestines (Fig. 6F). Likewise, the 50% CFF group had significantly increased (p=0.0003, Table 3) numbers of fat-filled vacuoles in the liver (Fig. 6 and 7). Curiously, unlike what was observed in the intestinal goblet cells, the females of the 50% CFF group did not have a dramatically increased number of fatty liver vacuoles compared to the males of the 50% CFF group (Fig. 6 and 7).

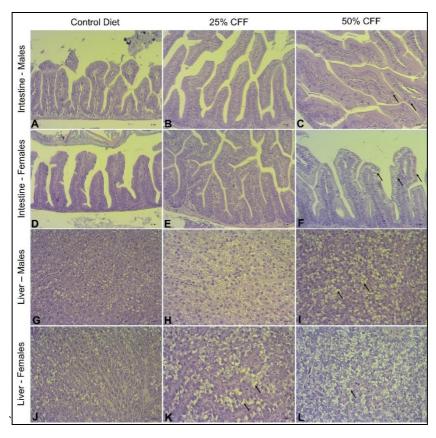


Fig 6: Representative images from sample of zebrafish fed with increasing proportions of food waste-based Converted Fish Flakes (CFF) for 32 days sent for histological analysis of intestine (paraffin embedding, 5 μm sections, stained with Hemotoxylin & Eosin and Sudan IV) of intestines of male (A-C); and female (D-F); liver of males (G-I) and liver of females (J-L)

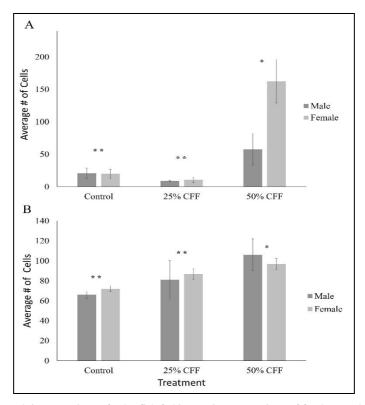


Fig 7: From manual counts of selected tissue sections of zebrafish fed increasing proportions of food waste-based Converted Fish Flakes (CFF) for 32 days. Average number of goblet cells in intestine sections (A) and average number of hepatocytes with vacuolar lipid accumulation in liver sections (B). Error bars represent ± one standard deviation, n=3. Groups with the same asterisk (* or **) share the same level of statistical significance

Discussion

This study demonstrated the feasibility of recycling preconsumer food waste into feed for fish, diverting it from landfills. As hypothesized, food waste was successfully processed into flakes with suitable protein levels for zebrafish using straight forward methods and simple equipment, and

this process could easily be scaled up or down depending on the amount of feed required. This should be useful in aquaculture, where fish feed is sometimes produced on-site (Tacon and Metian 2015) [21]. Food waste, which can be acquired without cost from institutional waste streams, as demonstrated in this study, is an attractive alternative to traditional feed ingredients. The demand and prices of these ingredients are likely to increase as the demand for aquaculture products also rises (Naylor 2020). A challenge to widespread incorporation of food waste-based feed is the need to separate and sort the food waste at the source to formulate feeds with acceptable nutrient profiles; for instance, unsorted food waste contains only about 20% protein, which is too low for even low trophic level fish, which have a lower protein requirement (Mo et al. 2018). There is a need for more information on different usage and processing methods to recycle food waste into feed, such as the use of fermentation (Mo et al. 2018). It has also been suggested that polytrophic culture, utilizing species from more than one biological niche, is a profitable and sustainable future direction for aquaculture. For instance, culturing channel catfish with paddlefish and freshwater mussels may offset the nitrogen output of the fish, which would otherwise decrease water quality (Wurts 2000)

The high survival rates and growth of zebrafish fed CFF in this study may indicate that food waste could be used in small-scale aquaculture of freshwater herbivorous or omnivorous fish without adversely affecting mortality or growth. Here, zebrafish survival was not significantly affected by diets supplemented with 25% or 50% CFF, or totally replaced with 100% CFF, for 32 days (Fig. 2). These high survival rates may indicate that food waste could supplement or replace fish feed without affecting survival, as long as the protein level remains adequate. Similarly, a diet formulated with a corn by-product and 28% protein was found to have no effect on the survival of channel catfish, another freshwater omnivore (Li 2013). In a different study with channel catfish, the traditional protein source (fishmeal) was replaced with a pork by-product without effect on survival (Li et al. 2020). Likewise, in this study food waste-based diets did not seem to adversely affect weight gain. Zebrafish fed CFF diets exhibited an average percent change in weight comparable to, or in the case of 25% CFF higher than, those fed a commercial diet (Fig 3). Food waste-based diets were also found to result in favorable weight gain in grass carp and Nile tilapia in other studies, suggesting that food waste could be an alternative feed ingredient without affecting fish growth (Mo et al. 2020, Bake et al. 2013).

Histological analysis of zebrafish fed a 50% CFF diet revealed changes in the intestine and liner (Fig. 6) that can be expected from a high-fat diet. For example, a different study with zebrafish reported a 24% fat diet resulted in fat accumulation in the liver (Dai 2015) [5]. The high-fat content of the CFF diets (Table 1) may indicate that excess fats should be removed from food waste, or that enzymes should be used to pre-digest the food waste to improve lipid utilization when processing into aquaculture feed (Choi 2016) [25]. A long-term study is needed to determine if the alterations in the digestive system seen in this study are harmful. It is unclear if the histological changes observed in this study negatively impacted the 50% CFF group, as this group also exhibited high survival and reproduction (Fig. 2, 4) The robust reproduction of zebrafish fed CFF suggest that food waste could be used to supplement or totally replace feed for freshwater, omnivorous fish without adversely affecting reproduction. Viscerosomatic index (VSI) is a measure of whether weight is distributed in muscle, which is more desirable for foodfish, or organs. While the 50% CFF group had a higher VSI than the 25% CFF group, the difference was not significant (Table 3, Figure 5).

Food waste-based feeds will not only divert food waste from landfills but should also decrease the industry's dependence on fish meal and oil, as well as expensive food crops that are in demand to feed humans. There are several promising methods to increase the nutrition provided by food waste to incentivize a transition to alterative feed ingredients. For example, additions such as yeast or enzymes, fortification with vitamins and minerals, or the fermentation of food waste before processing into feed have been shown to increase fish growth (Wong et al. 2016, Lateef et al. 2008) [25]. Another method to utilize food waste and food by-products involves using food waste as a substrate for black soldier fly larvae, which are then processed into fish feed (Zarantoniello et al. 2020) [27]. Recycling food waste into feed could increase the sustainability of aquaculture without compromising fish survival, growth, or reproduction.

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