

Original Article

Fermented calamansi (*Citrus microcarpa*) waste as a functional feed additive: Nutritional, antioxidant, and anti-nutritional insights for tilapia (*Oreochromis* sp.)

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Abstract: This study investigates the potential of fermented calamansi, *Citrus macrocarpa*, waste powder (FCWP), as a sustainable feed additive for tilapia (*Oreochromis* sp.). Calamansi waste, a byproduct of citrus processing, is often discarded, contributing to environmental pollution. To address this issue, the waste was repurposed as a fish feed additive through fermentation, which enhances nutrient content and bioavailability while reducing anti-nutritional factors. Fermentation trials were conducted with varying molasses concentrations (0, 5, 10, 15, and 20%) and durations (0, 14, and 28 days). The results revealed improved proximate composition (protein: 6.85-10.17%, fiber: 4.96-20.30%, fat: 2.49-3.95%, ash: 5.84-9.02%, carbohydrates: 62.87-77%) and antioxidant activity (TPC: 4.46-12.34 mg GAE/g, TFC: 0.56-1.56 mg catechin/g, DPPH: 5.68-41.57%) while reducing anti-nutritional factors (phytate: 0.06-0.067%, tannin: 11.25-23.97 mg TAE/g), with fermentation optimized at 5% molasses, 28 days. A 30-day feeding trial evaluated the effects of FCWP (0 and 3% inclusion levels) on tilapia growth performance, survival, water quality, gut microbiota, and stress tolerance. The results showed significant improvements in weight gain, specific growth rate (SGR), survival rate, and resilience to osmotic stress in the FCWP-fed group, with no adverse effects on water quality or gut microbiota. FCWP demonstrates potential as a cost-effective and sustainable alternative to conventional feed additives in aquaculture, enhancing fish growth and health while addressing environmental concerns by converting agricultural waste into valuable resources.

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Introduction

Aquaculture has emerged as a vital component of global food systems, meeting the increasing demand for fish and supporting the livelihoods of millions worldwide (FAO, 2022). A major factor in aquaculture success is feed quality, as it directly impacts fish growth, health, and performance (Cruz, 1996). However, the high costs of health-promoting feed additives, particularly immunostimulants and prebiotics, have presented challenges for the industry. Additionally, environmental concerns related to the disposal of fruit processing wastes have intensified the

need for sustainable waste management solutions (Leong and Chang, 2022). A promising solution lies in the utilization of fruit processing by-products, such as calamansi (*Citrus microcarpa*) peels, which are typically discarded as waste after juice extraction (Zalameda et al., 2014). This disposal contributes to environmental pollution and resource wastage, directly impacting Sustainable Development Goal (SDG) 12, which advocates for responsible consumption and production. Citrus peelings, however, are rich in bioactive compounds, including flavonoids and limonoids (Kumar, 2020), which have

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been shown to offer beneficial effects on fish performance and health status (Kesbic et al., 2022). Transforming citrus waste into valuable feed additives presents a dual benefit: it addresses waste management challenges while also providing a natural source of immunostimulants and prebiotics that support fish health (Aya et al., 2021; Râpă et al., 2024).

Fermentation, particularly through solid-state fermentation (SSF), is a transformative bioprocess that significantly enhances agro-industrial waste's nutritional profile and bioavailability. By breaking down complex macromolecules into simpler, more digestible compounds, SSF improves the overall utility of these byproducts as feed ingredients (Pandey, 2000; Ikusika et al., 2024). This process reduces anti-nutritional factors (ANFs) and elevates the levels of bioactive metabolites, such as antioxidants, thereby improving the digestibility and functional value of agricultural waste. These attributes make fermented byproducts a promising and sustainable feed additive for aquaculture (Dawood and Koshio, 2019). For example, dietary supplementation with fermented lemon peel has been shown to enhance immune responses, as evidenced by increased lysozyme activity and improved resistance to *Photobacterium damsela* infection in orange-spotted grouper (*Epinephelus coioides*) (Zhou et al., 2021). Furthermore, including fermented lemon peel in the diet of Asian sea bass (*Lates calcarifer*) significantly improved intestinal health, underscoring its potential to support both the nutrition and health of aquaculture species (Zhou et al., 2021).

In the case of calamansi waste, fermentation may reduce ANFs that hinder nutrient absorption and increase the concentration of bioactive compounds, potentially promoting better growth and disease resistance in fish (Salas-Millán and Aguayo 2024). Thus, this study aims to evaluate the nutritional profile, antioxidant properties, and anti-nutritional factors of calamansi waste fermented with different concentrations of molasses and fermentation periods. Additionally, it investigates the effects of fermented calamansi waste powder supplementation on the

growth, survival, water quality, gut microbiota, and osmotic stress tolerance of tilapia.

Materials and Methods

This research aligns with the United Nations Sustainable Development Goals (SDGs), particularly SDG 2 (Zero Hunger), SDG 12 (Responsible Consumption and Production), and SDG 14 (Life Below Water).

Sample collection and fermentation of calamansi waste: Fresh calamansi (*C. microcarpa*) waste materials, primarily peels discarded after juice extraction, were collected from a fruit processing plant in Siay, Zamboanga Sibugay. The peels with pulp and seeds were thoroughly washed to remove surface contaminants and then minced using a food processor (Kaisa Villa Electric Food Processor, 2L) to achieve a uniform particle size suitable for fermentation. Fermentation aimed to enhance the nutritional profile and digestibility of the calamansi peels, while reducing the anti-nutritional factors. Molasses was used as a substrate at concentrations of 0 (control), 5, 10, 15, and 20%. Minced peels were placed in fermentation containers with the corresponding molasses concentration and sealed to prevent external contamination. The fermentation was conducted at ambient temperature (~28-30°C) over 0 (control), 14, and 28 days. Post-fermentation, the samples were sun-dried for 48-72 hours to reduce the moisture content to 10%, ground into a fine powder by a grinder (BOBI Electric Herb Grinder 800 g stainless steel), and sieved (40 µm) for uniformity. The resulting fermented calamansi waste powder (FCWP) was stored in airtight containers for subsequent chemical analyses.

Determination of the proximate composition of FCWP and formulated diets: Representative samples (100 g) of FCWP and formulated diets were subjected to proximate analysis following the methods recommended by the AOAC (Association of Analytical Chemists, 1991). Moisture content was determined by the gravimetric-oven drying method, crude protein by the Kjeldal method, crude fat by the Soxhlet extraction method, crude fiber by the fritted glass crucible, and ash by combustion at 550°C.

Carbohydrates are calculated by difference using the formula: Carbohydrates (%) = 100 – (Moisture + Crude Protein + Crude Fat + Ash + Crude Fiber).

Determination of antioxidant activities: The extraction of phenolic compounds from FCWP was carried out following the protocol established by de Oliveira et al. (2009). Briefly, the FCWP was subjected to solvent extraction using methanol to isolate phenolic compounds. The methanolic extract was then concentrated and prepared for further analysis. To determine the total phenolic content (TPC) in the methanolic extract, the Folin-Ciocalteu reagent method was employed. This method involves the oxidation of phenolic compounds by the Folin-Ciocalteu reagent, leading to the formation of a blue complex. The intensity of the blue color, which correlates with the phenolic concentration, was measured spectrophotometrically at a wavelength of 760 nm. The results were expressed as milligrams of gallic acid equivalent per gram of extract (mg GAE/g extract). The total flavonoid content (TFC) of the methanolic extract was quantified using the aluminum chloride colorimetric method, as outlined by Hossain and Rahman (2011). In this method, flavonoids form a yellow complex with aluminum chloride, and the absorbance of the complex is measured at a specific wavelength (415 nm). The concentration of flavonoids in the sample was then calculated using a standard curve, and the results were expressed as milligrams of quercetin equivalent per gram of extract (mg QE/g extract). To evaluate the radical scavenging activity of the methanolic extract, the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was used, following the method described by Teraos et al. (1988), with some modifications. The DPPH method is based on the reduction of the DPPH radical, which has a deep violet color, by antioxidant compounds present in the extract. Upon reduction, the violet color fades to yellow, and the decrease in absorbance is measured at a wavelength of 517 nm. The radical scavenging activity of the extract was expressed as a percentage of inhibition.

Determination of anti-nutritional factors: The estimation of the total tannins followed the Folin-

Ciocalteu colorimetric method. This method is based on detecting the blue color produced by reducing phosphotungstomolybdic acid by tannin-like compounds in an alkaline medium. The tannin content is quantified by measuring the absorbance of the sample solution at 700 nm, with tannic acid used as a standard. The results are expressed as milligrams of tannic acid equivalent (TAE) per gram of extract. The determination of phytate followed the method described by Ekpa and Sani (2018). Briefly, 0.2 g of the sample was weighed and placed in a 250 ml conical flask. The sample was soaked in 100 ml of 20% concentrated HCl for 3 hours, then filtered. A 50 ml portion of the filtrate was transferred to a 250 ml beaker, and 100 ml of distilled water was added. Subsequently, 10 ml of 0.3% ammonium thiocyanate solution was added as an indicator, followed by titration with a standard iron (III) chloride solution, containing 0.00195 g of iron per ml.

Feeding trial

Experimental diet preparation: The experimental diets were formulated using high-quality ingredients, including fish meal, soybean meal, copra meal, rice bran, cassava flour, and a vitamin-mineral mix, following the diet formulation protocol of Alava (2002). The fermented calamansi peel powder (5% molasses, 28-day fermentation), identified as having the optimal nutritional profile in previous experiments, was incorporated as an additive. The composition of each diet is presented in Table 1. All ingredients were carefully weighed and prepared in a sanitized, dry environment to maintain quality and minimize contamination risks. Cassava flour was mixed with water and boiled under continuous stirring to create a uniform paste. After cooling, the paste was combined with the other ingredients and thoroughly mixed to form a homogeneous dough. The dough was processed through a pelletizer to produce uniform feed pellets. These pellets were steamed for 5 minutes to eliminate potential microbial contaminants, ensuring hygienic feed production. Finally, the steamed pellets were dehydrated in a controlled-temperature dehydrator (OneTwoFit Food Dehydrator Machine 5 Layers) at 50°C for 48 hours to achieve optimal

Table 1. Formulation and nutritional composition of the experimental diets.

Ingredients	Fermented calamansi waste powder (FCWP) in the diet	
	T1 (0%)	T2 (3%)
Fish Meal	18.25	18.25
SBM	25.00	25.00
Copra meal	10.00	10.00
Rice bran	6.00	3.00
FCWP	0.00	3.00
Cassava flour	36.42	36.42
Vitamin-mineral mix	4.33	4.33
Total (g)	100.00	100.00
Nutritional composition (%)		
Moisture	10.96	9.29
Crude protein*	27.77	29.50
Crude fiber*	3.56	4.01
Crude fat*	4.14	3.49
Ash*	16.51	15.83
Carbohydrates (Calculated by difference)	48.02	47.18

*Dry matter basis

moisture reduction and enhance shelf life.

Fish preparation and experimental conditions: The tilapia fingerlings were sourced from the Tilapia Hatchery of Mindanao State University at Naawan, Naawan, Misamis Oriental, where the feeding trial was conducted for thirty days. The fish were acclimated for one week and fed a standard basal diet. The experiment consists of two groups: Treatment 1 (control group) with a 0% inclusion level of FCWP and Treatment 2 with a 3% inclusion level of FCWP in the diet, with three replicates arranged in a completely randomized design. A total of 120 tilapia fingerlings with an average body weight of 1.2 grams were randomly distributed to six circular tanks (100L-capacity) at a stocking rate of 20 fish per tank. The fish were fed experimental diets twice daily, at 7:00 am and 4:00 pm. Daily feed ratio (DFR) was calculated based on 10% of their average body weight (ABW). To accommodate changes in weight and fish population, the daily feed ratio was adjusted every two weeks. Minimal (5%) water exchange was performed weekly, solely to replace the water collected for water quality analysis before sampling. Water temperature was recorded at 29.6°C, pH at 6.86, and salinity at 16 ppt.

Growth performance and index values: The initial weight and length of the fish were recorded at the start of the experiment to ensure baseline measurements. At the end of the experiment, all the data collected,

including the body weight and feed intake, were used to evaluate the growth performance. The parameters were calculated using the following formulas: weight gain = final body weight (g)– initial body weight (g); specific growth rate (SGR)% = $100 \times [(\ln W_t - \ln W_0)/t]$ (%), where W_0 and W_t are the initial weight and final weight of fish (g), and t is the culture period in days; feed conversion ratio (FCR) = feed intake (g)/ Weight gain (g) and survival rate = (number of remaining fish/initial number of fish) x 100.

Water quality and microbial analysis: Monitoring of water quality parameters such as nitrites, nitrates, ammonium, and phosphates was carried out weekly before feeding the fish. Water samples were analyzed in the Chemistry Laboratory, Research Division of MSU Naawan. The culture water and gut microbial profile were assessed after the 30-day feeding trial by a culture-based method previously described by Tropea et al. (2021). For the culture water microbiota, water samples were collected from each culture tank; for the gut microbiota, the collected intestine samples were homogenized in sterile distilled water and both samples from culture water and gut were serially diluted to 10^{-7} dilution, and 100 μ l of each diluted sample was spread in triplicates into the nutrient agar and MRS agar plates for enumeration of the total heterotrophic and lactic acid bacteria. After microbiological inoculation, the Petri dishes were incubated for 24 hours at a room temperature of 25°C,

Table 2. Proximate composition (dry matter basis) of FCWP fermented in different concentrations of molasses and different fermentation periods.

Treatment	Concentration of molasses (%)	Fermentation period (days)	Protein (%)	Fiber (%)	Fat (%)	Ash (%)	Carbohydrates (%)
1	0	0	8.43±0.11 ^e	20.30±0.29 ^a	2.56±0.07 ^{cd}	5.84±0.24 ^a	62.87±0.32 ^a
2	0	14	10.17±0.07 ^a	11.89±0.45 ^b	2.70±0.15 ^c	5.89±0.14 ^a	69.34±0.56 ^b
3	0	28	9.67±0.05 ^b	10.08±0.07 ^c	3.95±0.10 ^a	6.28±0.01 ^{ab}	70.02±0.06 ^b
4	5	14	8.63±0.05 ^e	9.39±0.18 ^d	2.70±0.00 ^c	6.83±0.21 ^{bc}	72.44±0.37 ^c
5	5	28	9.25±0.06 ^d	7.75±0.12 ^e	2.94±0.10 ^{bc}	8.02±0.09	72.03±0.25 ^c
6	10	14	7.63±0.04 ^f	7.36±0.16 ^{ef}	2.68±0.15 ^c	6.41±0.06 ^{bc}	75.91±0.34 ^d
7	10	28	9.48±0.05 ^c	6.83±0.28 ^g	3.78±0.27 ^a	7.52±0.04 ^{de}	72.39±0.50 ^c
8	15	14	7.23±0.04 ^g	7.35±0.09 ^f	2.69±0.08 ^c	7.01±0.36 ^{cd}	75.72±0.52 ^d
9	15	28	7.24±0.04 ^g	6.88±0.05 ^{fg}	2.25±0.07 ^d	7.65±0.07 ^e	75.97±0.16 ^d
10	20	14	6.85±0.04 ^h	6.22±0.06 ^g	3.17±0.16 ^b	6.77±0.20 ^{bcd}	77.00±0.18 ^d
11	20	28	6.94±0.04 ^h	4.96±0.10 ^h	2.49±0.09 ^{cd}	9.02±0.20 ^f	76.60±0.15 ^d
<i>P</i> -value			<0.000	<0.000	<0.000	<0.000	<0.000

Data are represented as mean ± SE. Means with different superscript letters within the same column are significantly different ($P < 0.05$).

and colony-forming units (CFU) ml⁻¹ were counted and log transformed (Kesbiç et al., 2024).

Osmotic stress test: After 30 days of feeding with experimental diets, an osmotic stress test was conducted to evaluate the tolerance of tilapia to high salinity conditions (Pedroso and Moquera, 2023). This test was conducted in a separate setup, using containers with a 10 L capacity, also in triplicate. Ten fish survivors from each treatment tank were abruptly transferred to the 10 L container with water prepared to a salinity of 60 ppt by dissolving sodium chloride (NaCl). The water was aerated for 24 hours prior to the test to ensure uniform salinity. Mortality was monitored and recorded at 15-minute intervals during the exposure period. Average cumulative mortality across the different treatments was reported at each time interval.

Statistical analysis: A one-way analysis of variance (ANOVA) was employed to determine the significance of the effects of molasses concentration and fermentation period on the nutritional, antioxidant, and anti-nutritional properties of the FCWP, while an independent T-test, at a significance level of 0.05, was used to assess the impact of FCWP supplementation on fish growth, survival, and microbial counts in the culture water and fish gut. Post hoc comparisons were conducted using Duncan's Multiple Range Test (DMRT) to identify significant differences among treatments. All statistical analyses were analyzed using IBM SPSS Statistics (version 26,

IBM Corporation, USA). All data were presented as mean±SE.

Results

The proximate composition of fermented calamansi waste powder (FCWP) subjected to different concentrations of molasses (0, 5, 10, 15, and 20%) and varying fermentation periods (0, 14, and 28 days) is presented in Table 2. The results show significant changes in the nutritional profile, including protein, fiber, fat, moisture, ash, and carbohydrate content, across the treatments ($P < 0.05$). Protein content ranged from 6.85 to 10.17%, with the highest level observed in the 0% molasses treatment after 14 days of fermentation. Protein levels generally decreased as molasses concentration increased, with the lowest value recorded in the 20% molasses treatment after 14 days. Fiber content significantly declined with higher molasses concentrations and extended fermentation periods, ranging from 20.30% in the unfermented sample (0% molasses, 0 days) to 4.96% in the 20% molasses treatment after 28 days. Fat content varied between 2.49 and 3.95%, with the highest level observed in the 0% molasses treatment after 28 days. Fat levels were generally lower in treatments with higher molasses concentrations and longer fermentation periods. Carbohydrate content, calculated by difference, significantly increased with higher molasses concentrations and prolonged fermentation, ranging from 62.87% in the

Table 3. Antioxidant properties of FCWP fermented in different concentrations of molasses and different fermentation periods.

Treatment	Concentration of molasses (%)	Fermentation period (days)	Total Phenolic Content (mg GAE /g sample)	Total Flavonoid Content (mg Catechin/g sample)	DPPH Scavenging Activity (% inhibition)
1	0	0	4.46±0.46 ^a	0.56±0.02 ^a	33.43±0.03 ⁱ
2	0	14	4.62±0.36 ^a	0.56±0.03 ^a	16.13±0.03 ^e
3	0	28	8.37±0.10 ^c	0.76±0.01 ^{bc}	5.68±0.03 ^a
4	5	14	7.58±0.45 ^{bc}	0.60±0.01 ^{ab}	10.19±0.01 ^b
5	5	28	12.34±0.58 ^d	1.56±0.08 ^e	41.57±0.03 ^k
6	10	14	7.27±0.52 ^{abc}	0.67±0.05 ^{bc}	12.40±0.02 ^c
7	10	28	7.52±0.16 ^{bc}	0.96±0.10 ^d	23.53±0.03 ^g
8	15	14	5.06±0.34 ^a	0.50±0.05 ^a	13.64±0.02 ^d
9	15	28	5.29±0.31 ^a	0.59±0.06 ^a	26.41±0.03 ^h
10	20	14	6.81±0.64 ^b	0.65±0.04 ^{ab}	19.90±0.01 ^f
11	20	28	6.92±0.28 ^b	0.82±0.03 ^{cd}	37.92 ± 0.04 ^j
<i>P</i> -value			<0.000	<0.000	<0.000

Data are represented as mean ± SE. Means with different superscript letters within the same column are significantly different ($P < 0.05$).

unfermented sample to 76.60% in the 20% molasses treatment after 28 days.

The antioxidant properties of FCWP are shown in Table 3. The Total Phenolic Content (TPC) of FCWP varied significantly across treatments. The lowest TPC was observed in the unfermented control (0% molasses, 0 days) at 4.46 mg GAE/g, while the highest value was recorded in the 5% molasses treatment after 28 days of fermentation at 12.34 mg GAE/g. TPC generally increased with longer fermentation time. Similarly, Total Flavonoid Content (TFC) showed significant variation among treatments. The lowest TFC was found in the unfermented control (0% molasses, 0 days) at 0.56 mg catechin/g, while the highest was in the 5% molasses treatment after 28 days of fermentation at 1.56 mg catechin/g. TFC also exhibited an increasing trend with prolonged fermentation, particularly in treatments with 5% molasses. The DPPH scavenging activity, an indicator of antioxidant capacity, was significantly affected by both molasses concentration and fermentation period. The lowest activity was observed in the unfermented control (0% molasses, 28 days) at 5.68% inhibition, while the highest was in the 5% molasses treatment after 28 days of fermentation at 41.57% inhibition. DPPH scavenging activity generally increased with higher molasses concentrations and extended fermentation periods, with the 5% molasses treatment demonstrating the most significant improvement over time. The antioxidant properties of FCWP, including

TPC, TFC, and DPPH scavenging activity, were significantly enhanced by fermentation and the addition of molasses. Among all treatments, the 5% molasses concentration with a fermentation period of 28 days consistently exhibited the highest antioxidant values, highlighting its potential as an optimal treatment condition.

The anti-nutritional factors of FCWP, specifically phytate and tannin content, were significantly influenced by varying concentrations of molasses and fermentation periods (Table 4). The phytate content of FCWP showed significant variation among treatments. The unfermented control (0% molasses, 0 days) had a phytate content of 0.063%, while the lowest phytate level (0.060%) was observed in the 0% molasses treatment after 14 days of fermentation. Overall, phytate levels remained relatively stable across treatments, ranging from 0.060 to 0.067%, suggesting minimal reductions due to fermentation or molasses addition. In contrast, the tannin content exhibited more pronounced changes across treatments. The highest tannin content (23.97 mg TAE/g) was recorded in the unfermented control, while the lowest tannin level (11.25 mg TAE/g) occurred in the 20% molasses treatment after 14 days of fermentation. Tannin content generally decreased with increasing fermentation time and molasses concentration. However, some treatments, such as the 15 and 20% molasses groups at 28 days, showed slightly higher tannin levels compared to their 14-day

Table 4. Anti-nutritional factors of FCWP fermented in different concentrations of molasses and different fermentation periods.

Treatment	Concentration of molasses (%)	Fermentation period (days)	Phytate (%)	Tannin (mg TAE/g)
1	0	0	0.063±0.00 ^b	23.97±0.03 ⁱ
2	0	14	0.060±0.00 ^a	15.20±0.17 ^e
3	0	28	0.062±0.00 ^{ab}	18.19±0.07 ^h
4	5	14	0.061±0.00 ^{ab}	12.30±0.17 ^b
5	5	28	0.061±0.00 ^{ab}	15.34±0.02 ^e
6	10	14	0.064±0.00 ^{bc}	13.94±0.02 ^c
7	10	28	0.062±0.00 ^{ab}	14.60±0.02 ^d
8	15	14	0.067±0.00 ^c	15.93±0.06 ^g
9	15	28	0.062±0.00 ^{ab}	12.41±0.03 ^b
10	20	14	0.067±0.00 ^c	11.25±0.06 ^a
11	20	28	0.067±0.00 ^c	15.67±0.02 ^f
<i>P</i> -value			<0.000	<0.000

Data are represented as mean ± SE. Means with different superscript letters within the same column are significantly different ($P<0.05$).

Table 5. Growth performance of performance Tilapia fed with FCWP-supplemented diets after 30 days.

Growth parameters	Control	with FCWP (3%)	p-value
Initial weight (g)	1.20±0.00	1.20±0.00	--
Final weight (g)	3.06±1.53 ^a	5.53±0.02 ^b	0.023
Weight gain (g)	2.26±1.13 ^a	4.47±0.14 ^b	0.026
SGR (%)	2.98±1.49 ^a	5.17±0.08 ^b	0.020
FCR	1.46±0.13 ^a	1.31±0.09 ^a	0.403
SR (%)	77.50±12.50 ^a	95.00±2.89 ^a	0.386

Data are represented as mean ± SE. Means with different superscript letters within the same column are significantly different ($P<0.05$).

counterparts. The results confirmed significant differences ($P<0.05$) in both phytate and tannin levels across the treatments, emphasizing the influence of fermentation conditions on the antinutritional factors of FCWP.

The feeding trial investigated the effects of including 3% FCWP in the diets of tilapia fingerlings over a 30-day culture period. The results showed that tilapia fed with FCWP-supplemented diet exhibited significantly improved growth performance compared to the control group (Table 5). Specifically, the fish in the FCWP group achieved a higher final weight and greater weight gain than the control, with both parameters showing statistical significance. The specific growth rate (SGR) was also significantly enhanced in the FCWP group, indicating faster growth over the experimental period. The feed conversion ratio (FCR) was lower in the FCWP group than in the control group, but did not differ significantly despite the improved growth, suggesting similar feed efficiency. The survival rate (SR) was numerically higher in the FCWP group than in the control group, though the difference was not statistically significant.

The water quality parameters of the culture water during the 30-day feeding trial demonstrated notable differences between the control and FCWP-supplemented groups (Table 6). Ammonia (NH_3) concentrations increased over time in both groups, with the FCWP group consistently exhibiting slightly higher levels, peaking at 2.31 ppm on Day 28 compared to 1.03 ppm in the control group. Phosphate (PO_4^{3-}) levels fluctuated across the study period, with the FCWP group generally maintaining higher values, reaching 0.59 ppm on Day 30, significantly above the control's 0.09 ppm. Nitrate (NO_3^-) levels were initially higher in the FCWP group during the early stages (Day 7 and Day 14) but were lower than the control at later stages, indicating a shift in nutrient dynamics. Nitrite (NO_2^-) concentrations remained low throughout the trial, with minor variations between the groups.

The study also assessed the microbial composition in tilapia's culture water and gut microbiota in a system with minimal water exchange. Both culture water and gut microbiota showed consistent trends observed in the control and the FCWP-supplemented

Table 6. Water quality parameters of the culture water of tilapia fed with FCWP-supplemented diets.

Parameters (ppm)	Day 7		Day 14		Day 21		Day 28	
	Control	with FCWP	Control	with FCWP	Control	with FCWP	Control	with FCWP
Ammonia	0.286±0.00	0.290±0.00	0.695±0.00	0.411±0.00	1.023±0.00	1.199±0.00	1.030±0.00 ^a	2.309±0.00 ^a
Phosphate	0.323±0.00	0.357±0.00	0.572±0.00	0.466±0.00	0.346±0.00	0.366±0.14	0.093±0.00 ^a	0.591±0.00 ^a
Nitrate	0.038±0.04	0.226±0.07	0.113±0.00	0.213±0.00	1.812±0.82	0.111±0.00	0.326±0.25 ^a	0.086±0.00 ^b
Nitrite	0.001±0.00	0.021±0.01	0.004±0.00	0.003±0.00	1.190±1.19	0.003±0.00	0.003±0.00 ^a	0.006±0.00 ^a

Data are represented as mean ± SE. Means with different superscript letters within the same column are significantly different ($P < 0.05$).

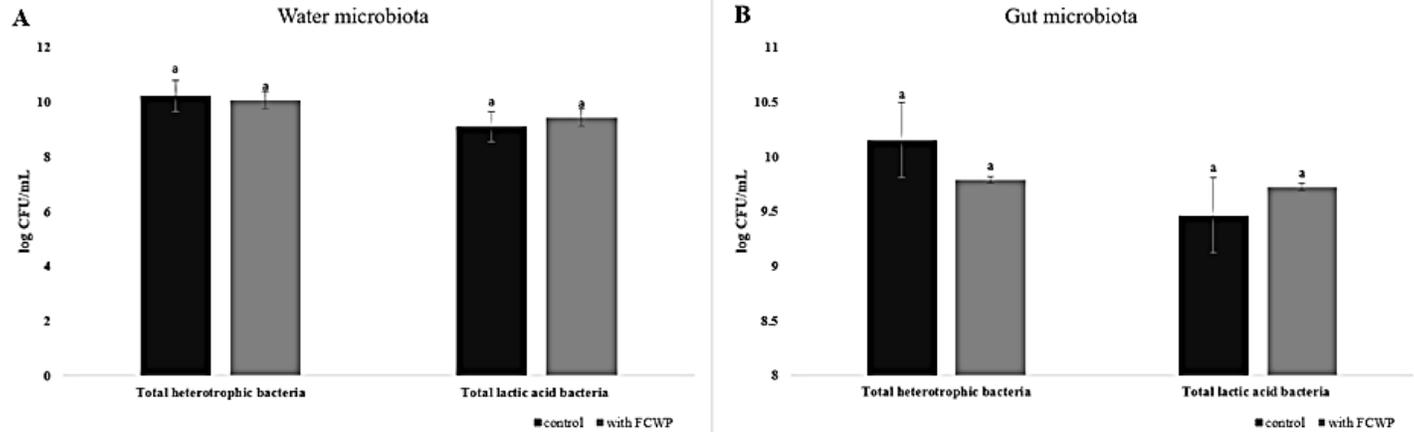


Figure 1. Microbial composition in culture water and gut microbiota.

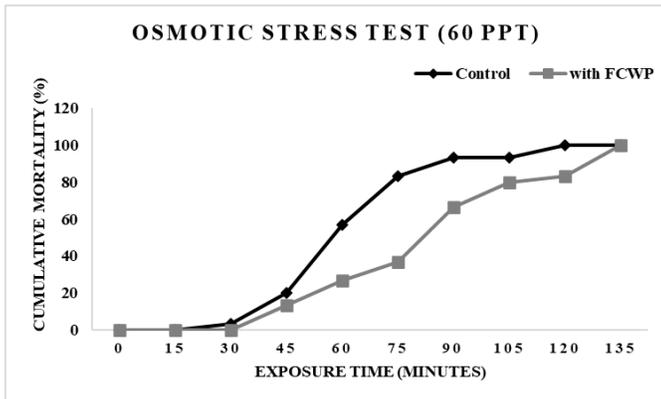


Figure 2. Cumulative mortality of tilapia fingerlings exposed to 60 ppt salinity.

groups for total heterotrophic and lactic acid bacteria. The microbial analysis of the culture water revealed no significant differences between the control and the FCWP-supplemented group for both microbial parameters (Fig. 1A). The control group had a mean count of 10.21 log CFU/ml for total heterotrophic bacteria, while the FCWP group showed a slightly lower mean of 10.05 log CFU/ml. Similarly, the FCWP group exhibited a slightly higher mean value of 9.42 log CFU/ml for total lactic acid bacteria compared to 9.10 log CFU/ml in the control group. The gut microbiota analysis also indicated no

significant differences between treatments for total heterotrophic bacteria, with the control group showing a mean value of 10.15 log CFU/ml and the FCWP group slightly lower at 9.79 log CFU/ml (Fig. 1B). For total lactic acid bacteria, the FCWP group exhibited a slightly higher mean (9.72 log CFU/ml) compared to the control (9.46 CFU/ml).

The study further examined the cumulative mortality of tilapia subjected to an osmotic stress test (60 ppt salinity) to evaluate the resilience of tilapia-fed diets with and without FCWP. Mortality was recorded at 15-minute intervals up to 135 minutes, providing a detailed temporal profile of stress response. In the control group (without FCWP), mortality began as early as 30 minutes (3.3%) and escalated rapidly, reaching 56.7% at 60 minutes, 93.3% at 90 minutes, and 100% at 120 minutes (Fig. 2). This rapid progression highlighted the susceptibility of tilapia to acute osmotic stress under standard feeding conditions. In contrast, the group fed FCWP-supplemented diets displayed significantly enhanced resilience. No mortality was observed until 45 minutes, indicating a delayed onset of stress-induced fatalities. At 60 minutes, cumulative mortality

in the FCWP group was markedly lower (26.7%) compared to the control group (56.7%). At 75 minutes, the FCWP group had significantly lower cumulative mortality at 36.7% compared to the control group at 83.3%. Even at 90 minutes, the FCWP group maintained a reduced cumulative mortality rate of 66.7%, substantially lower than the control's 93.3%. Mortality in the FCWP group ultimately reached 100% by 135 minutes, reflecting similar long-term outcomes as the control, but with a consistently better survival rate during the critical early exposure period.

Discussions

The global aquaculture industry faces increasing pressure to develop sustainable, cost-effective feed additives that enhance fish health and productivity. By-products such as citrus waste, particularly calamansi (*C. microcarpa*) peels, are abundant yet underutilized. Rich in bioactive compounds like antioxidants and fiber, these peels have potential as functional feed additives with immunostimulatory and prebiotic properties (Saleem et al., 2023). This study employed fermentation to enhance the nutritional and functional profile of calamansi waste, converting it into a value-added feed ingredient for aquaculture. The results in the proximate composition analysis of FCWP revealed several key components that are essential for fish nutrition. Protein is crucial for the growth and maintenance of fish, providing the necessary amino acids for tissue repair, enzyme function, and overall development (Ali et al., 2022). The protein content of FCWP ranged from 6.29 to 9.34%. The variation in protein levels across treatments may be attributed to the differing nutritional requirements and metabolic activities of the microbes, which are affected by the availability of carbon sources such as molasses. Nadar et al. (2024) highlighted that the type of fermentation process and the microbial strains utilized significantly influence protein content. Fiber plays a vital role in the digestive health of fish, aiding in the proper functioning of the gastrointestinal tract (Nafees et al., 2023). However, excessive fiber can reduce the digestibility of other nutrients. The fiber content ranged between 4.55 and

18.63%, decreasing significantly with fermentation. This trend is consistent with a study by Singhanian et al. (2013) on the fermentation of fruit and vegetable wastes, which found that fiber content often reduces due to microbial degradation during fermentation, dropping from initial values of around 20% to lower levels after fermentation. The significant reduction in fiber content through fermentation observed in this study enhances the digestibility of FCWP, making it more efficient as a feed additive.

Fat is a concentrated energy source, and essential fatty acids, which are important for fish's energy metabolism, immune function, and reproductive health (Kujoana et al., 2024). The fat content varied from 2.07 to 3.62%, which is within the typical range for fermented agricultural wastes, contributing to the energy density of fish diets. This is comparable to a study from Shyu et al. (2014), who reported fat contents of 1.8 to 1.9% in fermented citrus peels, and Yiğit et al. (2018), who noted a fat content of 1.28% in fermented soybean meal. Carbohydrates are an important energy source for fish, supporting their growth and activity (Krogdahl et al., 2005). The carbohydrate content ranged from 58.64 to 72.39%, exceeding values reported by Oboh et al. (2012) for fermented citrus peels (57.2-64.2%). The high carbohydrate content indicates that FCWP can serve as a significant energy source in fish feed, reducing the need for other carbohydrate-rich ingredients. These results emphasize FCWP's potential as a functional feed additive that improves digestibility and offers prebiotic benefits, addressing aquaculture's need for sustainable, eco-friendly solutions while enhancing fish health and performance.

Antioxidant properties are critical in aquaculture as they protect fish from oxidative stress, which can lead to various health issues, including compromised immunity and reduced growth rates (Marmelo et al., 2024). The antioxidant properties of FCWP, fermented under various molasses concentrations and fermentation periods, show promising results compared to similar studies. Notably, the best treatment was observed with 5% molasses and a 28-day fermentation period. The total phenolic content of

FCWP ranged from 4.46 to 12.34 mg GAE/g. This range aligns with findings from Mamy et al. (2022), who observed an increase in *Citrus reticulata* peel waste phenolic content from 10.77 to 18.31 mg GAE/g after a 192h fermentation period. Flavonoids, another group of antioxidants with anti-inflammatory properties, showed a total flavonoid content (TFC) ranging from 0.50 to 1.56 mg Catechin/g, with the highest levels also associated with 5% molasses and a 28-day fermentation period. These findings are comparable to those of Li et al. (2024), who found TFC in *C. reticulata* peel powder of 1.08 and 1.31 mg/g after fermentation. The presence of flavonoids in FCWP suggests that they could help improve fish's health and resilience, potentially reducing the need for synthetic antioxidants in fish feed. DPPH scavenging activity, a measure of the ability of antioxidants to neutralize free radicals (Baliyan et al., 2022; Gulcin and Alwasel, 2023), ranged from 5.68 to 41.57%, with the highest activity observed under the 5% molasses and 28-day fermentation period. This surpasses Li et al. (2024), who reported DPPH scavenging activity in fermented citrus peels from 3.31 to 3.87 $\mu\text{mol/g}$. The high DPPH activity in FCWP indicates its strong antioxidant potential, which can help mitigate oxidative stress in fish, promoting better health and growth.

Anti-nutritional factors, such as phytates and tannins, can interfere with nutrient absorption and reduce feed efficiency (Hamid et al., 2017; Samtiya et al., 2022; Singh et al., 2023). Therefore, it is crucial to minimize these components in fish feed. Phytate content showed a slight but significant reduction during fermentation. This modest reduction aligns with the findings of Sharma et al. (2020), who noted that phytate degradation is facilitated by microbial phytase enzymes during fermentation, although the extent of reduction depends on the fermentation conditions. Tannin content exhibited a more pronounced reduction, aligning with the findings of Sawant et al. (2025), who found that microbial fermentation significantly reduced tannin levels by breaking down tannin-protein complexes, thus improving the bioavailability of nutrients and their

utilization in feed formulations. The observed reduction in phytate and tannin levels highlights the effectiveness of fermentation in mitigating anti-nutritional factors, as also noted in the study of Anyiam et al. (2023), who observed that increasing fermentation time reduced phytate and tannin levels in *Macrotermes nigeriensis*-Cassava mahewu, leading to improved digestibility.

Fruit processing by-products, rich in bioactive compounds such as phenolics, terpenes, dietary fibers, and saponins, offer a sustainable solution for aquafeed development by promoting growth, health, and immunity in aquatic species (Dawood et al., 2022). Citrus waste, particularly peels and pulp, contains dietary fiber, antioxidants like flavonoids and vitamin C, and essential minerals, making it a valuable feed ingredient (Rafiq et al., 2018; Czech et al., 2020). Studies have shown that citrus waste enhances growth performance, feed efficiency, and immunity in fish, as evidenced by improved outcomes in species like Nile tilapia, Rainbow trout, and European sea bass (Acar et al., 2019; Chekani et al., 2021). In this study, the 30-day feeding trial demonstrated the significant benefits of supplementing tilapia fingerlings' diet with 3% FCWP, which notably improved growth performance, water quality, culture water and gut microbiota, and stress tolerance of tilapia fingerlings. These findings align with the growing body of research on the use of fermented plant- or fruit-based waste as feed additives in aquaculture, which has shown improvements in growth, gut health, and stress resilience due to their bioactive components and fermentation-induced improvements in nutrient bioavailability (Baba et al., 2016; Gonzales-Plasus et al., 2022; Sulaiman et al., 2022; Chotolli et al., 2023). The inclusion of FCWP significantly improved final weight, weight gain, and specific growth rate (SGR) of tilapia. These findings are consistent with those of Zhuo et al. (2021), who reported similar benefits of fermented lemon peel in Asian sea bass (*Lates calcarifer*), showing no adverse effects on growth and improved health markers. Zhuo et al. (2021) also observed enhanced lysozyme activity and increased disease resistance in orange-spotted grouper (*Epinephelus coioides*), further emphasizing

the benefits of fermented fruit waste in aquaculture. Zhang et al. (2023) found that fermented soybean meal enhanced the growth, serum biochemistry, digestion, antioxidation, immunity, and mTOR signaling pathway of juvenile Coho salmon (*Oncorhynchus kisutch*), with fermentation improving protein digestibility and reducing anti-nutritional factors. The superior performance of the FCWP-fed group compared to the control can be attributed to several factors. The reduction in anti-nutritional compounds minimized nutrient-binding effects, allowing for better protein utilization and growth. This aligns with the findings of Dawood and Koshio (2019), who highlighted that fermentation reduces anti-nutrients in plant-based materials, improving nutrient bioavailability in aquafeeds. Additionally, the elevated levels of phenolic and flavonoid compounds in FCWP acted as antioxidants, reducing oxidative stress and improving cellular function, further supporting better growth and health outcomes. The fermentation process also introduced beneficial microbial metabolites, promoting a balanced gut microbiome and enhancing digestion (Dawood and Koshio, 2019). Together, these improvements likely contributed to better growth, feed efficiency, and greater stress resilience in the FCWP-fed fish.

Water quality parameters, including ammonia, phosphates, nitrates, and nitrites, are vital in tilapia culture systems (Abd El-Hack et al., 2022). Ammonia (NH_3), a byproduct of fish metabolism, can reach toxic levels, harming fish health, reducing growth, and increasing mortality if unmanaged. Phosphates from feed and waste contribute to eutrophication, depleting oxygen and affecting fish performance. Nitrates (NO_3^-) and nitrites (NO_2^-) reflect nitrogen cycling; while nitrates are less harmful, elevated nitrite levels hinder oxygen transport in fish, causing stress and potential health issues (Verma et al., 2023). In the current study, the water quality analysis indicated no significant effect of FCWP supplementation on any of the measured parameters. While ammonia (NH_3) and phosphate (PO_4^-) levels were slightly higher in the FCWP-supplemented tanks, these differences were not statistically significant. Similarly, nitrate (NO_3^-)

levels showed a slight reduction, and nitrite (NO_2^-) levels exhibited a marginal increase in the FCWP group. Importantly, all parameters remained within the safe limits for tilapia as established by Boyd (2015). These suggest that the inclusion of FCWP in the diet does not compromise water quality in tilapia culture systems. The results further emphasize that FCWP supplementation maintains environmental safety and stability in the aquaculture setup, aligning with sustainable aquaculture practices.

The microbial analysis of the water column revealed no significant differences in total heterotrophic and lactic acid bacteria between control and FCWP-supplemented tanks under minimal water exchange. However, tanks with the control diet developed a foul odor, whereas those with FCWP did not, highlighting its potential in maintaining water quality. Feed additives like FCWP may enhance digestion and reduce waste accumulation, thereby limiting the production of foul-smelling compounds such as ammonia and hydrogen sulfide. The study also noted increased lactic acid bacteria levels in the gut of fish fed FCWP, consistent with its prebiotic effect. Bioactive compounds enhance nutrient absorption by stimulating amylase production, which aids in breaking down feed more effectively and reduces the amount of undigested material reaching the large intestine, thereby curbing the growth of harmful bacteria (Caipang et al., 2019). Similar mechanisms may explain the positive impact of FCWP on gut health and water quality, as bioactive compounds from fermented citrus peels could enhance digestion and reduce waste excretion, indirectly benefiting the surrounding microbial environment.

The enhanced stress tolerance observed in tilapia fed with FCWP, demonstrated by delayed onset and gradual progression during salinity exposure, aligns with the findings of Pedroso and Moquera (2023). Their study reported a significant improvement in the salinity tolerance of pompano larvae when their diets were enriched with *Schizochytrium*, highlighting the potential of functional dietary supplements to enhance resilience against environmental stressors. Similarly, research by Xu et al. (2020) examined the role of β -

glucan supplementation in the diet of Nile tilapia under hypersaline conditions. The study found that β -glucan enhanced growth performance, hematological parameters, immune gene expression, and modulated intestinal microbiota, thereby mitigating the negative impacts of hypersaline stress. These results indicate that β -glucan can effectively improve the health status of tilapia in challenging salinity environments. Additionally, Herrera et al. (2019) reviewed various dietary additives used in fish diets to mitigate stress responses. The review highlighted that supplements such as amino acids, fatty acids, and vitamins play crucial roles in enhancing fish resilience to environmental stressors, including salinity changes. These studies suggest that incorporating functional dietary supplements can bolster fish resilience against environmental stressors like salinity fluctuations. The observed benefits in tilapia fed FCWP may be attributed to similar mechanisms, where bioactive compounds in the supplement enhance stress response systems, leading to a delayed onset of mortality and performance under salinity stress.

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