Pakistan J. Zool., pp 1-10, 2023. **DOI**: https://dx.doi.org/10.17582/journal.pjz/20221216061205

Improved Growth Performance, Innate Immune Response, and Gut Microbes by Dietary Inclusion of Polyphenol Rich Agricultural Waste in Hybrid Tilapias

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Article Information
Received 16 December 2022
Revised 25 May 2023
Accepted 20 June 2023
Available online 09 September 2023
(early access)

Authors' Contribution
AK conceptualization, methodology, manuscript writing. RK resources, supervision. MAG project administration, supervision. CC resources, supervision, software, data analysis.

Key words
Rice husk, Sugarcane bagasse, Corn cobs, Phenolics, Growth performance, Gut microbes, Innate immune response, Red tilapia, Agricultural waste, Hybrid tilapia, *Oreochromis aureus, Oreochromis mossambicus*

ABSTRACT

Pathogen and stress-related morbidities are the main challenges in tilapia farming. Improving the immune health is an effective approach to protect tilapia against these pathogens. Phenolic content and antioxidant activity of rice husk (RH), sugarcane bagasse (SB), and corn cob (CC) evaluated by Folin-Ciocalteau assay and DPPH assay revealed high total phenolic content and antioxidant activity in RH compared to CC and SB. LCMS analysis of RH, SB, and CC exhibited the presence of hydroxybenzoic acid and hydroxycinnamic acid in agricultural waste samples. Effects of RH, SB, and CC on growth performance, gut microbial flora, innate immunity, and hematology of hybrid red tilapia (*Oreochromis aureus X Oreochromis mossambicus*) were evaluated in 91 days feeding trial with a test diet supplemented with 10% RH, SB, CC to basal diet (BD) ingredients in optimum conditions of growth. Growth performance data was recorded at an interval of 13 days. At the end of feeding trial, fish gut microflora, skin mucus bactericidal activity, blood serum lysozyme, and bactericidal activity against *Shigella sonnei*, and hematological parameters were evaluated. Significant difference in growth performance of red tilapia fed with experimental diets was observed. RGR (relative growth rate) was lowest in BD (380.79 ± 46.26) and observed highest in RH (554.01 ± 83.24). Lactobacillus count was increased in all experimental groups. Decrease in *Salmonella* sp., *Shigella* sp., and *E. coli* count was observed in CC. All experimental groups showed enhanced serum bactericidal activity against *Shigella sonnei*. SB group showed the highest serum bactericidal activity. Skin mucus bactericidal activity was also highest in SB. Least skin mucus bactericidal activity was presented by BD. Results of research work indicated that RH, SB, and CC have bioactive ingredients that positively influenced the growth performance, innate immune response, and hematology of red tilapia. Agricultural by-products are abundant biomass and rich in bioactive and nutraceutical compounds, therefore these are good candidates for maintaining redox balance, health status and growth performance in fish.

INTRODUCTION

Aquaculture is one of the fastest growing sectors in the global livestock industry. Tilapia, a large group of cichlid fish, is suitable for aquaculture due to its high adaptability, stock density, breeding capacity, disease resistance, and low production cost. As the second most farmed fish, tilapia accounts for approximately 40% of fish in aquaculture (Renuhadevi *et al*., 2019).

Pathogen and stress-related morbidities are the main challenges in tilapia farming. Pathogenic viruses and bacteria have led to massive loss in cultured fish farming including tilapia. For example, tilapia lake virus causes mortality by infecting the vital organs of tilapia, including liver and brain (Jansen *et al*., 2019) while *Streptococcus* infections decimate Nile and red tilapia at all growing

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0030-9923/2023/0001-0001 $ 9.00/0

Abbreviations
FCR, feed consumption ratio; RGR, Relative growth rate; DPPH, 2,2-diphenyl-1-picrylhydrazyl; LCMS, Liquid chromatography mass spectrometry; UHPLC-HR-TOFMS, Ultra-high performance liquid chromatography/high-resolution time of flight mass spectrometry

stages (Jantrakajorn *et al*., 2014). Improving the immune health is an effective approach to protect tilapia against these pathogens. Besides pathogens, acute and chronic stresses from diverse sources, including environmental conditions (temperature, pH, pollution), stocking density, malnutrition, transport, and handling, can inflict damages to tilapia (El-Khaldi, 2010). Therefore, improved antioxidant and immune systems provide better protection against these stresses.

Plant-derived phenolics, with their antibacterial, antioxidative, and anti-inflammatory activities (Russell and Duthie, 2011), are the good candidates for maintaining redox balance and immune functions. Many oil seeds, and vegetables in human and animal food chain are good sources of phenolic compounds but the extensive usage of these ingredients exploited for phenolic production could be very costly and could impact the feeding requirements of people in developing and under developing countries.

Agricultural by-products are abundant biomass with diverse nutritional values such as economic sources of protein and calories for animal production (Ajila *et al*., 2012). Many agricultural by-products such as RH, SB, and CC are also rich in bioactive and nutraceutical compounds including phenolics, carotenoids, and dietary fibers. However, the utilization of these agricultural by-products for tilapia production has not been well explored.

The aim of this study was to identify the best and least expensive sources of bioactive compounds and feed ingredients that serve as growth enhancers and immune stimulants and assist in solid waste management by recycling agricultural byproducts, considering current economic problems, disease outbreaks and environmental problems.

MATERIALS AND METHODS

Sample collection and its analysis

Rice husk (RH), corn cobs (CC), and sugarcane bagasse (SB) were collected from the local farming practices of Punjab, Pakistan. These byproducts were cleaned, air dried, and grinded in an electric mill prior to chemical analysis and feed preparation. These samples were analyzed for total phenolic content and antioxidant. The Folin-Ciocalteau method for total phenolic content was followed with little modification (Gutfinger, 1981).

Free radical scavenging capacity was evaluated with minor amendments in previously reported procedure (Ali *et al*., 2018). Powdered plant samples were extracted in pure methanol (1:10 w/v) and mixed with 0.1mM DPPH solution by 1:10 ratio (v/v). The reaction was conducted in the dark at ambient temperature for 30 min. The decrease in absorbance was recorded at 515 nm against a blank consisting of pure methanol and butylated hydroxytoluene (BHT) as positive control.

% Radical scavenging activity = [E (control)- E (sample)/ E (control)], where E is the extinction coefficient of DPPH

LC-MS analysis

Grounded and sieved agricultural waste samples were added to 50% Acetonitrile (0.01g/1ml) after vertexing for 2 min, the samples were sonicated for 10 min and centrifuged at 14000 rpm for 10 min. Supernatant was collected and diluted 100 times in 50% acetonitrile.

A 2 µL aliquot of the prepared sample was injected into an Acquity ultra performance liquid chromatography (UPLC) system (Waters, Milford, MA) and separated on a BEH C18 column (Waters, Milford, MA) by a gradient of mobile phase ranging from 0.1% formic acid in water to 0.1% formic acid in acetonitrile over a 10 min run. LC eluant was introduced into a quadrupole time-of-flight mass spectrometry system (Xevo G2S, QTOFMS system Waters) for accurate mass measurement and ion counting. Capillary voltage and cone voltage for electrospray ionization was maintained at 3 kV and 30 V for positive-mode detection, or at -3 kV and -35 V for negative-mode detection, respectively. Source temperature and desolvation temperature were set at 120°C and 350°C, respectively. Nitrogen was used as both cone gas (50 L/h) and desolvation gas (800 L/h), and argon as collision gas. For accurate mass measurement, the mass spectrometer was calibrated with sodium formate solution (range m/z 50–1,200) and monitored by the intermittent injection of the locked mass leucine enkephalin ($[M+H]^+$ = m/z 556.2771 and ($[M+H]^-$ = m/z 554.2615) in real time. Mass chromatograms and mass spectral data were acquired and processed by Mass Lynx software V4.2 (Waters, Milford, MA, USA) in centroided format. The chemical identities of interested compounds were determined by accurate mass measurement, elemental composition analysis, databased search using Metlin database (https://metlin.scripps.edu) and human metabolome database (https://hmdb.ca/) on May 22, 2022.

Feed preparation and feeding trial

Test diets were prepared by adding each powdered sample (RH, CC, SB) by 1:10 w/w of basal diet feed ingredients. The mixture was pelleted at ambient temperature, air dried, and stored at 4°C. Feed ingredients are listed in Table I.

One hundred and twenty healthy red tilapias (*Oreochromis aureus* x *Oreochromis mossambicus*) of average body weight 4.0g ± 1.5 g were collected from Fisheries Department of National Agricultural

Research Centre (NARC), Islamabad, Pakistan. They were acclimatized for 14 days and after acclimatization all fingerlings were divided into four groups with one replicate. All experimental and basal diet groups were fed twice a day by feed 3% of their body weight**.** For maintaining better water quality conditions in aquarium, 50% water of each aquarium was exchanged daily and the whole water was exchanged after every six days. The water quality parameters were monitored daily. Temperature was maintained between 26-30 °C, pH 7.5-8.1, dissolved oxygen 4.2-6.9 mgL^{-1}, and conductivity fluctuated between 0.361-0.557m.

Table I. Ingredients and nutrient composition of experimental diets (as-fed base).

Ingredients (g/Kg)	BD	RH	SB	CC
Fish meal	200	170	170	170
Soya bean meal	150	140	140	140
Sunflower meal	100	90	90	90
Canola seed meal	100	90	90	90
Rice polish	100	90	90	90
Gluten-30	100	90	90	90
Gluten-60	100	90	90	90
Wheat bran	100	90	90	90
Vitamin premix	25	25	25	25
DCP	20	20	20	20
Soya bean oil	5	5	5	5
Rice husk	----	100	----	---
Sugarcane bagasse	----	----	100	----
Corn cobs	----	----	----	100
Proximate composition				
Moisture (%)	7.55	6.76	7.40	6.95
Fats/Oil (%)	4.02	3.78	4.06	3.99
Crude fiber (%)	7.56	7.56	7.04	7.92
Crude protein (%)	33.85	33.21	32.59	32.41
Ash % (mineral matter)	13.28	12.43	13.26	12.53
TDN (%)	69.03	70.16	69.89	69.89
Metabolizable energy (Kcal/Kg)	2728.0	2756.4	2738.5	2741.5

BD, basal diet; RH, rice husk; CC, corn cobs; SB, sugar cane bagase.

Growth performance and survival rate of fish

Total number of fingerlings, their weight (g), and total length (cm) were recorded on day 1 and after every 13 days that continued till 91 days for evaluating total biomass, feed adjustment and growth performance.

Microbial analysis of gut

Fish was euthanized with rapid chilling in ice water and dissected to get intestinal content for microbial analysis under sterile conditions. Intestinal content from each treatment was diluted 1:10 w/v in sterile saline solution (0.9% NaCl), and 100 µl of each dilution was spread on the specific solid medium such as Salmonella–Shigella Agar (CM0099, Oxoid Ltd), Sorbitol MacConkey agar (CM0813, Oxoid Ltd**),** and MRS (CM0361, Oxoid Ltd) to check the load of *Salmonella* spp., *Enterobacter* sp., *Shigella* sp., *E. coli* and lactic acid bacteria, respectively. These cultures were incubated at 30 °C for lactic acid bacteria and 37 °C for others. After 48 h, the load of each bacterium was evaluated by enumeration method in the petri dishes and express in log $CFUg^{-1}$.

Bactericidal activity of skin mucus

Fish were starved for 24 h and bathed with 4 % potassium permanganate prior to mucus and blood collection at the end of the feeding trial. Mucus was collected by carefully scraping the fish on its dorsal side from anterior to posterior direction with a sterile spatula. The mucus samples were centrifuged at 5000 rpm for 5 min and the supernatant was collected and stored at 4 °C (Kumari *et al*., 2019).

Skin mucus bactericidal activity against *Shigella sonnei* was carried out by Kirby-Bauer-Disk method. *Shigella sonnei* 1T1 was adjusted to 1×10^{8} CFUml-1 using 0.5 McFarland standard and cultured on nutrient agar. Sterile discs were soaked in skin mucus and placed on culture plates and incubated at 35 °C for 24 h and growth inhibition zones were measured (Bragadeesw and Thangaraj, 2011).

Hematological parameters

Blood samples were collected from the lateral vein and one part of the blood was dropped into the EDTA tubes and proceeded for automatic hematology analyzer, and the second part of blood was dropped into non-EDTA tubes. Serum in non-EDTA tubes was separated by centrifugation at 4000 rpm for 15 min and proceeded further for serum bactericidal and lysosome activity.

The blood samples in EDTA tubes were used immediately for analysis with an automatic hematology analyzer (Beckman Coulter Ac-T, Germany). Complete blood count (CBC) such as red blood cells (RBC), white blood cells (WBC), platelets (PLT), hemoglobin (Hb), mean platelet volume (MPV), mean corpuscular hemoglobin (MCH), neutrophils, eosinophils, monocytes, lymphocytes were determined. Blood iron, glucose, alanine transaminase (ALT), C-reactive protein (CRP) levels were also analyzed.

Table II. Bioactive compounds in rice husks, corn cobs, and sugarcane bagasse and their tentative identification.

Peak ID	RT	m/z (-)	Metlin/ hmdb ID	Mass	ppm	Name	Formula	Detection mode	Detected in
1	0.52	153.0193	267460	154.0193	3	3,4 Dihydroxybenzoic acid	C7H6O4	[M-H]-	RH
2	1.86	121.0295	335656	122.0368	4	p-Hydroxybenzaldehyde	C7H6O2	[M-H]-	RH
3	1.98	194.0506	Hmdb 0240705	194.0579	2	Cis-Ferulic acid	C10H10O4	[M-H]-	CC
4	2.1	179.0350	265302	180.0423	3	Caffeic acid	C9H8O4	[M-H]-	RH
5	2.96	163.0401	354145	164.0473	3	p-Coumaric acid	C9H8O3	[M-H]-	RH, CC, SB
6	3.22	147.0452	386653	148.0524	3	Cinnamic acid	C9H8O2	[M-H]-	CC
7	3.58	563.1406	48783	564.1479	0	Apigenin 7-alpha-L-arabion-opyranosyl glucoside	C26H28O14	[M-H]-	RH, CC, SB
8	3.90	609.1461	HMDB 0003249	610.1534	2	Rutin	C27H30O16	[M-H]-	RH, CC, SB
9	4.24	491.1195	49289	492.1268	0	Tricin 5-glucoside	C23H24O12	[M-H]-	RH, CC,
10	4.44	312.1241	HMDB 0029365	313.1314	1	N-trans-Ferulotyramine	C18H19NO4	[M-H]-	RH, CC, SB
11	4.78	463.0882	50599	464.0955	0	Quercetin 3-glucoside (Quercetin derivatives)	C21H20O12	[M-H]-	CC

Serum bactericidal activity

1T1 of fresh pure culture of *shigella sonnei* was adjusted to 1×10^8 CFUml-1 using 0.5 McFarland standard. Bacterial culture and serum samples were mixed 1:1 ratio and incubated for 90 min at 35 °C, the mixture was diluted by 1:20 ratio with lysogeny broth, plated on LB agar and incubated for 24 h at 35 °C and viable colonies were counted. For negative control PBS was used (Kausar *et al*., 2020).

Serum lysozyme activity

Turbidimetric assay (Kausar *et al*., 2020) was used with some modifications to determine serum lysozyme activity. *Shigella sonnei* suspension 1T1 in 0.05 molL-1 PBS (pH 5.2) was added to the serum sample (1:1) in flat-bottomed 96-well microtiter plate and vortexed immediately. The optical density (OD) reading was taken at 450 nm using a spectrophotometer at start and after every 15 min interval for one hour. A unit of lysozyme activity was expressed as the decrease in absorbance of 0.001 min^{-1} by serum and bacterial samples. Hen egg-white lysozyme (Sigma, USA) was considered as standard.

Statistical analysis

Two-way ANOVA was done using GraphPad Prism version 8.0.2 (GraphPad Inc., La Jolla, CA, USA) to determine the statistical significance of growth performance among different treatment groups. Bonferroni's multiple comparison test was performed to see the difference among groups, significance level was considered when $P<0.05$.

RESULTS

Components of waste material

The results from Folin-Ciocalteau assay showed that RH extract had the highest phenolic content (2.913g in 100g of RH), followed by CC (1.896g in 100g of CC) and SB (1.315g in 100g of SB). Similarly, DPPH assay showed that RH extract had the highest radical scavenging activity (18.16 %) compared to CC (3.63 %) and SB (0.97 %).

The presence of phenolic compounds in RH, CC, and SB extracts was further examined by the LCMS analysis. The phenolic compounds and their conjugates analyzed belong to two families of phenolics i.e., hydroxybenzoic acids and hydroxycinnamic acids. Mainly p-coumaric acid, N-trans-ferulotyramine were detected in RH, CC, and SB. 3,4 dihydroxybenzoic acid, p-hydroxybenzaldehyde, p- coumaric acid, caffeic acid, and tricin 5-glucoside were characterized in RH extracts. Cis-ferulic acid, cinnamic acid, and tricin 5-glucoside were identified in CC extracts. Along with phenolic compounds some flavonoids such as quercetin, apigenin, and rutin were also identified (Table II, Fig. 1).

Effect of growth performance

Statistical analysis showed that fish fed with RH, SB and CC demonstrated no significant difference in RGR in the initial 26 days. After 39 days, only RH group showed

significant difference compared to BD group. Significant difference in RGR was revealed in all experimental groups on day 91. Total length increase percentage was significant from 39-91 days in RH and CC and non-significant in SB, however, the total length increase was significant in SB from days 26-91, in CC from days 39-91 and RH from days 52-91 (Figs. 2, 3). Fish in all experimental groups showed no mortality that shows fish adaptability to experimental feed. Fish weight gain in SB (23.56±14.63 g) was highest as compared to CC (22.78±8.64 g), RH (22.07±6.80 g) and (BD 16.03±4.08 g). Feed consumption ratio in RH, SB, CC, and BD was 1.06, 1.10, 1.06, and 1.13, respectively.

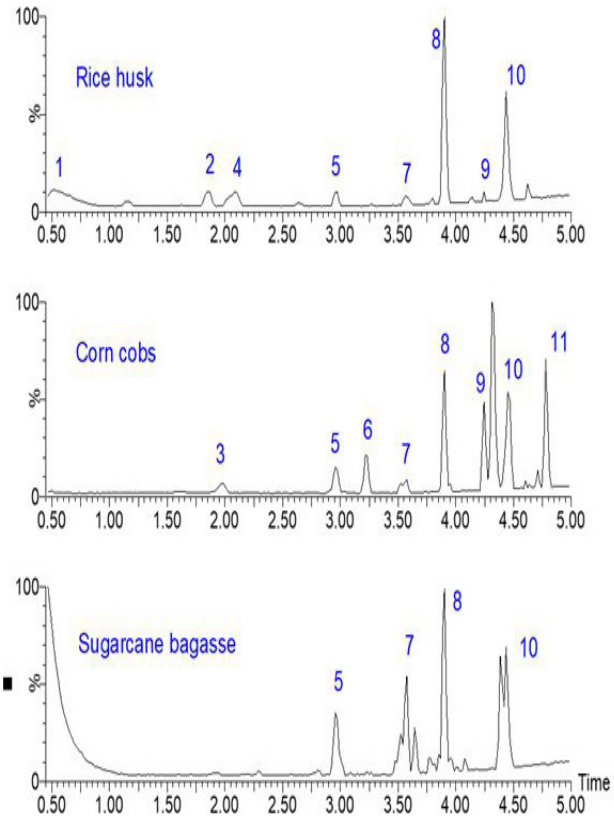

Fig. 1. Chromatographs of rice husk, corn cobs and sugar cane bagase showing peaks of phenolic compounds.

Effect on gut microbiota

Table III shows that the highest *Lactobacillus* loads were observed in SB (2.91×10^{12} /ml) followed by CC (2.81×10^{12} /g), RH (2.07×10^{12} /g) and BD (2×10^{12} /g). The increase in *Lactobacillus* load indicates the positive effect of test diet on these microbes. Reductions in loads of *E. coli*, *Salmonella* sp., *Shigella sonnei*, *Enterobacter* sp., were seen in all experimental groups that may be due to acidic pH and release of hydrolytic enzymes by gut beneficial microbes such as *Lactobacillus* sp.

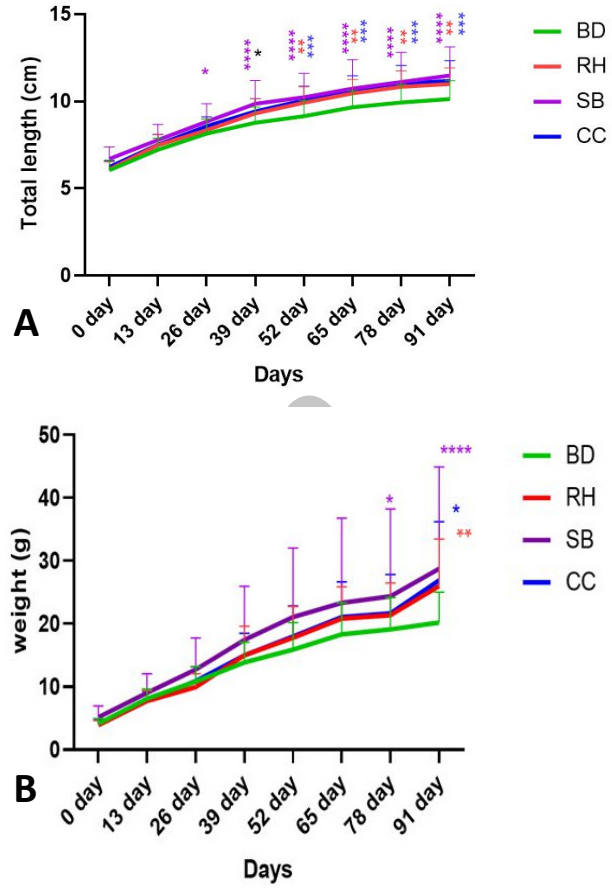

Fig. 2. A, Total length measured (cm) at an interval of 13 days. B, Weight measured (g) at an interval of 13 days. * = $p < 0.03$, ** = $p < 0.002$, *** = $p < 0.0002$, **** = $p < 0.0001$. For abbreviations see Table I.

Table III. CFU/g of microbes in experimental groups (RH, SB, CC) and Basal diet group (BD).

Microorganism	BD	RH	SB	CC
Lactobacillus sp.	12.29	12.30	12.45*	12.44*
E. coli	12.12	11.61*	11.84*	11.80*
Salmonella sp.	12.16	11.92*	12.01	11.85*
Shigella sp.	11.10	10.77*	11.11	10.36*
Enterobacter sp.	11.94	11.89	11.85	11.74*

Values are represented as mean Log10 formation. Asterisk in same row show significant difference at $p < 0.05$. For abbreviations see Table I.

Effect on innate immune response

Results of skin mucus bactericidal activity showed clear zones of inhibition of *Shigella sonnei* (4-6 mm) in SB, partial zones of inhibition (2-4 mm) were seen in

CC and (2-3 mm) RH. BD represented least bactericidal activity of skin mucus (1-2 mm).

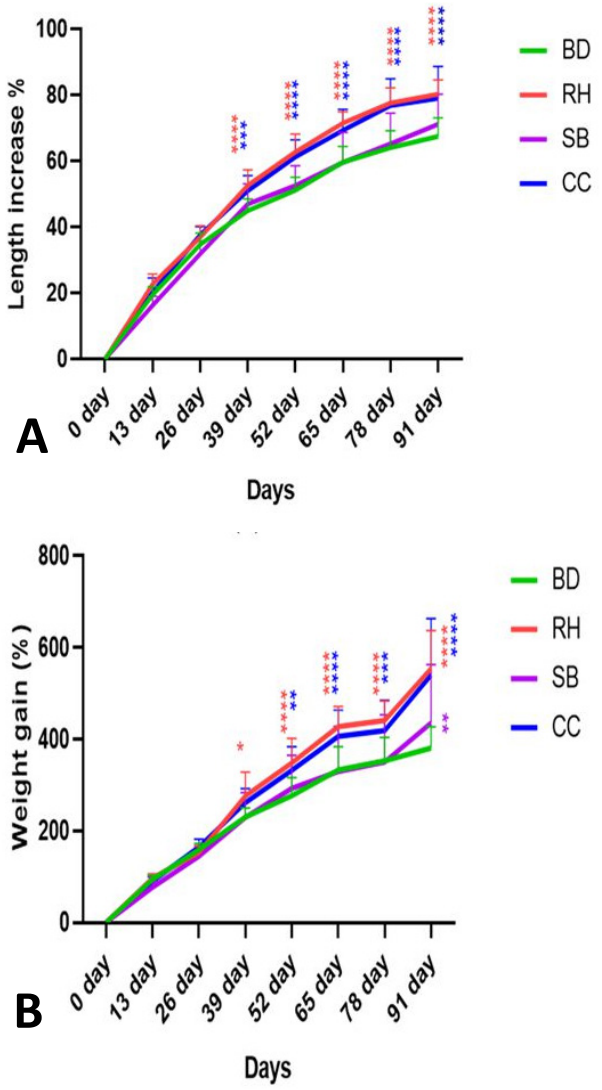

Fig. 3. A, Length increase % = Final length - Initial length/ Initial length × 100); B, Relative growth rate (RGR) = Final weight - Initial weight/Initial weight × 100 (Lugert *et al*., 2016) * = $p < 0.03$, ** = $p < 0.002$, *** = $p < 0.0002$, **** = $p < 0.0001$. For abbreviations see Table I.

Viable colonies of *Shigella sonnei* during serum bactericidal activity were 15 ± 3 in SB and CC which was the lowest count compared to other groups. The highest count of viable colonies was 90 ± 3 seen in BD, which represents its least bactericidal activity against *Shigella sonnei*. Viable colonies in group RH were 17 ± 2.

Turbidimetric assay results represented a decrease in OD values of groups RH, SB, CC, and BD which were 0.33, 0.42, 0.42, and 0.15, respectively. Decrease in OD values represents lysozyme activity. Lysozyme activity per minute in group RH, SB, CC, and BD was 5 Units ml^{-1}, 7 Units ml^{-1}, 7 Units ml^{-1}, and 2 Units ml^{-1}, respectively.

Effect on hematological parameters

Hematological parameters are presented in Table IV. Significant difference in WBC and platelets count was observed in RH, SB, and CC compared to BD. RBC count was significantly higher in CC and lower in SB. Significantly high Iron and alanine transaminase (Alt) concentrations were seen in BD. Some abnormal red blood cells (sickle shaped) were also observed in BD. Blood glucose level was significantly higher in RH and SB. All these values differ between groups, however, came within the normal range of blood parameters (Hrubec *et al*., 2000).

Table IV. Hematological parameters. Values are represented as mean values. Asterisk in the same row represent significant difference at $p < 0.05$.

Parameters	BD	RH	SB	CC
WBC (/µl)	3.93×10^7	4.61×10^{7}*	5.32×10^{7}*	4.45×10^7
RBC (/µl)	1.58×10^6	1.54×10^6	1.21×10^{6} *	1.87×10^{6}*
RDW (%)	28.1	24.7*	29.2	27.7
PLT (/µl)	1.75×10^4	2.79×10^{4}*	5.75×10^{4} *	6.48×10^{4}*
MCH (Pg)	44.90	47.36 *	49.6*	46.89*
MPV (Fl)	6.29	7.09*	6.11	6.23
Neutrophils (%)	31.30	44.80*	37.70*	50*
Lymphocyte (%)	53.5	49.40*	44.60*	45*
Monocyte (%)	4.9	3	6.4*	3.2
Eosinophils (%)	8	3*	4.9*	4.2*
Hb (g/dl)	7.1	7.46	6.1*	8.54*
Iron (mg/dl)	349	227.2*	243*	272.6*
ALT (IU/L)	76.8	46.6*	41.8*	47*
Glucose (mg/L)	67.3	85.80*	77.40*	72.80*
CRP (mg/L)	negative	negative	negative	Negative

WBC, white blood cells; RBC, red blood cells; RDW, red blood cells distribution width; PLT, Platelet, MCH, Mean corpuscular hemoglobin; MPV, Mean platelet volume; Hb, Hemoglobin; ALT, Alanine transaminase; CRP, C-reactive proteins. For abbreviations see Table I.

DISCUSSION

Increasing population day by day demands more food and at the same time combating with serious health and environmental issues is a huge challenge worldwide.

Large food production to feed huge population ends with billion tons of agricultural and food processing waste which makes this situation more daunting. There is dire need to explore the inner potential of agriculture and food wastes to reuse them. Agricultural wastes have valuable components and it is truly a waste to discard any of them (Lai *et al*., 2017).

Nutraceutical foods have medicinal effects, they are helpful in improving health and well-being, enhance immunity, and therefore useful in the treatment and prevention of specific diseases (Kumar *et al*., 2017). The plant bioactive components effects in multiple ways by improving feed consumption ratio, minimizing nitrogen excretion, and alteration in gut flora and overall health status of organisms (Alemayehu *et al*., 2018). In fish, dietary polyphenols or polyphenol- rich diet's beneficial effects on antioxidant defenses, disease resistance, immune response, reproductive and growth performance have been reported in many species such as black carp (Zhang *et al*., 2020), European sea bass (Magrone *et al*., 2019), common carp (Hoseinifar *et al*., 2020), convict cichlid (Hoseinifar *et al*., 2020), and beluga sturgeon (Safari *et al*., 2020). Corn husk meal (CHM), as a source of antioxidants, mainly ferulic acid and coumaric acid modulated the antioxidant response and prevented the damage elicited by hypoxia in Nile tilapia as reported earlier (Galeana-Lopez *et al*., 2021) Therefore, the improvement in growth performance and immune status of fish fed on RH, SB and CC in current study could be the effect of potential bioactive compounds present in these agricultural waste samples.

Oreochromis mossambicus fed with mixture of *Astragalus membranaceus*, *Codonopsis pilosula, Atractylodes macrocephala, Rheum palmatum, Isatis tinctoria, Scutellaria baicalensis,* and *Chrysanthemum indicum* showed enhanced phagocytic, superoxide dismutase, serum lysozyme and catalase activities and NBT-positive cell count in blood (Pu *et al*., 2017). Growth performance of yellow catfish fed with *Glycyrrhiza uralensis* extracts was significantly improved, with increased weight gain and specific growth rate but decreased feed consumption ratio as compared with fish fed with basal diet (Wang *et al*., 2020). The present findings correlate with the previous findings of Wang *et al*. (2020).

In Pakistan, fish production has increased many folds to fulfill fish demand, livelihood, and economy (Shah, 2018). In aquaculture, bacterial infections are a serious threat and cause considerable loss to economic production. Fish skin mucus acts as barrier and prevents fish from pathogens. It is a reservoir of antimicrobial components and innate antibacterial ability (Kumari *et al*., 2019). In the present study fish skin mucus bactericidal activity was enhanced in all experimental groups compared to Basal diet group. Lysozyme and complement protein molecules are constituents of fish immune system, acting non-selectively against bacteria and antigens. These components can be stimulated by feed additives (Paray *et al*., 2020). Increased in serum bactericidal activity and lysozyme activity was seen in all experimental groups compared to basal diet group. Lactic acid bacteria are well known for improving growth performance, regulating pathogens, and production of short chain fatty acids and lysozyme (Van Doan *et al*., 2018). Higher count of *Lactobacillus* sp. and improved growth performance in fish fed with sugarcane bagasse and corn cobs in the current study has well proved this fact.

In animal fitness evaluation, hematology is an appropriate observation which could discover changes provoked through illnesses or body physiology. The erythrocyte count and leucocytes profile can be altered by intrinsic or extrinsic elements like pathogen infections, water contaminants, and immunostimulant supply (Cavalcante *et al*., 2020). Significant differences ($p < 0.05$) in white blood count, platelets, iron, alanine transaminase, and glucose levels were observed between experimental group and basal diet group corelate with previous findings.

Nrf2 is a transcription factor that regulates redox homeostasis in fish, however, oxidative stress in fish prevents regulation of Nrf2 pathway and enhance the hepatic injuries induced by free radicals (Mohammadi *et al*., 2022). The antioxidant systems glutathione and thioredoxin, as well as the phase I and phase II detoxification of exogenous and endogenous products, NADPH regeneration, and heme metabolism, are all regulated by the transcription factor Nrf2. As a result, it serves as an important regulator of the cellular mechanisms for protecting against oxidative and xenobiotic stress. Nrf2 participates in several physiological activities besides antioxidant responses, including autophagy, and intermediate metabolism (Tonelli *et al*., 2018). Natural polyphenols not only play an important role in regulation of Nrf2 related pathway but they also inhibit Keap1-Nrf2 protein–protein interaction, degrade Keap1 and reduce production of reactive oxygen species (Zhou *et al*., 2019). Polyphenols in agriculture byproducts proved to have promising effects on growth performance and immune regulation in red tilapia.

CONCLUSION

Inclusion of RH, SB, and CC (10%) in hybrid red tilapia feed proved to have beneficial effects on its growth performance, gut microflora, innate immunity, and

hematology. Very few previous records of the inclusion of RH, SB, and CC in its crude form in fish feed have been recorded. Attention should be made to further explore the agricultural waste products for their beneficial dietary and health effects and their mode of action, and the journey from waste to wealth should never stop.

ACKNOWLEDGEMENTS

The authors acknowledge Wisdom Laboratory and Ahad Veterinary Clinical Laboratory for providing lab facilities.

Funding

COMSATS University Islamabad, Higher Education Commission, Pakistan, National Agriculture Research Centre, Islamabad, Pakistan and Department of Food Science and Nutrition, University of Minnesota provided essential funding for the work.

IRB and ethical approval

Feeding protocol was approved by Animal research ethics committee at ASI (Animal Sciences Institute) National Agriculture Research Centre, Islamabad, Pakistan (approval number 221003). Fish was euthanized with rapid chilling in ice water at the end of the feeding trial. No chemical anesthesia was applied during the study.

Data availability

All required data is provided with the manuscript.

Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20221216061205

Statement of conflict of interest

The authors have declared no conflict of interest.

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Pakistan J. Zool., pp 1-10, 2023. **DOI**: https://dx.doi.org/10.17582/journal.pjz/20221216061205

Supplementary Material

Improved Growth Performance, Innate Immune Response, and Gut Microbes by Dietary Inclusion of Polyphenol Rich Agricultural Waste in *Oreochromis aureus X Oreochromis mossambicus*

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Supplementary Table I

Chemicals and reagents	Vendor
Butylated hydroxytoluene (BHT),	Aldrich
Methanol LC-MS grade	Avantor performance materials (Radnor, PA)
Chloroform, Acetonitrile (MeCN), water	Fisher Scientific (Houston, TX)
Ethanol	Pharmaco-AAPER (Brookfield, CT)
2,2-diphenyl-1-pierylhydrazyl (DPPH),	ThermoFisher Scientific
Sodium carbonate, Folin-Ciocalteu reagent	Sigma
Formic acid	Fisher
p-Coumaric acid	Sigma

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0030-9923/2023/0001-0001 $ 9.00/0