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Effect of Urea Treated Sugarcane Bagasse on Growth, Proximate Composition, Microbial Flora and Digestive Enzymes Activities of Grass Carp (*Ctenopharyngodon idella*)

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Authors' Contribution
AK conducted the research trial and drafted the manuscript. NK and MF conceptualised the experimental design. NK did project administration and fund acquisition. MF and NK supervised the project. KJI, FR, MF, KMA, HA, AK, SN, SB, SA, MA and SS wrote, reviewed and edited the manuscript. SN and SB, MA assisted in sample analysis.

Key words
***C. idella*, Sugarcane bagasse, Body composition, *L. fermentum*, Digestive enzymes**

ABSTRACT

A 90 days experimental trial was conducted to evaluate the effect of urea treated sugarcane bagasse on the growth, gut microflora and digestive enzymes of grass carp (*Ctenopharyngodon idella*). Fish were fed to 3% wet body weight per day with experimental diets having 0% (CTRL), 0.7% (T1), 1.4% (T2) and 2.1% (T3) urea, with each group having two replicate tanks. Fish fed on 2.1% urea treated sugarcane bagasse (T3) showed significantly ($P<0.05$) higher growth as compared to T2, T1 and CTRL. The feed conversion ratio and specific growth rate were also significantly higher in T3 fish group, followed by the T2, T1 and CTRL groups. Proximate analysis of fish showed that the level of crude protein was higher significantly in T2 fish than other groups and crude fat level was significantly higher in T1 fish, followed the T2, CTRL and T3 fish groups. The percentage level of dry matter was significantly higher in T3 fish and ash percentage was significantly higher in T2 fish relative to T3, T1 and CTRL groups. Amylase and Lipase concentration was significantly higher in T2 fish compared with T3, T1 and CTRL groups. Protease concentration was significantly higher in T3 fish, followed by the T1, T2 and CTRL fish groups. Lastly, the presence of *Lactobacillus fermentum* was confirmed in the T1, T2 and T3 fish groups. Overall, the results showed that urea treated sugarcane bagasse can be used as a feed ingredient for *C. idella* and has no adverse effect on the nutritional value of fish.

INTRODUCTION

Fish are rich source of high-quality protein, fatty acids and various micronutrients (Tacon and Metian, 2013) and contribute a higher nutritional value than other basic foods like vegetables (Leroy and Frongillo, 2007). In addition, fish fat contains omega-3 fatty acids that are important for growth, cardiovascular activity and mental development in humans, particularly during the childhood and prenatal period and (FAO, 2003). The aquaculture sector plays an increasingly important role in providing fish for human consumption, with a current growth rate of 17% annually (FAO, 2018). Relative to world fish production, aquaculture provides 40.1% of fish for the table, and in 2010 produced 59 million tonnes of seafood. The estimated value of farmed fish is USD 130 billion.

Grass carp (*Ctenopharyngodon idella*) is an herbivorous exotic fish that was introduced into Pakistan

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to control aquatic weeds in lakes and reservoirs. They can eat more plant materials than their own body weight under suitable conditions, which constitutes 24% of their diet (Pipalova, 2006). Although a freshwater fish species native to China, they are now cultured in more than a hundred countries, mostly for food and controlling aquatic weeds or grasses in farms. Under natural conditions grass carp production plays an important role in aquaculture products around the world (Wu *et al*., 2012).

Artificial feed has a vital role to increase fish growth and production. However, the increasing prices of cereals and oilseed feed ingredients impact the cost of finfish feed and this needs to be addressed. One solution may be the inclusion of waste agro-industrial products such as sugarcane bagasse for herbivorous fish species. According to business records, sugarcane production in Pakistan was estimated at 67.17 million tons in 2018-2019.

Bagasse is produced by the sugar industry and can be used in aqua feed for herbivorous fish species. By utilizing the waste products of the sugarcane industry, like bagasse cane top and bagasse pith etc., the cost of feed can be reduced (Wang, 1986). Bagasse does not alter water quality standards but does increase the growth of zooplankton. Therefore, the substrate supply will be useful for developing microbial biofilms. Sugarcane bagasse is low in protein (< 3%) and rich in carbohydrates, such as cellulose (40%), hemicellulose (35%) and lignin (15%), and has a low digestibility of about 20-30% (Da Costa *et al*., 2015).

The protein content of sugarcane bagasse must be amplified by whole soybean meal and urea treatment when used as a feed since it can only provide a basal diet. Among all of the supplements, molasses and urea are widely used due to the low cost of urea and ready availability of molasses (Anandan and Sampath, 2012). Urea can also reduce the fiber content of sugarcane bagasse. Urea treatment of bagasse gives a high level of digestibility and microbial mass for feed (Gunun *et al*., 2017). The current study investigates the potential use of urea treated sugarcane bagasse as a feed ingredient for *C. idella*, specifically to examine its effect on fish growth, body composition, digestive enzyme (protease, amylase and lipase) activities and gut microbial flora (*Lactobacillus fermentum*).

MATERIALS AND METHODS

Experimental site and design

The experiment was conducted at Fish Hatchery, Department of Fisheries, Government of the Punjab in District Dera Ghazi Khan grass carp (*Ctenopharyngodon idella*) were used as experimental fish, having an average body weight of 30-50 g and stocked in cement tanks. Total body weight of selected fish samples was recorded before stocking. Fish were fed on daily basis with experimental diets 3% of wet body weight on daily basis (average CP 30.89%), containing 0% (CTRL), 0.7% (T1), 1.4% (T2) and 2.1% (T3) urea treated sugarcane bagasse, each having two replicate tanks.

Feed ingredients and formulation

Sugarcane bagasse was treated with urea ready for inclusion into the experimental feeds for fish. The ingredients of the feed included fish meal, canola meal, wheat flour, bagasse, minerals, vitamins premix, fish oil, molasses and urea, as outlined in Table I.

Table I. Formulation and composition of experimental feeds.

Ingredients	C	T1	T2	T3
Fish meal	35	35	35	35
Canola meal	20	20	20	20
Bagasse	25	25	25	25
Wheat flour	12	11.3	10.6	9.9
Urea	0	0.7	1.4	2.1
Fish oil	6	6	6	6
Vitamin premix*	1	1	1	1
Mineral mixture**	1	1	1	1
Total	100	100	100	100
Proximate composition				
Moisture contents (%)	11.26	12.15	11.9	13.77
Crude protein (%)	30.54	30.94	31.3	30.8
Crude fat (%)	4.35	4.75	5.1	4.6
Ash (%)	8.7	9.35	9.2	8.3
Crude fiber (%)	9.35	9.7	11.4	10.6
Starch (%)	27.45	27.8	29.5	30.2

*Each Kg of Vitamin premix contains: Vitamin A 15 M.I.U; Vitamin D3 3 M.I; Nicotinic acid 25000mg; Vitamin B1 5000 mg; Vitamin E 6000 IU; Vitamin B2 6000mg; Vitamin K3 4000 mg; Vitamin B6 4000 mg; Folic acid 750 mg; Vitamin B12 9000 mg; Vitamin C 15000mg; Calcium pantothenate 10000mg. **Each mineral mixture contains: $MnSO_4.5H_2O$ 116.67 mg/g; KH2PO4 479 mg/g; $MgSO_4.7H_2O$ 153 mg/g; $CoCl.6H_2O$ 0.0816 mg/g; $AlCl_3.6H_2O$ 0.255 mg/g; NaCl 51mg/g; $CuSO_4.5\ H_2O$ 210.67mg/g; $FeSO_4.H_2O$ 100.6mg/g; $CaCO_3$ 316 mg/g $ZnSO_4.7H_2O$ 121.33 mg/g.

Growth parameters

At the time of initial stocking the individual body weights of the fish were measured. Subsequently, every 15-days random samples of fish from all replicates were netted to record their total weight and total length for estimation of gain in these parameters. The fish were released back to their respective tanks after recording

the data. Growth parameters like net weight gain, feed conversion ratio (FCR) and specific growth rate (SGR) were calculated according to the following formulae:

Net weight gain: (NWG) = Final body weight (g) – Initial body weight (g)

Feed conversion ratio: (FCR) = Feed intake (g) / Net weight gain (g)

Specific growth rate: (SGR) %/day = ln(W1) - ln(W2) × 100 / Number of days

Where W1 is initial weight and W2 is final weight.

Proximate analysis

The formulated feed and fish samples were analyzed in the Fish Nutrition Laboratory, Department of Fisheries and Aquaculture, University of Veterinary and Animal Sciences, Ravi Campus, Pattoki, according to the Association of Analytical Chemist (AOAC, 2006).

Microbial analysis

Isolation of *L. fermentum* was done following the procedure of Ghanbari *et al*. (2009), after the fish samples were cleansed and disinfected. The molecular detection of *L. fermentum* was done by polymerase chain reaction (PCR) to confirm the species identity, according to the method of Dickson *et al*. (2005), with DNA isolated by the boiling method of Ahmed and Dablool (2017). For this purpose, the specific primers LF-1 (5´-AATACTGCAACTTTG-3´) and LF-2 (5´-GGTCAAATATCATCAACGTA-3´) to *L. fermentum* were used, that generate a 700 base pair product. The products of PCR were analyzed by gel electrophoresis and visualized on a Master video documentation system.

Digestive enzymes activity

After completion of the feeding trial, intestines were collected from fish in each replicate group. They were degutted and washed with fresh water, then homogenized using an electrical homogenizer and centrifuged for 15 min at 12,000 rpm. The supernatant was collected for assay of digestive enzymes. The casein digestion method was used to analyze the protease enzyme activity while, amylase enzyme activity was assayed by the Dinitro-salicylic acid (DNS) method and lipase activity was assayed by the titrimetric method of Thongprajukaew *et al*. (2010).

Statistical analysis

Data were analyzed by one-way ANOVA followed by Duncan's multiple range (DMR) test, to assess significant differences in growth, body composition and correlation matrix of physico-chemical parameters, using IBM SPSS statistical software.

RESULTS

Growth performance

The average initial body weight (g), final body weight (g) and net weight gain (g) of the C, T1, T2 and T3 groups are shown in Table II. The significant variations ($P<0.05$) were found in fish initial body weight. The average initial body weight was higher significantly in T1 fish comparatively to the C and T3 group. The final body weight was found different significantly ($P<0.05$) among the treatments. Comparatively, average final body weight was significantly higher in T3 fish.

Table II. Growth performance of grass carp (*C. idella*) fed urea treated sugarcane baggase based feed.

Parameters	C	T1	T2	T3
Initial weight (g)	32.40±1.27^{a}	35.95±1.20^{b}	33.45±1.06^{ab}	32.15±0.49^{a}
Final weight (g)	55.05±0.49^{b}	58.15±0.63^{a}	61.35±1.34^{a}	67.80±1.97^{c}
NWG[1](g)	20.30±0.47^{a}	22.20±0.80^{b}	27.90±1.09^{b}	35.65±1.26^{c}
FCR[2]	3.67^{a}	3.00^{ab}	2.98^{b}	2.80^{c}
SGR[3](%)	0.89^{a}	0.93^{ab}	1.06^{a}	1.21^{c}

*Different letters indicate significant differences ($P<0.05$) when different between treatment groups. [1]NWG, net weight gain; [2]FCR, feed conversion ratio; [3]SGR, specific growth rate.

Table III. Proximate composition of fish in the treatment groups fed sugarcane bagasse based feed.

Parameters	C	T1	T2	T3
Crude protein (%)	22.84±0.83^{a}	23.59±0.22^{c}	23.71±0.65^{bc}	23.67±0.43^{c}
Crude fat (%)	2.86±0.14^{ab}	3.76±0.20^{c}	3.11±0.17^{ab}	2.42±0.64^{a}
Dry matter (%)	23.22±1.88^{b}	19.43±1.12^{a}	21.33±1.76^{ab}	24.84±1.95^{c}
Ash (%)	1.55±0.03^{a}	1.20±0.08^{a}	2.52±0.06^{c}	2.12±0.14^{b}

Different letters indicate significant differences ($P<0.05$) when different between treatment groups.

Table IV. The average values of digestive enzyme activities of fish in the control and treated groups.

Enzymes	C	T1	T2	T3
Amylase (U/mg protein)	28.57±1.32[a]	27.69±1.07[a]	31.61±1.19[b]	29.84±1.78[ab]
Protease (U/mg protein)	19.16±0.18[a]	22.66±0.38[c]	20.52±0.38[b]	23.23±0.11[d]
Lipase (U/g protein)	0.79±0.06[a]	1.57±0.30[b]	2.64±0.42[c]	0.97±0.01[a]

Different letters indicated significant differences (P<0.05) when different between treatment groups.

The average net weight gain of fish also showed significant (P<0.05) differences among the treatment groups, with the average value of net weight gain significantly higher in T3 fish compared to T2, T1 and C fish, respectively. Similarly, the average FCR was comparatively better in T3 followed by T2, T1 than control. The SGR was significantly higher in T3 fish followed by the T2, T1 and C fish groups.

Proximate composition of fish

The average CP % was calculated and found to be significantly higher in the T2 group relative to all other groups (Table III). The average CF level was significantly higher in T1 fish, followed by the T2, C and T3 fish groups. The average percentage level of DM was significantly higher in T3 fish compared to the C, T2 and T1 fish groups. The average percentage level of ash was significantly higher in T2 fish relative to the T1, T3 and C groups, with T3 ash level also higher than T1 and C ash levels.

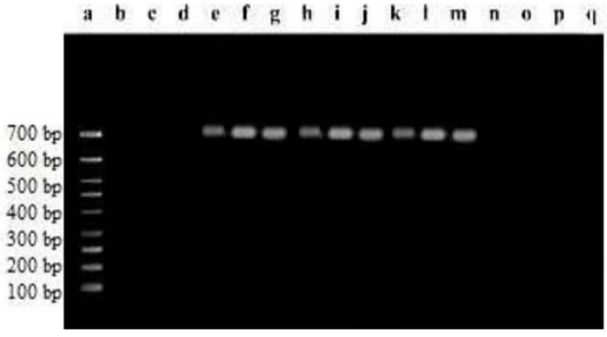

Fig. 1. *L. fermentum* was not found in *C. idella* C fish. However, the presence of *L. fermentum* was found in T1, T2 and T3 fish as evidenced by a 700 base pair PCR product. Lane (a) shows a base pair ladder; lanes (b-d) show the lack of PCR products from samples from the C group fish; lanes (e-g), (h-j) and (k-m) show the obtained PCR products from the T1, T2 and T3 fish groups, respectively.

Microbial analysis

The presence of *L. fermentum* in the gut of experimental fish was confirmed on extracted DNA by PCR, with products obtained shown in Figure 1.

Digestive enzymes activity

The average amylase activity was significantly higher in T2 fish relative to T1 fish and C fish, as shown in Table IV. However, the average protease activity was significantly higher in all of the treatment groups relative to the C fish, with T3 fish having the highest values, followed by T1 and then T2 fish. Lastly, the average lipase activity was significantly higher in treatment groups T2 and T1 relative to CTRL and T3 fish, with T2 fish activity also significantly higher than in T1 fish.

DISCUSSION

Healthy growth and production of *C. idella* was obtained by using urea treated sugarcane bagasse as a feed ingredient. Plant materials are commonly used in commercial fish feeds, as they improve the health status of the fish as well as enhancing food consumption proficiency and growth (Setiawati *et al*., 2016). However, digestibility of bagasse is known to increase when it is treated with urea (Gunun *et al*., 2017), which increases the level of microbial mass in the feed (Gunun *et al*., 2017). This likely accounts for the highest growth seen in fish fed with a high level of urea treated fermented sugarcane bagasse in the current study, where the average final body weight (67.80±1.97 g) and net weight gain (35.65±1.26 g) was significantly higher in T3 fish compared to other treatment groups. Similar results were reported by Hossain *et al.* (2020) who used Asian water grass as feed for *C. idella* with different levels of urea supplementation. The average FCR and SGR was also significantly higher in the T3 fish group.

A significant difference was observed in proximate composition of fish fed the varying treatment levels. The maximum average percentage of CP, 23.71±0.43 %, was found in T2 fish fed 1.4% urea treated sugarcane bagasse whilst the minimum average percentage of CP, 22.84±0.83 %, was found in the control group fed a sugarcane bagasse diet without urea treatment. Proteins make all of the structural components of the body (e.g., *C. idella*), are required for repair of damaged tissue and can be used as an energy source. Hence, such fish serve as a source of high-quality protein for the nourishment and growth of humans

(Tacon and Metian, 2013).

Crude fat% was also significantly higher in fish fed on urea treated bagasse relative to fish of the control group. This result indicates that urea influences the CF of fish if used to treat the feed. Fish fat contains omega-3 fatty acids that are important for normal growth, cardiovascular activity and mental development, particularly during the prenatal period and childhood (FAO, 2003) and so a higher CF level may be beneficial for the consumer. In contrast, the percentage value of ash and moisture content in fish of all treatments showed significant variation.

The study of microbial flora in *C. idella* fed the different diets showed well developed bacterial colonies on nutrient agar plates with samples from all of the groups. The presence of *L. fermentum* in the gastrointestinal tract of fish is known to regulate the gut and improve host health (Hoseinifar *et al*., 2019). The existence of *L. fermentum* was confirmed using extracted DNA from the colonies as template for PCR with specific primers for *L. fermentum*. No *L. fermentum* was identified in samples from the control group but was confirmed in T1, T2 and T3 samples. Microbial communities show competition in the middle and fore gut but have a joint relationship in the hind gut. The microbial communities of the fish gut similarly show competition and functional changes along the intestine, with interspecies interactions enhancing fermentation of microbiota in the intestine of grass carp (Yang *et al*., 2019).

Digestive enzyme activity in fish can increase with particular feed compositions (Wu *et al*., 2020). Hence, digestive enzymes were also examined in the present study, and their activity showed significant differences between the treatments. Amylase, protease and lipase activities were increased in fish given the urea treated sugarcane bagasse diets versus the control group. These feeds had a low lipid content with a high protein and carbohydrate percentage, explaining why the protease and amylase activities in *C. idella* were relatively high compared with the lipase activity. Feed is a most important factor in commercial aquaculture, and costs almost 60% of total farming expenses. Over the last ten years or so, efforts have been made for the development of cheap and easily available nutritious feeds to enhance the digestibility and digestive enzyme activities in cultured fish and shellfish (Hoseinifar *et al*., 2017). Aslam *et al*. (2018) studied intestinal enzyme activities using experimental feeds with duckweed and soybean meal in *C. idella* and silver carp (*Hypophthalmichthys molitrix*), and showed a significant impact on intestinal cellulose, protease and amylase enzymes in both species. Wu *et al*. (2020) used experimental feeds with different protein and fat levels and found higher protease and lipase enzyme activities in fish fed these diets relative to a control group. However, no previous experiments on grass carp have tested the effect of feeding urea treated sugarcane bagasse on intestinal enzyme activities. Thus, the present study serves as a baseline for future investigations aiming to develop better feeds for grass carp using constituents that can affect intestinal enzyme activities and growth.

CONCLUSIONS

Sugarcane bagasse is introduced as a new and low-cost ingredient for fish feed preparation. It is a palatable and preferable feed ingredient for herbivorous fish such as grass carp. The growth performance of grass carp, together with their gut micro flora and digestive enzyme activity showed significant effects, with no adverse changes in nutritional value and body composition of fish found.

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IRB approval

The experiment was conducted after the approval of the Ethical Review Committee of the University of Veterinary and Animal Sciences, Lahore.

Ethical statement

The fish were cultured and harvested according to the ethical guideline, i.e. the fish were anesthetized with MS-222 and samples were collected for different analysis.

Statement of conflict of interest

The authors have declared no conflict of interest.

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