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Impacts of Protein-, L-Tryptophan-, Carbohydrate-, Oil-Rich Diets on Growth Performance, Levels of Melatonin, Oxidative Stress, Antioxidative Agents, and Vital Digestive Enzymes in the Gut of Juvenile Carp (*Catla catla*)

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Abstract

The dietary protein, tryptophan, carbohydrate, and oil content of fish feed has many vital roles in the growth performances, stress management, and digestive physiology of fish. However, in this context, the functions of gut melatonin, which depends on the availability of food, timing of food supply, frequency of feeds/day, quality of food, and growth stages of carp, still need to be clarified. The present study aimed to investigate the impact of different experimental diets on growth performances, melatonin, oxidative stress and its essential antioxidants in the gut, and vital digestive enzymes of juvenile carp, *Catla catla* (mean body weight ~50g). The fish were fed any one of the seven diets viz. (i) a standard diet (SD/control) (with 34.99% protein, 14.56% carbohydrate, 9.84% oil, and 0.36% L-tryptophan) (ii) two protein (PRD1 with 41.02%, and PRD2 with 50.55% protein), (iii) two L-tryptophan (TrpRD1 with 0.96%, and TrpRD2 with 1.36% tryptophan), (iv) one carbohydrate (CRD with 24.62% carbohydrate), and (v) one oil (ORD with 14.68% oil) - rich diets for 30 days. Results indicated that the growth performance was better in PRDs, TrpRDs, and CRD compared to SD but not in ORD-fed carp. Further, PRDs and TrpRDs stimulated gut melatonin and suppressed oxidative stress by enhancing all the studied antioxidant levels. Upregulated digestive enzyme activities were also recorded after the PRDs and TrpRDs supply. However, CRD and ORD-fed groups exhibit less/no impact on most studied parameters, except digestive physiology. Nonetheless, the current study reports for the first time that PRDs and TrpRDs can modulate gut melatonin, oxidative stress, different antioxidants, and digestive efficacy.

Keywords: Digestive Efficacy, Fish Feeds, Gut Melatonin, Juvenile Carp, Oxidative Stress Management

1. Introduction

The environmental synchroniser of melatonin (5-methoxy-*N*-acetyltryptamine) within the gut tissue and the pineal organ/gland of a vertebrate is not identical[1-11]. Unlike the melatonin synthesised by vertebrates' pineal organs/glands, gut melatonin synthesis is not an environmental light-dark-dependent phenomenon[4,5]. However, the availability, timing, and frequency of feed(s) per day[4,5,9,12-16], and even the quality of food supplied[7,17,18] might influence the melatonin content within the gut, at least in fish species[1,19]. A recent study has demonstrated that gut melatonin levels may also vary with the different growth stages of carp, as varied feeding intensities were recorded in their different developmental stages[20]. In the current decade, research in gut melatonin is gaining attention because, in addition to its endocrine functions, the molecule is playing a significant role as an autocrine, or paracrine, or luminal effector, or detoxifying modulator[19] in the context of regular activities of digestion of food

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particles for its better nutritional assimilation by the animal.

The ingested food, until processed for absorption following digestion, can be considered a foreign particle for body physiology. The gut wall provides the first barrier following the activation of different mechanisms[21], of which the antioxidative defence mechanism provides a critical safeguard[22,23]. A significant relationship between gut melatonin, detoxifying enzymes, and vital digestive enzymes in fish has been noted during the past decades. A possible physiological interplay was reported among gut melatonin, antioxidative agents, and digestive enzymes to manage the stress developed either by the entry of the food particle within the gut lumen[1,16] or by maintaining the fish under feeding-fasting cycle[12,24] or by the introduction of pathogenic bacteria in the gut wall[15]. Further, a regular rhythmic feeding-fasting cycle is essential to maintain optimum metabolism and gut melatonin levels. The altered manifestation of gut melatonin titre, its biosynthesising enzymes, different core clock genes, and diverse appetite-regulating hormones (such as leptin, ghrelin, etc.) was reported by altering the feeding-fasting cycle[25,26].

Numerous studies have shown that 30% to 55% protein is required for optimum growth rate in fish depending on species, size, dietary protein sources, and environmental conditions[27]. Several studies are also available about the role of dietary carbohydrates[28-31] and lipids[32-35] on fish growth. Recent investigations demonstrate that proteins[36-39], carbohydrates[40,41], and lipids[42-44] content of feed have a role in oxidative stress and the digestive physiology of fish. Apart from protein, carbohydrate, and oil content of the diet, to date, the role of L-tryptophan (Trp) as a nutraceutical component of the diet in the modulation of melatonin synthesis in the gut, growth performance, oxidative stress, antioxidant enzymes, intestinal enzyme activities[45-48] in different animals have also been investigated, indicating that the Trp availability in food not only be one of the decisive factors of melatonin synthesis in the digestive tract but also can enhance growth, antioxidant capacity and digestibility. However, the response may vary with the amount of Trp administered[17,18,45,46,48-50]. However, from the perspective of gut melatonin in carp, apart from the Trp content of the diet, no study has been performed to understand the role of dietary protein, carbohydrate, and oil content in its regulation.

Reports on gut melatonin as a gut health indicator and its effect on oxidative stress and digestive enzymes are available in the literature[16,24]. However, the present study sounds unique when the role of gut melatonin in oxidative stress management and digestive functions comes under the context of seven different quality dietary groups. Different dietary criteria have been provided by altering the nutrient matters of the experimental diets to the juveniles of the fastest-growing Indian major carp (IMC), *Catla catla*, which achieves reproduction capability in just two years[20]. Moreover, being a tropical surface-dwelling carp, it also retains tremendous potential to act as the model organism for melatonin-related studies[8,9]. For this investigation, the early stage of juveniles has been selected due to its accelerated growth rate during this phase and easy maintenance (Figure 1).

2. Materials and Methods

2.1 Chemicals and Reagents

Melatonin ELISA kits were purchased from IBL International, Germany. Casein, dextrin, L-tryptophan (Trp), leupeptin hemisulphate, 2-thiobarbituric acid (TBA), and hydrogen peroxide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trichloroacetic acid (TCA), carboxymethyl cellulose (CMC), tyrosine, citric acid, nitro blue tetrazolium (NBT), nicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide phosphate (NADPH), reduced glutathione (GSH), 3,5-dinitro salicylic acid (DNSA), sodium hydroxide (NaOH), phenyl methyl sulphonyl fluoride (PMSF), ethylenediamine tetraacetic acid (EDTA), 5, 5'-dithiobis (2-nitrobenzoate) (DTNB), 5-methylphenazinium methyl sulfate (PMS), o-phenylenediamine (OPD), 1-chloro-2, 4-dinitrobenzene (CDNB), glutathione oxidized (GSSG), glycine, alanine, butylated hydroxyanisole (BHA) and all other chemicals and reagents of the highest commercially available purity were purchased from SRL (Mumbai, India).

2.2 Compositions of Diets

2.2.1 Standard Diet (SD)

Standard diet (SD) for control carp was prepared using 21% rice bran, 4.6% dextrin, 32% fish meal, 25% mustard oil cake, 4% soybean meal, 5% casein, 1.2% each sunflower and cod liver oils, 2% each multivitamin-mineral premix (OsMin, Biostadt India Ltd., Mumbai, India) and CMC, 0.995% each glycine and alanine and 0.01% butylated hydroxyanisole. This composition of SD is formulated

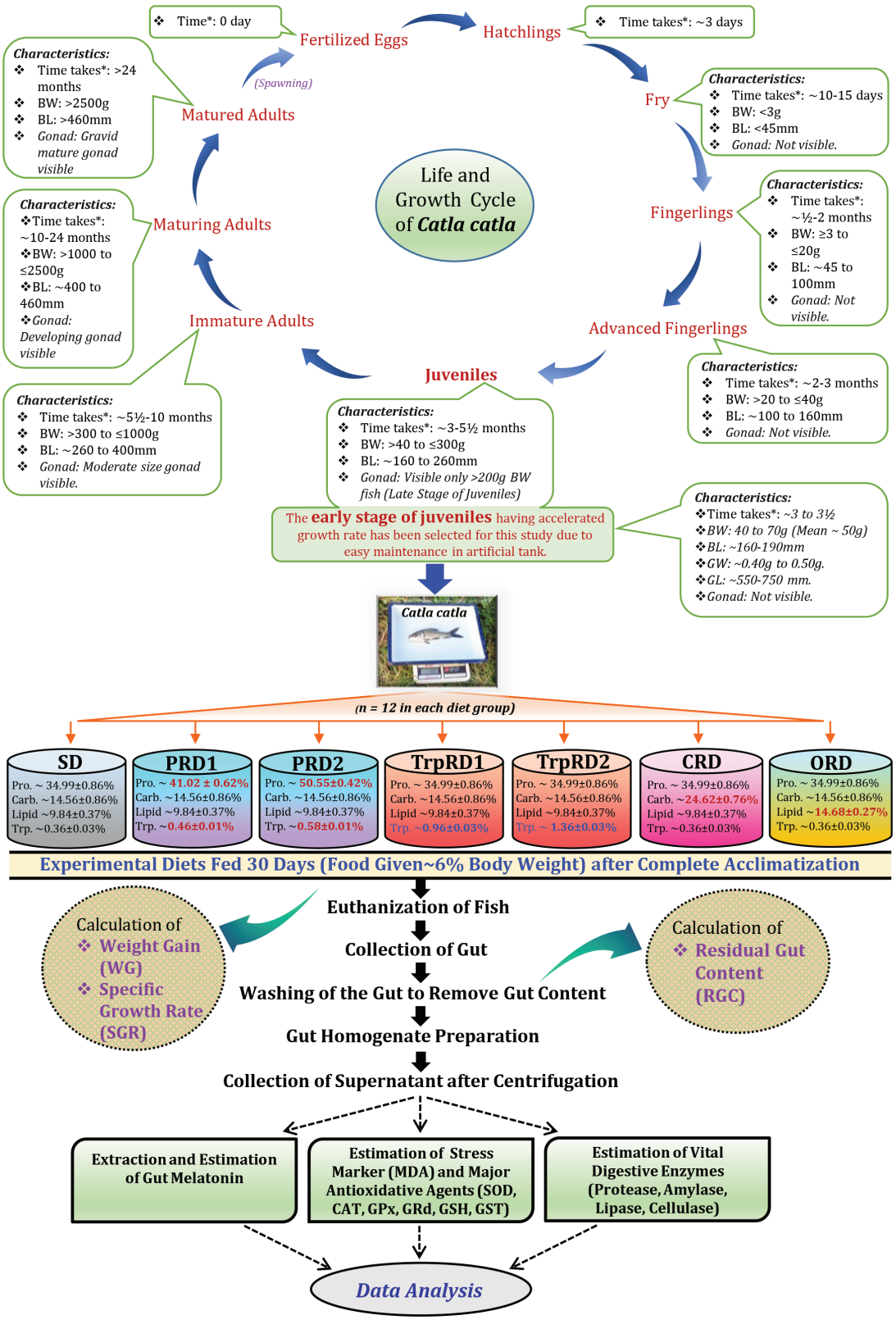

Figure 1. Life and growth cycle of the IMC, *Catla catla*, with key characteristics of different growth stages. The investigation was done with the early stage of juvenile carp, and the undertaken experimental workflow has been described. *The time required after hatching to reach the respective growth stages varies depending on various environmental factors.

with ~14.56±0.86% carbohydrate, ~34.99±0.86% protein, ~9.84±0.37% lipid substances, and ~0.36±0.03% Trp content (Figure 1).

2.2.2 Protein-Rich Diets (PRDs)

Two different laboratory-made protein-rich diets (PRDs) were used in this study, namely PRD1 (isocaloric with SD) and PRD2 (non-isocaloric with SD), which were prepared by enhancing the protein content significantly of the SD. In the case of PRD1, the protein content was increased to ~41.02 ± 0.62% from ~34.99±0.86% as in standard diet. While during the preparation of PRD2, the protein content was further uplifted to ~50.55±0.42%. The Trp contents were noted at ~0.46±0.01% in PRD1 and ~0.58±0.01% in PRD2, equivalent to 1.28 times and 1.61 times more Trp than in the SD (3.6 g/kg, expressed as a gram of total Trp per kg of dry feed), respectively (Figure 1). Other than the significant variations ($P < 0.001$) in the particular percentage of protein and Trp content either in PRD1 or in PRD2, and gross energy content only in PRD2, no such significant variations in terms of carbohydrate and lipid composition were noted between both the PRDs and SD used in this investigation.

2.2.3 L-Tryptophan-Rich Diets (TrpRDs)

For experimental purposes, two different laboratory-made L-tryptophan-rich diets (TrpRDs) (isocaloric) have been used in this study. Both the diets were prepared by enhancing only the Trp content of the SD with the addition of 6.00 g/kg and 10.00 g/kg (expressed as a gram of total Trp per kg of dry feed) of Trp for TrpRD1 and TrpRD2, respectively. TrpRD1 contained ~0.96±0.03% Trp, and TrpRD2 had ~1.36±0.03% Trp, equivalent to 2.67 times and 3.78 times more Trp than in the balanced diet (3.6 g/kg, expressed as a gram of total Trp per kg of dry feed), respectively (Figure 1). Apart from the amount of Trp, the diets used for the SD and TrpRDs-fed fish were identical in composition.

2.2.4 Carbohydrate-Rich Diet (CRD)

In comparison to the SD, one laboratory-made carbohydrate-rich diet (CRD) was prepared by enriching significantly ($P < 0.001$) the carbohydrate content of the diet ~24.62±0.76% (Figure 1). Other than the amount of carbohydrates used in the CRD, no significant changes in protein and lipid compositions and gross energy content of the ingredients used in SD and CRD were noted.

2.2.5 Oil-Rich Diet (ORD)

In comparison to the SD, one laboratory-made oil-rich diet (ORD) was prepared by elevating significantly ($P < 0.001$) the oil content ~14.68±0.27% (Figure 1). Other than the amount of oil used and gross energy retained in the ORD, no such significant changes were noted in terms of the protein and carbohydrate compositions of diets used in SD and ORD-fed fish.

2.3 Collection, Care, and Feeding of Juvenile Fish

252 healthy juvenile carp, *Catla catla* (Cyprinidae, Cypriniformes) with a mean body weight of ~50g, were captured in the early hours of the day with the help of local fishermen from the large water body of Fisheries Research and Training Centre (FRTC), Department of Zoology, North Bengal University (NBU), situated within the NBU Campus, Dist. Darjeeling, India (lat. 26°71' N, Long. 88°36' E), where they were reared under natural conditions. The live fish was quickly transported to the nearly located Aquaculture Unit of the Department of Zoology, NBU, and then randomly distributed in the circular cement tanks, each measuring 1.2 m (D) × 0.75 m (H), at a stocking density of 12 fish per tank with three replicates for each experimental diet for acclimatization to ambient photo-thermal and physicochemical conditions for two weeks. During the acclimatization period, fish were fed daily at ~10:00 h with an adequate amount equivalent to 6% body weight (BW) of the SD[4,5]. After complete acclimatization to the artificial environment, except for the SD group (control), the rest of the diet-fed groups were fed any altered quality diets, viz. PRD1, or PRD2, or TrpRD1, or TrpRD2, or CRD, or ORD by replacing the SD with the same delivery schedule for an additional 30 days of the experimental period (Figure 1). However, the control carps continued to feed the SD for the same daily duration and frequency. All the dietary experiments were performed from September to October (duration of mean natural photoperiod: ~12h 00min, sunrise approximately at 05:30 h; water temperature: max. 28.0°C, min. 27.0°C) corresponding to the autumnal equinox of the annual year. Throughout the culture period of study, each fish had free access to food for a sufficient duration, and no apparent changes in their

feeding and locomotor behaviour were noted between the fish held in separate tanks during the experimental period. Throughout the study, no mortality was recorded, and all the fish were found healthy and free from any apparent signs of injury or disease. No changes in lighting and other physical conditions, including temperature, dissolved O_2 and CO_2, and water pH, were noted among the different dietary fish tanks, eliminating the possible influences of these environmental components on the studied variables. Throughout the investigation, constant running and well-aerated water were supplied to ensure the hygienic conditions of the fish. Laboratory care of fish and adopted study schedules agreed with international standards and duly approved (No. IAEC/NBU/2022/19) by the Institutional Animal Ethics Committee (IAEC) (Reg. No.: 840/GO/Re/S/04/CPCSEA) of the Department of Zoology, University of North Bengal following the guidelines of CPCSEA, New Delhi and funding agency Council of Scientific and Industrial Research (CSIR) [37(1737)/19/EMR-II dated 19.07.2019], Govt. of India, New Delhi.

2.4 Collection and Preparation of Gut Samples

At the end of each experiment, 12 fish of each group from each replicate were caught using a hand net. Then, the fish were anaesthetized with MS-222 before being euthanized. Quick dissection was followed for the collection of gut tissues. Since melatonin levels do not differ with the anatomical segments of carp gut[4,5], the tissues from a particular region of the gut (~20 cm behind the oral aperture) corresponding to the small intestine were sampled at mid-day (12:00 h) when gut melatonin levels in carp are known to attain daily peak[4,5]. The required amount of gut tissue (~600 mg) for a single gut sample of each replicate was obtained from two individuals. On each occasion, the gut tissues were washed thoroughly in ice-cold phosphate buffer saline (PBS) to remove food/faecal matter, and 10% homogenate was prepared using the glass homogenizer with chilled 0.1 (M) phosphate buffer (pH 7), 0.1 mM PMSF and 10μM leupeptin hemisulfate. The supernatant was obtained after centrifugation at 8,000×g (4°C, 10 min) and was stored at -20°C until used for quantitative assay of gut melatonin, different antioxidative agents, and digestive enzymes (Figure 1). The protein content in the supernatant was determined by Bradford reagent using BSA as standard protein.

2.5 Evaluation of Growth Performance and Residual Gut Content

The following parameters were calculated according to the formulae[12,51]:

$$\text{Weight gain (WG, \%)} = \frac{\text{final weight} - \text{initial weight}}{\text{initial weight}} \times 100$$

$$\text{Specific growth rate (SGR, \% day}^{-1}\text{)} = \frac{\ln \text{final body weight} - \ln \text{initial body weight}}{\text{total number of experimental days}} \times 100$$

Residual gut content (RGC) = (weight of the intact whole gut containing the undigested food and faecal matter) - (the weight of washed gut tissue).

All the above parameters were calculated for each group with replicates.

2.6 Estimation of Gut Melatonin Concentrations

Melatonin concentration was quantified from the gut tissue extracts using the commercially available ELISA kit (Melatonin ELISA, IBL, Germany), following the manufacturer's instructions[20,52,53]. The absorbance was taken at 405 nm in a Bio-Rad iMark Micro Plate Absorbance Reader.

2.7 Estimation of the Levels of Stress Marker (Malondialdehyde/MDA)

The homogenates of gut tissue were centrifuged at 3000×g for 15 minutes, and the supernatants were used to measure the level of MDA by thiobarbituric acid reactive substances assay according to Draper and Hadley[54] with minor modifications. The method involved heating samples (1 mL) with TBA reagent (20% trichloroacetic acid, 0.5% TBA, and 2.5 N HCl; 2 mL) for 20 minutes in a boiling water bath. After cooling, the solution was centrifuged at 500×g for 10 minutes, and the precipitate was discarded. The spectrophotometric absorbance (Shimadzu UV-1900i) of the supernatant of samples was determined at 532 nm. The MDA equivalents of the sample were calculated using an extinction coefficient of 1.56×10^5/Mcm.

2.8 Estimation of the Levels of Antioxidative Agents

2.8.1 Superoxide Dismutase (SOD)

The SOD activity was measured using a microplate reader (Bio-Rad iMark Micro Plate Absorbance Reader)

to monitor the absorbance at 560 nm[55]. In brief, a 25 µL enzyme sample was pipetted into a microtiter well containing freshly prepared 200 µL of 50 mM phosphate buffer (pH 7.4) containing 0.1 mM EDTA, 62 µM NBT, and 98 µM NADH. The reaction was initiated by adding 25 µL of 33 µM PMS in 50 mM phosphate buffer containing 0.1 mM EDTA at pH 7.4. The assay was validated using serial dilutions of substrates (NADH and PMS). The test samples without substrates showed negligible changes in absorbance.

2.8.2 Catalase (CAT)

The activity of CAT in the gut tissue samples was estimated using a standard method[56]. Briefly, 40 µL of the enzyme sample was added and mixed quickly with H_2O_2 phosphate buffer (2 mM H_2O_2 in phosphate buffer) in a cuvette, and absorbance was recorded at 240 nm using a UV-Vis spectrophotometer (Shimadzu UV-1900i) up to 90s at 15s intervals. To validate the assay, the gut samples were treated with sodium azide, an inhibitor of CAT activity[57].

2.8.3 Glutathione Peroxidase (GPx)

The GPx activity was measured following a spectrophotometric method[58]. In brief, 1 mL OPD in phosphate citrate buffer (pH 5.0) was added to a 100 µL enzyme sample, then appropriately mixed with 0.9 mL 0.013% H_2O_2 and incubated at room temperature for 30 min. The absorbance was recorded at 492 nm (Shimadzu UV-1900i) against a blank (100 µL extra OPD solution instead of the sample). The enzymatic activity was expressed in units where one unit of enzyme activity increases absorbance (A492) by 1.0 under the standard assay conditions. Serial dilutions of OPD as the substrate of GPx were used to validate the assay.

2.8.4 Glutathione Reductase (GRd)

The GRd activity was determined by monitoring the glutathione-dependent oxidation of NADPH in a reaction mixture containing 950 µL of 0.15 mM NADPH, 0.5 mM glutathione, 3 mM $MgCl_2$ in 50 mM Tris (pH 7.5), and 50 µL extract, at 340 nm (Shimadzu UV-1900i)[59]. Corrections for NADPH oxidation were made in the absence of glutathione.

2.8.5 Glutathione S-Transferase (GST)

The GST activity was measured following a standard method[60]. GSH (2.4 mM/L) and CDNB (1 mM/L) were used as substrates. The absorbance was measured at 340 nm (Shimadzu UV-1900i) at a regular interval of the 60s for 5min. Enzymatic activity was expressed as U/mg protein. To validate the assay, serial dilutions of substrates (GSH and CDNB) were used to record their optimum concentrations for the maximum GST activity.

2.8.6 Reduced Glutathione (GSH)

The level of GSH was estimated following the method described by Ellman[61]. An equal volume (100 µL) of 5% perchloric acid was added to the gut homogenate supernatant and centrifuged at 800×g for 10 min at 4°C. The reaction mixture (2 mL) [containing supernatant (100 µL), 1.88 mL of 0.1 mol/L potassium phosphate buffer (pH 8.0), and 0.02 mL 4% DTNB] was incubated at room temperature for 3 min. Then absorbance was measured at 412 nm (Shimadzu UV-1900i) at a regular interval of the 60s for 5 min. The level of GSH in each sample was calculated by extrapolating the data from the standard graph prepared using GSH.

2.9 Estimation of the Activities of Vital Digestive Enzymes

2.9.1 α-Amylase Activity

The α-amylase activity in each gut sample was measured following the method described by Bernfeld[62]. Briefly, 1 mL of the enzyme extract (sample) was added to 1 mL of the substrate (1% soluble starch), and the mixture was incubated at 37°C for 20 min. Blank was prepared by replacing the sample with PBS. After that, 2 mL of DNSA reagent (containing 1% DNSA, 30% sodium potassium tartrate in 0.4 N NaOH solution) was added to each tube and kept in a boiling water bath for 5 min, after which the tube was cooled. The intensity of the colour developed was recorded at 560 nm using maltose as the standard. The activity was expressed as mg maltose liberated per mg protein per hour.

2.9.2 Protease Activity

The protease activity was estimated using the caseinase assay method[63], in which the substrate was prepared by dissolving casein (0.6%) in a Tris-HCl buffer (0.1 M, pH 7.8). The absorbance was recorded at 273 nm. One unit of enzyme activity in each sample was expressed as the amount of enzyme required to liberate 1 mg of tyrosine per mg protein per hour under standard assay conditions.

2.9.3 Lipase Activity

The lipase activity in the gut samples was measured following the standard method described by Bier[64], using olive oil as substrate. The enzyme activity was titrated with 0.01 M NaOH, where 1 mL of NaOH was equivalent to 100 µM of free fatty acid. Lipase activity was expressed as µM of fatty acid liberated per mg of protein per hour.

2.9.4 Cellulase Activity

The cellulase activity in each gut tissue sample was measured following the DNSA method[65], where the substrate was prepared using 1% CMC in sodium citrate buffer (0.1 M, pH 5.0). The intensity of colour in the reaction product was measured at 540 nm using glucose as the standard. One unit of cellulase activity was expressed as mg glucose liberated per mg of protein per hour.

2.10 Statistical Analysis

The means and the standard error of means (SEM; n = 6; average of three replicates in different tanks) of the data WG, SGR, gut melatonin concentration, RGC, levels of MDA, different antioxidative agents, and digestive enzymes were calculated. The Shapiro-Wilk normality test was performed to check the normal distribution of data sets. As the data sets passed the test ($p > 0.05$), the analysis of variance (ANOVA) was done. Each data set was used for simple (one-way) ANOVA considering the type of diets fed as the independent variable, where F values indicated significance, and means were compared by a post hoc Duncan's multiple range test (whenever applicable). The correlations were sought among gut melatonin, RGC, WG, and SGR following a simple correlation coefficient test. Further, the correlations were sought among gut melatonin, MDA, antioxidant agents, and digestive enzymes following the simple correlation coefficient test. Significance was always considered at the $p < 0.05$ level[66]. Statistical analysis and data presentation were carried out using MS Excel and SPSS.

3. Results

3.1 Growth Performance Parameters

The PRD1-fed group had the highest ($p < 0.001$) values of WG and SGR, while those parameters were also elevated significantly in PRD2, TrpRD1, TrpRD2, and CRD but not in ORD-fed fish in comparison with the SD-fed group (Table 1).

3.2 Residual Gut Content (RGC)

Compared to the standard diet, all experimental diets resulted in a noteworthy decrease in RGC values ($p < 0.001$) (Figure 2a)

3.3 Gut Melatonin Concentration

Compared to the SD, the PRD2-fed group showed the highest levels of gut melatonin ($p < 0.001$), followed by the PRD1, TrpRD1, and TrpRD2-fed groups. No significant differences were observed in the CRD and ORD-fed groups (Figure 2b).

3.4 Levels of Stress Marker

The supply of PRDs compared to SD caused a significant reduction ($p < 0.001$) in the gut MDA levels. A further drop in the gut MDA levels was noted when the fish fed with any of the TrpRDs. Notably, the supply of CRD and ORD exhibited no significant changes in the MDA levels compared to the SD-fed group (Figure 2c).

3.5 Levels of Antioxidative Agents

When compared with the SD-fed group, a significant increase ($p < 0.001$) in gut SOD activities was found following the supply of all of the experimental diets; e.g., the highest level of SOD activity was noted in TrpRD1, and TrpRD2-fed groups, followed by PRD1, PRD2, CRD, and ORD-fed groups (Figure 3a). The highest levels ($p < 0.001$) of CAT activities were found in the gut of the TrpRD1 and TrpRD2-fed group, followed by the PRD2, PRD1, CRD, and ORD compared to the values in the SD-fed group (Figure 3b). The highest levels of GPx were exhibited ($p < 0.001$) by the TrpRD1 and TrpRD2-fed group, moderate by PRD2 and PRD1-fed group, followed by CRD and ORD-fed group, compared to the SD-fed group (Figure 3c). Significantly higher GRd levels ($p < 0.001$) were noted in the PRD2-fed group, followed by the PRD1, TrpRD1, and TrpRD2-fed group compared to the SD/CRD/ORD-fed group (Figure 3d). The highest level ($p < 0.001$) of GST was found in the gut of TrpRD1 and TrpRD2-fed groups, moderate in PRD2 and PRD1-fed groups, followed by CRD and ORD-fed groups, compared to SD-fed group (Figure 3e). Compared to the SD-fed group, the highest amount of GSH ($p < 0.001$) was found in the gut of CRD, PRD2, and PRD1-fed groups and moderate in the gut of TrpRD1 and TrpRD2-fed groups. The ORD-fed group

Table 1. Data of growth performances (mean ± SE; n = 6) in juvenile carp, *Catla catla,* which were regularly fed a Standard Diet, followed by either Protein-Rich Diets (PRD1 and PRD2), or Tryptophan-Rich Diets (TrpRD1 and TrpRD2), or Carbohydrate-Rich Diets (CRD), Oil-Rich Diets (ORD) at ~ 10:00 h in the morning for 30 days.

Growth Performances Parameters	Experimental Diets						
	SD	PRD1	PRD2	TrpRD1	TrpRD2	CRD	ORD
Weight gain (%)	10.86 ± 1.20^{c}	63.30 ± 8.23^{a}	20.42 ± 3.24^{b}	22.06 ± 4.31^{b}	25.41 ± 3.06^{b}	26.31 ± 2.12^{b}	8.00 ± 1.70^{c}
Specific Growth Rate (% day^{-1})	0.34 ± 0.04^{c}	1.61 ± 0.17^{a}	0.61 ± 0.09^{b}	0.65 ± 0.12^{b}	0.75 ± 0.08^{b}	0.78 ± 0.06^{b}	0.25 ± 0.05^{c}

Different letters in the same row indicate significant (p<0.05) differences between different laboratory-made diets.

does not exhibit any variation in the gut GSH levels from the SD-fed group (Figure 3f).

3.6 Activity of Vital Digestive Enzymes

Significantly higher α-amylase activity (p < 0.001) was noted in the CRD-fed group, followed by the PRD1/PRD2/TrpRD1/TrpRD2-fed group compared to the SD and ORD-fed group (Figure 4a). Significantly higher (p < 0.001) protease activity was noted in the PRD2-fed group, followed by the PRD1/TrpRD1/TrpRD2-fed group compared to the SD/CRD/ORD-fed group (Figure 4b). Significantly higher (p < 0.001) lipase activity was noted in the ORD-fed group, followed by PRD1/PRD2/TrpRD1/ TrpRD2-fed group compared to the SD and CRD-fed group (Figure 4c). Significantly higher cellulase activity (p < 0.001) was noted in the PRD1, PRD2, CRD, and ORD-fed group, followed by the TrpRD1 and TrpRD2-fed group compared to the SD-fed group (Figure 4d).

3.7 Correlation among the Gut Melatonin Concentrations, RGC, WG, and SGR of Fish

When correlating the values after the supply of different diets (Table 2), gut melatonin showed a significant positive correlation with WG and SGR, but no correlation with RGC.

3.8 Correlation among the Gut Melatonin Concentrations and the Activity of Enzymatic and Non-enzymatic Antioxidative Agents and Intra-Cellular Stress Marker

Gut melatonin levels exhibited a significant positive correlation with the activities/levels of SOD, CAT, GPx, GST, GSH, and GRd. However, the levels of MDA displayed a significant negative correlation with the levels of gut melatonin and all the antioxidative agents except the levels of GSH (Table 3).

3.9 Correlation between Gut Melatonin Concentrations and the Activity of Digestive Enzymes

Gut melatonin levels exhibited a significant positive correlation with the activities/levels of protease and cellulase but no correlation with α-amylase and lipase (Table 4).

4. Discussion

By introducing better quality food, such as PRDs, TrpRDs, CRD, and ORD, growth performances, gut melatonin levels, antioxidative agents, and digestion enzymes are affected differently. In our study, the WG and SGR were significantly higher in PRD1 compared to SD. WG and SGR reduced significantly in PRD2 compared to PRD1. After the supply of a higher rate of protein in the diet compared to the standard range, evidence of retarded or inhibited growth was also noted not only in carnivorous[67] but also in herbivorous fish[37,68]. SGR was increased in the TrpRD1 and TrpRD2-fed group compared to SD-fed carp. The optimal dietary Trp level could modulate the growth performance and feed efficiency, as reported earlier in several fish species[69-74].

After supplying any PRDs and TrpRDs, a significant reduction in the RGC was recorded compared to the SD. According to a recent report, amino acid sensing receptors can sense the aromatic amino acid, like Trp[75]. This increase in Trp levels in the diets may increase the cholecystokinin (CCK) group in the gut, as found in rainbow trout (*Oncorhynchus mykiss*)[75], hence decreasing the hunger level by reaching early satiety. The supply of CRD and ORD showed lower values in RGC. These

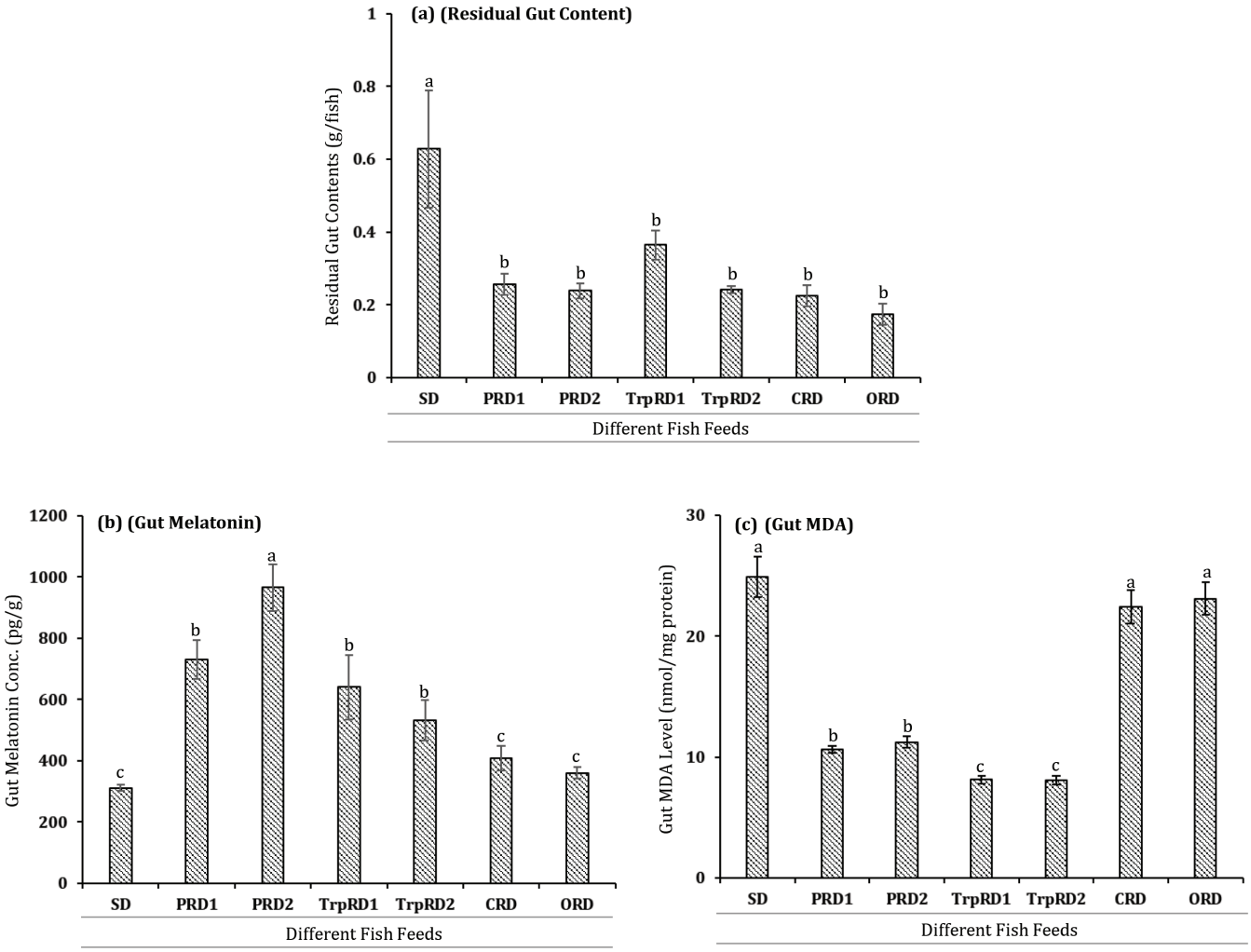

Figure 2. Graphical presentation of the data (mean ± SE shown in vertical bars; n = 6) on **(a)** amount of residual gut content (RGC), **(b)** concentrations of gut melatonin, and **(c)** malondialdehyde (MDA) in juvenile carps, which were supplied with standard diet (SD), or protein-rich diet 1 (PRD1), protein-rich diet 2 (PRD2), or Trp-rich diet 1 (TrpRD1), or Trp-rich diet 2 (TrpRD2), or carbohydrate-rich diet (CRD), or oil-rich diet (ORD) at ~10:00 h for 30 days. Different letters indicate significant (p<0.05) differences between different laboratory-made diets.

data indicate that when the quality of fish feed increases employing CRD[76] or ORD[77], the required amount of energy can be absorbed by the gut from the small amount of fish feed. Either CRD or ORD increases the respective enzymes for better digestion, but higher nutritional quality of the diets may delay the gastric emptying for more extended assimilation and suppress the hunger for a longer duration. Hence, satiety reaches speedily[78].

The fish exhibited different responses on the levels of gut melatonin when modulated by dietary ingredients. In the present study, the highest levels of gut melatonin were noted after the supply of PRD2, not in TrpRD2. This indicates that the carp gut may have different rates of AANAT and ASMT enzyme expression[79]/activity[80,81] or serotonin levels for melatonin synthesis. If the serotonin synthesis in the TrpRD2-fed group is higher, that may cause reduced Food Transmit Time (FTT)[82]. The reduced level of RGC in carp supports the finding. Further investigation of the AANAT and ASMT enzyme activity[80,81] and the measurement of serotonin levels in the different diet-fed groups in the future may solve the conjecture. The supply of CRD and ORD did not exhibit any significant changes in melatonin levels.

In our study, the level of different antioxidative enzymes (SOD, CAT, GPx, GST, and GRd) and a non-enzymatic antioxidative agent (GSH) in the gut have been significantly increased. However, the level of MDA, a

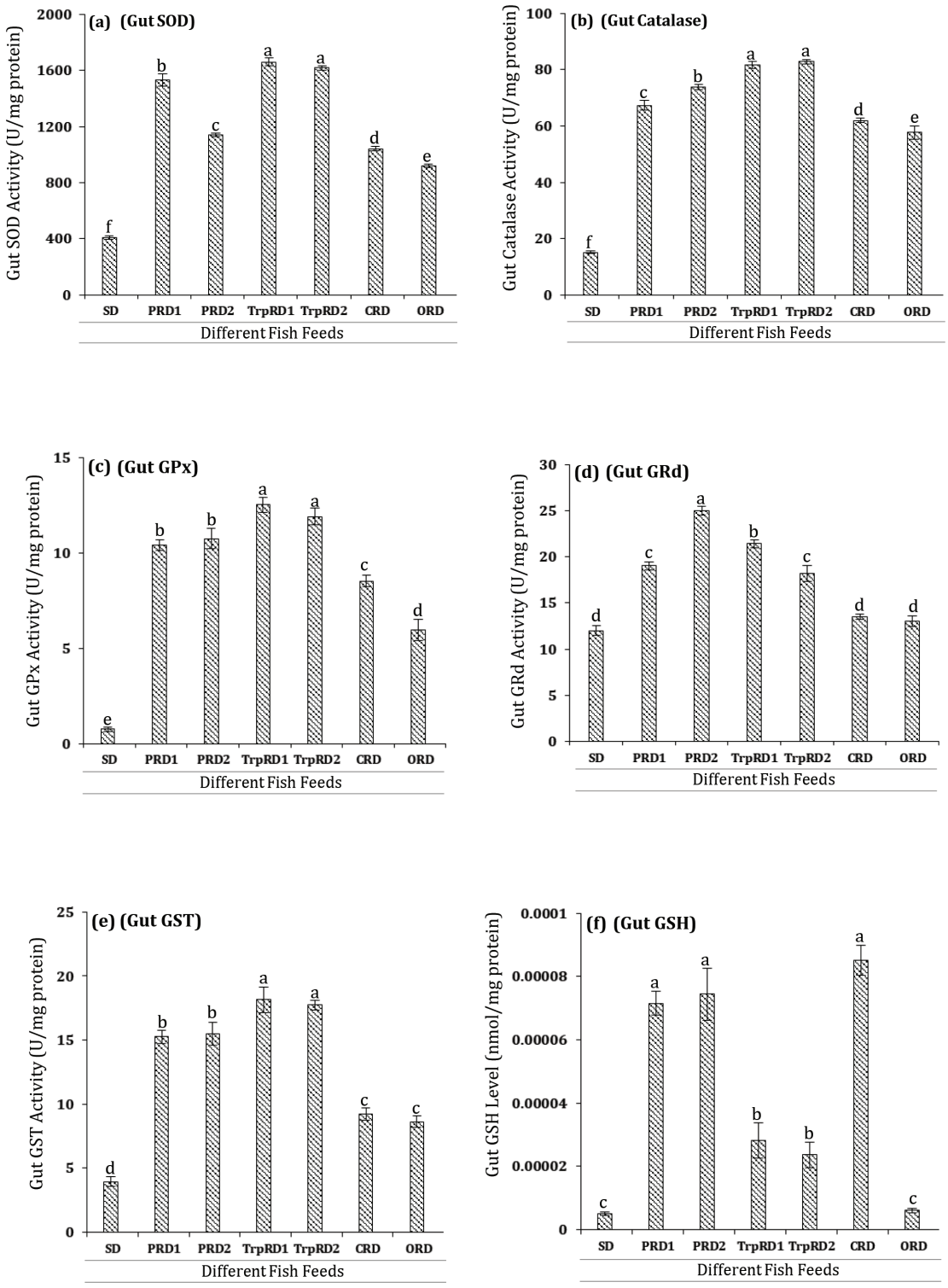

Figure 3. Graphical presentation of the data (mean ± SE shown in vertical bars; n = 6) on **(a)** superoxide dismutase (SOD), **(b)** catalase (CAT), **(c)** glutathione peroxidase (GPx), **(d)** glutathione reductase (GRd), **(e)** glutathione *S*-transferase (GST), **(f)** reduced glutathione (GSH) in juvenile carps, which were supplied with standard diet (SD), or protein-rich diet 1 (PRD1), protein-rich diet 2 (PRD2), or Trp-rich diet 1 (TrpRD1), or Trp-rich diet 2 (TrpRD2), or carbohydrate-rich diet (CRD), or oil-rich diet (ORD) at ~10:00 h for 30 days. Different letters indicate significant (p<0.05) differences between different laboratory-made diets.

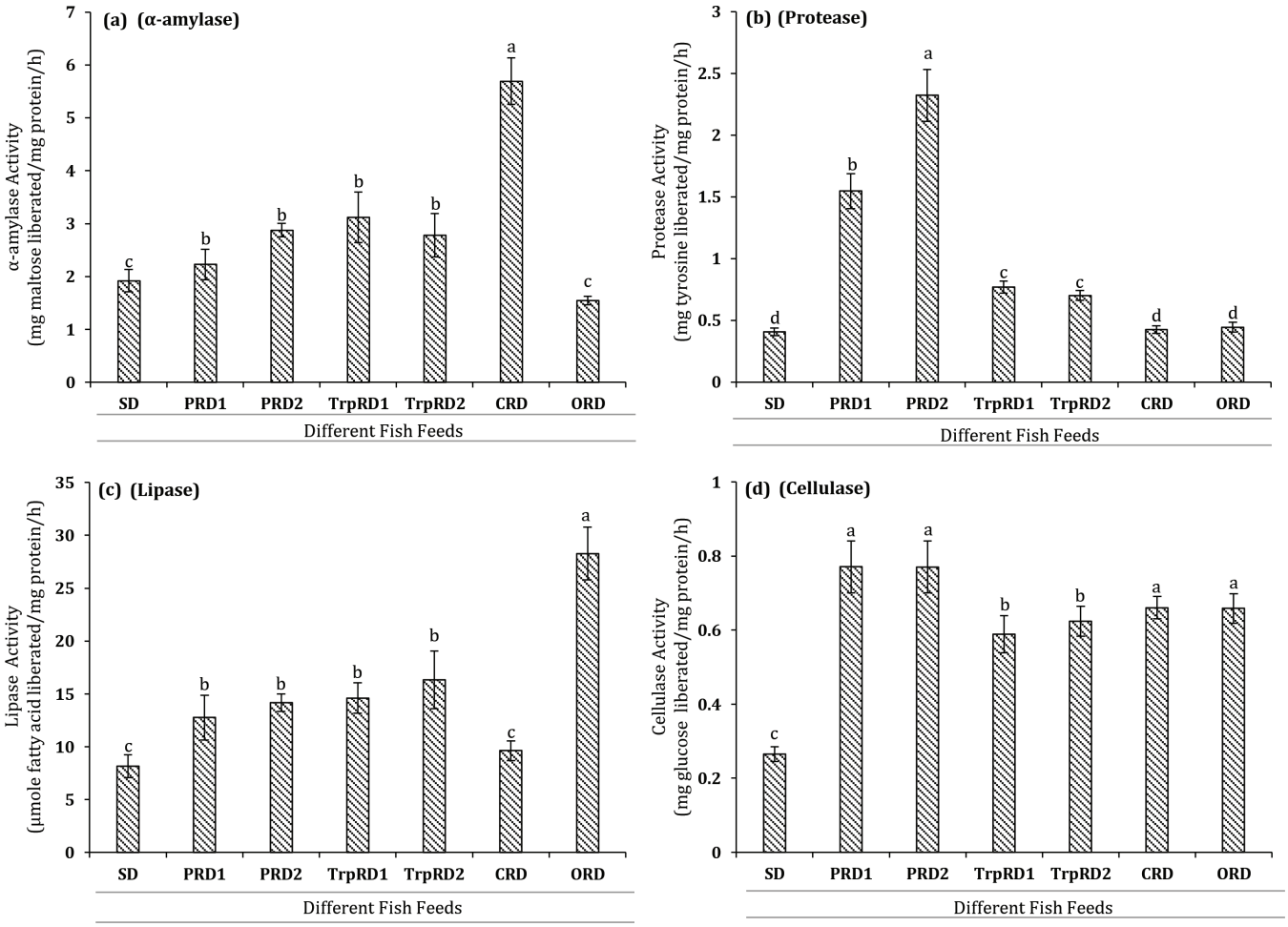

Figure 4. Graphical presentation of the data (mean ± SE shown in vertical bars; n = 6) on **(a)** α-amylase activity, **(b)** protease activity, **(c)** lipase activity and **(d)** cellulase activity in juvenile carps, which were supplied with a standard diet (SD), or protein-rich diet 1 (PRD1), protein-rich diet 2 (PRD2), or Trp-rich diet 1 (TrpRD1), or Trp-rich diet 2 (TrpRD2), or carbohydrate-rich diet (CRD), or oil-rich diet (ORD) at ~10:00 h for 30 days. Different letters indicate significant (p<0.05) differences between different laboratory-made diets.

Table 2. Values of 'r' were revealed from simple correlation coefficient analysis of the values of gut melatonin concentrations (Mel), residual gut contents (RGC), weight gain (WG), and specific growth rate (SGR) of carp, which were regularly fed a Standard Diet, followed by either Protein-Rich Diets (PRD1 and PRD2), or Tryptophan-Rich Diets (TrpRD1 and TrpRD2), or Carbohydrate-Rich Diet (CRD), Oil-Rich Diet (ORD) at ~ 10:00 h in the morning for 30 days.

Parameters	Mel	RGC	WG
RGC	-0.174	-	-
WG	**+0.353[c]**	-0.102	-
SGR	**+ 0.360[c]**	- 0.114	**+0.996[a]**

[a] $p < 0.001$, [b] $p < 0.01$, [c] $p < 0.05$

reliable intracellular stress marker, was reduced in both the PRDs and TrpRDs-fed groups, where melatonin was increased. Melatonin is known to play a vital role in regulating intracellular antioxidative molecules[16,24,83]. According to our data, the activities of all the studied antioxidative agents, viz. SOD, CAT, GST, GPx, GRd, and GSH have been promoted after increasing the gut melatonin levels following the modulation of food ingredients. However, the upregulation of gut melatonin was more prominent with GRd and GSH, indicating more xenobiotics detoxification activity by the gut[84] and activates different antioxidative enzymes, viz. SOD, CAT, GPx, and GST by inducing translational changes in their production, which decreases free radicals[85]. The GRd

Table 3. Values of 'r' were revealed from simple correlation coefficient analysis of the values of melatonin concentrations (Mel), malondialdehyde (MDA), and different antioxidant agents in the gut of carp, which were regularly fed a Standard Diet, followed by either Protein-Rich Diets (PRD1 and PRD2), or Tryptophan-Rich Diets (TrpRD1 and TrpRD2), or Carbohydrate-Rich Diet (CRD), Oil-Rich Diet (ORD) at ~ 10:00 h in the morning for 30 days.

Different redox parameters in the gut tissues							
	Mel	**MDA**	**SOD**	**CAT**	**GPx**	**GRd**	**GST**
MDA	**- 0.594[a]**	-	-	-	-	-	-
SOD	**+ 0.447[a]**	**- 0.850[a]**	-	-	-	-	-
CAT	**+ 0.510[b]**	**- 0.759[a]**	**+ 0.900[a]**	-	-	-	-
GPx	**+ 0.526[a]**	**- 0.805[a]**	**+ 0.919[a]**	**+ 0.937[a]**	-	-	-
GRd	**+ 0.734[a]**	**- 0.790[a]**	**+ 0.601[a]**	**+ 0.657[a]**	**+ 0.709[a]**	-	-
GST	**+ 0.612[a]**	**- 0.884[a]**	**+ 0.922[a]**	**+ 0.874[a]**	**+ 0.895[a]**	**+ 0.774[a]**	-
GSH	**+ 0.458[a]**	- 0.214	+ 0.273	**+ 0.359[c]**	**+ 0.423[a]**	**+ 0.406[b]**	+ 0.274

[a] $p < 0.001$, [b] $p < 0.01$, [c] $p < 0.05$

Table 4. Values of 'r' were revealed from a simple correlation coefficient analysis of the values of gut melatonin concentrations (Mel) and activity of digestive enzymes (α-amylase, protease, lipase, and cellulase) in the gut of carp, which were regularly fed a Standard Diet, followed by either Protein-Rich Diets (PRD1 and PRD2), or Tryptophan-Rich Diets (TrpRD1 and TrpRD2), or Carbohydrate-Rich Diet (CRD), Oil-Rich Diet (ORD) at ~ 10:00 h in the morning for 30 days.

Parameters	**The activity of digestive enzymes**			
	Mel	**α-Amylase**	**Protease**	**Lipase**
α-Amylase	-0.047	-	-	-
Protease	**+0.731[a]**	-0.107	-	-
Lipase	-0.083	**-0.345[c]**	- 0.068	-
Cellulase	**+0.423[b]**	+0.164	**+0.539[a]**	+0.245

[a] $p < 0.001$, [b] $p < 0.01$, [c] $p < 0.05$

values were highest in PRD2-fed carp, parallel with the melatonin profiles noted in these carp. The SOD, CAT, and GPx showed a positive stimulatory relationship with melatonin. Still, in such cases, the efficacy of melatonin in the regulation of the activity of the antioxidative enzymes is limited to a certain level[86], above which melatonin cannot stimulate their actions, as no further increase in their activity was noted when carp were fed with PRDs and having higher melatonin levels compared to the TrpRDs-fed groups. Under increased nutritional intake via PRDs, TrpRDs, CRD, and ORD compared to SD, the metabolic rate within the gut cells may increase, leading to higher production of free radicals. To reduce this stress, free radicals are dismutated by the increased levels of SOD and form a less reactive molecule, H_2O_2. Higher levels of H_2O_2 within the gut may be managed by the increased levels of CAT and GPx[16], with a positive correlation with gut melatonin. However, the mechanism of actions of melatonin on the antioxidative functions in fish gut tissues should remain speculative until further study.

In ORD, the oil content has been increased by cod liver oil (fish oil or FO) and sunflower oil (vegetable oil or VO). FO and VO are rich in long-chain polyunsaturated fatty acids (LC-PUFA), and it is well established that due to their high degree of unsaturation, LC-PUFA is more susceptible to peroxidation than PUFA, thus promoting the formation of more free radicals[87]. Thereby, it was evidenced that unaltered MDA levels in the gut following the replacement of ORD after SD were noted. Hence, oxidative stress was high in the ORD-fed group compared to PRDs and TrpRDs, along with the increased levels of different antioxidative agents to combat stressful situations. It was found that the levels of different

antioxidative agents were significantly increased after feeding with CRD compared with the SD-fed group. Dietary macronutrients substantially affect the metabolic pathway but can be achieved in several modes. Hence, those have various roles in regulating oxidative stress of different gut cells of the digestive tract. The beneficial effect of dietary carbohydrates on handling oxidative stress may be attained by increasing the level of different antioxidative agents[88-91].

The carp exhibited a gradual increase in protease levels. This indicates that the particular aged carp are developing their gut for better digestion. We have also found a significant increase in α-amylase levels in both the PRDs. Previous reports indicate that crude protein availability in diet may alter the *α-amylase* gene expression in red tilapia juveniles (*Oreochromis* sp.)[37], possibly because of the higher protein availability for the production of digestive enzymes. In our study, digestive enzyme activities were significantly increased in both the TrpRDs compared to SD. Similar kinds of observations were observed in hybrid catfish[46], Jian carp (*Cyprinus carpio* var. Jian)[74], Atlantic salmon (*Salmo salar*), and Coho salmon (*Oncorhynchus kisutch*)[92]. Optimal protein[93], Trp[94], carbohydrate[95], and oil[96] play a role in maintaining intestinal structural integrity and intestinal development, possibly by maintaining gut microflora. Further, the increase in cellulase activity following the supply of better-quality diets may be due to the contribution of the gut microflora. Existing reports indicate that better quality of nutrients in terms of protein, mainly focusing on the amino acid Trp, may play essential roles in maintaining gut microflora and intestinal health. The Trp, accumulated within the gut following a supply of PRDs or TrpRDs, by the formation of melatonin may increase the richness and diversity of the intestinal microbiota, perhaps partly because Trp promotes the growth of the intestinal villi, thus increasing the nutrients available to the intestinal flora[97,98]. On the other hand, in the present study, gut melatonin showed a positive correlation with protease but not with α-amylase and lipase. In contrast, a complete time-bound positive correlation between gut melatonin and different digestive enzymes has been seen previously in juvenile carp, *Catla catla*[16]. This contradiction invites us to think that protease activity may be associated with gut melatonin synthesis, and the cellulase activity is performed by the gut microflora, which develops during the early stage of the gut. However, α-amylase and lipase are related to food intake as higher levels of α-amylase and lipase were associated after the CRD and ORD diets supply. In our study, the level of α-amylase in the gut was significantly highest in CRD. Similar observations were noticed in various carp[99] and catfish (*Clarias batrachus*)[100] earlier after feeding CRD. All these observations help to speculate that the feeding preference/intensity differs with the growth of carp from fingerling to adult. In the present study, we worked on the early stage of juveniles, which exhibits a particular type of feeding preference and subsequent enzymatic actions. As noted in a recent publication[20] by our group, the higher α-amylase activity was recorded in the early stage (Fingerling), protease in the middle (Juvenile), and lipase and cellulase in later (Adult) stages of the growth phase. This indicates that similar kinds of food for all stages of carp is not suitable; instead, it is essential to understand the best food quality appropriate for a particular age group.

Finally, the present study generally reports that PRDs and TrpRDs can increase gut melatonin levels. In particular, the stimulation over gut melatonin synthesis is more after the PRDs supply than TrpRDs. It can be argued that fish feed's protein/Trp components have a significant role in maintaining gut melatonin synthesis. It may be hypothesized that a higher amount of Trp supplement can upregulate the melatonin production by the gut due to the additional availability of primary substrate (L-Trp) in the melatonin biosynthesizing pathway, but that can be achieved only up to optimum levels of the four melatonin biosynthesizing enzymes activity. However, additional supplementation of protein as a whole not only increases the Trp availability but also supplies all the raw ingredients required to upregulate the genes of melatonin biosynthesizing enzymes. Further studies are necessary to address the issue by estimating the expression patterns of vital melatonin biosynthesizing genes in the gut under altered-quality diets. Remarkably, increased gut melatonin, at least via Trp supplementation, can enhance digestive function by uplifting vital digestive enzyme activity, particularly protease and cellulase-mediated digestion, which results in better nutrition. Further, this feed-induced enhanced gut melatonin can reduce gut stress by increasing antioxidant activity, resulting in better gut health and promoting growth performance. In the future, it would be exciting and informative to investigate the role of feed-induced altered gut melatonin in regulating diverse physiological functions in fish.

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