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# Evidence of Microplastics in Gut Content of Grass Carp (*Ctenopharyngodon idella*) Fingerlings and their Effects on Growth Performance and Body Composition

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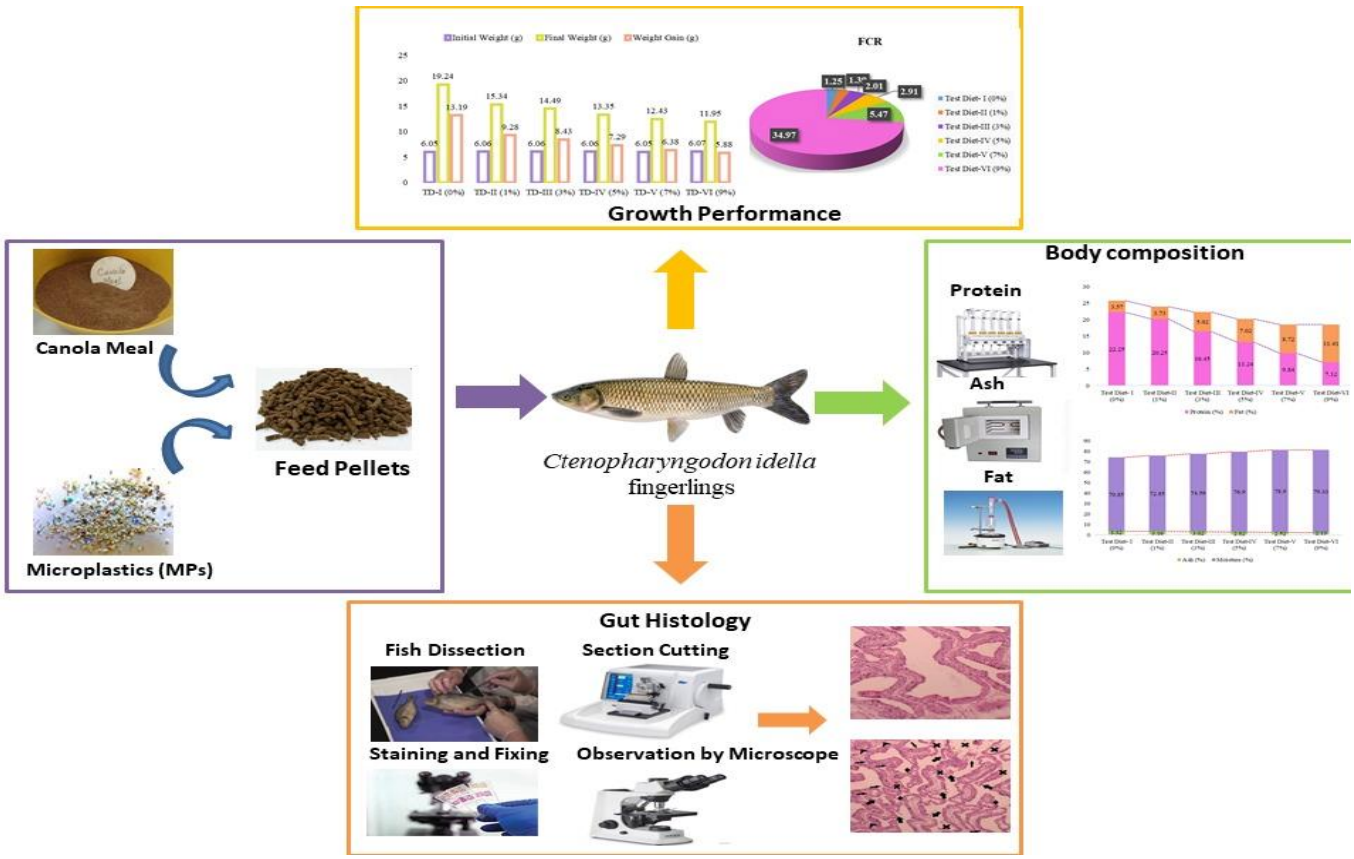
**HIGHLIGHTS**

- Growth, body composition and gut of grass carp were negatively impacted by microplastics (MPs).
- Dietary MPs up to 9% in canola meal based diet affected the well-being of grass carp.
- Grass carp fed control diet had highest values for growth, carcass and gut health.
- Fish gut causes severe damage from MPs, including tissue damage and enterocyte necrosis.

**Abstract:** Microplastics (MPs) are emerging pollutants that may affect the aquatic life, including fish, raised in aquaculture. The purpose of this study was to determine the effects of biodegradable-MPs mixed in canola meal based diet on the growth performance, body composition, nutrient digestibility and gut content of grass carp fingerlings. For this purpose, six canola meal based diets were prepared including one control diet (without addition of MPs) and five other diets containing 1%, 3%, 5%, 7% and 9% MPs. 270 fingerlings were fed twice for 60 days at a rate of 5% of their wet weight. Effect of each treatment on the weight gain, feed conversion ratio (FCR), nutrient digestibility, gut performance and body composition was calculated by standard procedures. Highest value for growth, nutrient digestibility and no damage to gut was observed in fish fed control diet. Results showed that there was significant decrease ($P$<0.05) in growth and increase in FCR in fish fed 9% biodegradable-MPs added diet. Nutrient digestibility and body composition of grass carp was highly affected by microplastics. Microplastics also caused severe damage to gut of fish including, tissue damage, vacuolization and necrosis of enterocytes.

**Keywords:** *Ctenopharyngodon idella*; microplastic; growth performance; carcass composition; gut histology.

**GRAPHICAL ABSTRACT**




## INTRODUCTION

Aquatic products and animals are essential nutrients in the human diet and are also present in the global aquatic product industry for consumers [1]. Therefore, we need to protect our aquatic environment against to pollution on various environmental and ecological effects. The aquatic ecosystems and living organisms suffer from environmental impact by emissions of volatile organic substances, and pollution of water by oil chemicals and many various hazardous agents [2,3].

Emerging contaminants known as microplastics (MPs) have the potential to pollute the environment of aquatic organisms. Plastics with a size of less than 5 mm are microplastics [4]. They are so widely used in recent times, due to which their production has almost been doubled and around 33 billion tonnes of plastic will have been produced by 2050 [5]. MPs are available in a wide range of sizes, forms, chemical nature and sources [4]. Plastics currently cause significant environmental damage, but they are still used. They have been found in all aspects of our ecosystem, especially in the water [6]. In 2018, global plastic output was 360 million tonnes, with an estimated 80,000 tonnes reaching the oceans [7]. Anthropogenic activities are the primary source of plastics in the water [8]. Around 10% of all plastic waste produced is detected in the ocean, making it a major hazardous factor [7].

Plastic waste decays into micro fragments in aquatic environments due to chemical and physical interactions with aquatic organisms, waves, and sunlight [9]. MPs are prevalent in estuarine and marine habitats while both marine and freshwater fish species acquire various MPs [10]. MPs in aquatic habitats could vary in a wide range of colors, indicating a wide range of sources. Aquatic species may mistakenly ingest particles that breakdown to the MP level [9]. MPs accumulate in the fish body and move to the lymphatic and circulatory systems via cells, where they are dispersed in the body. Due to their bioavailability, they deposit in organs and tissues via blood circulation and ultimately cause damage to intestines. Moreover, fish gills can also be blocked with microplastics [11]. Tissue accumulation of these MPs can affect fish growth and development, as well as oxidation, immunity and metabolism. There are several ways in which MPs may be absorbed by fish: through the mouth, gills, and also through skin absorption [5,12].

Fish species need to be protected and conserved from the adverse effects of MPs because they not only harm the fish but also the whole food web [13]. One of freshwater exotic carp, Grass carp (*Ctenopharyngodon idella*), is frequently farmed in Southeast Asia due to its rapid development and excellent nutritional values. It has become one of the four biggest freshwater fish in China [14]. China has seen a tremendous growth in the aquaculture output of grass carp in recent years, going from 4.222 million tons in 2015 to 5.571 million tons in 2020 [15]. In recent years a lot of work was done on effects of MPs in aquatic organisms but less attention was paid to effects of MPs added in fish diet especially grass carp (freshwater fishes). So, this is the need of the hour to investigate the effects of MPs added in diet on different parameters of fish.

## MATERIAL AND METHODS

This study was carried out with the aim of determining the effects of microplastics on the gut, growth performance, body composition and nutrient digestibility of grass carp fingerlings. The experiment was conducted at the Fish Nutrition Laboratory, Department of Zoology, Government College University, Faisalabad.

### Fish sampling and trial setup

Grass carp fingerlings (average weight: 6.05±0.01 g) were purchased from the Government Fish Hatchery on Satiana road in Faisalabad. In the research lab, cemented tanks were used to acclimatize fingerlings to the experimental conditions for a period of two weeks. The health of fingerlings was maintained by monitoring several water quality measures on a regular basis, such as the temperature of the water (25.2-28.8°C), the amount of dissolved oxygen (5.7-7.5 mg/L), and pH (7.0-8.5). The capillary technique was used throughout the experiment to provide continuous aeration to all of the experimental tanks.

### Experimental diets

Feed components were bought from a commercial feed mill and tested for chemical composition using the right procedures prior to creating experimental diets [16]. Microplastics were obtained from Department of Environmental Biology, Government College University, Faisalabad. Six canola meal based experimental diets were formed with addition of 0%, 1%, 3%, 5%, 7% and 9% of biodegradable-MPs, respectively, in which 0% was the control (without MPs inclusion) (Table 1).

**Table 1.** Ingredients composition (%) of test diets (TD)

| **Ingredients** | **TD-** I$_{(Control)}$ | **TD-** II | **TD-** III | **TD-** IV | **TD-** V | **TD-** VI |
|---|---|---|---|---|---|---|
| MPs* (%) | 0 | 1 | 3 | 5 | 7 | 9 |
| Canola meal | 54 | 54 | 54 | 54 | 54 | 54 |
| Fish meal | 10 | 10 | 10 | 10 | 10 | 10 |
| Wheat flour | 17 | 16 | 14 | 12 | 10 | 8 |
| Rice polish | 8 | 8 | 8 | 8 | 8 | 8 |
| Fish oil | 7 | 7 | 7 | 7 | 7 | 7 |
| Vitamins premix | 1 | 1 | 1 | 1 | 1 | 1 |
| Minerals premix | 1 | 1 | 1 | 1 | 1 | 1 |
| Ascorbic acid | 1 | 1 | 1 | 1 | 1 | 1 |
| Chromic oxide | 1 | 1 | 1 | 1 | 1 | 1 |

*Biodegradable-Microplastics.

### Pellets formation

The feed contents were grinded finely enough to pass through a 0.5 mm sieve. The concentrations of MPs (1%, 3%, 5%, 7% and 9%) were added as feed additive as described by [17]. All these contents were combined in an electric mixer for 5 minutes and fish oil was added gradually. The gradual addition of water produced a homogeneous dough and after that, pellets were formed by pelletizer. These pellets were dried at 105°C for 2 hours and fed to fingerlings for period of 60 days.

### Feeding protocol and sampling

A total 270 grass carp fingerlings were divided into replicas, and 15 fish were placed in each replicate in V-shaped tanks. They were fed at the rate of 5% of live wet weight on daily basis. The uneaten diet was emptied out of each tank by opening the valves after the two-hour feeding period. Each tank was thoroughly cleaned to get rid of any leftover feed before it was refilled with water. After collecting fecal material, it was dried in oven and stored for chemical analysis. The experimental trial was carried for 60 days.

### Growth assessment

Fish in each tank was bulk weighed at the start and end of experiment to assess the growth. Growth performance of fingerlings was evaluated based on standard formulae.

$$\text{FCR} = \text{Total dry feed intake (g)} / \text{Wet weight gain}$$

$$\text{Weight gain \%} = (\text{Final weight} - \text{Initial weight}) \times 100 / \text{Initial weight}$$

$$\text{SGR} = (\text{Final weight} - \text{Initial weight}) \times 100 / \text{No. of days}$$

### Chemical analysis of whole body and feed

Whole body and experimental diet samples were homogenized separately with a motor and pestle before being examined using standard techniques [16]. Moisture was determined by oven drying at 105°C for 12 hours; ash was determined by ignition at 650°C for 12 hours in an electric furnace (Eyela-TMF 3100); crude fat by petroleum ether extraction method through Soxtec HT2 1045 system and crude protein (CP) (N×6.25) by micro Kjeldahl apparatus. Chromic oxide (inert marker) was measured in feed and feces by spectrophotometer at 350nm for digestibility analysis [18].

### Calculation of digestibility

The apparent nutritional digestibility coefficients (ADC%) for the experimental diets were calculated using this standard formula [19].

$$\text{ADC\%} = 100 - 100 \text{ x } (\text{Percent marker in diet x Percent nutrient in feces}) / (\text{Percent marker in feces x Percent nutrient in diet})$$

### Gut histology study

At the completion of the experimental phases, fish were removed from the test tanks and immediately given 75 l/L of clove oil to anaesthetize them. After being exposed to MPs for 60 days, fish were dissected, and intestinal samples were obtained. Finally, the sections were stained for microscopic examination using the hematoxylin and eosin technique. We took tissue samples, which we then fixed in formalin solution. The procedure for histological examination was as follows: embedding, sectioning, mounting, and staining with H&E, all after dehydrating the tissue with ethanol 96%. Each of these procedures was performed utilizing a tissue processor under fixed program (Tissue processor, Triangle biomedical sciences, USA). A photomicroscope from Olympus was used to capture images of histological lesions [20].

### Statistical analysis

Data of growth performance, body composition and nutrient digestibility of fish fed with control and test diets was subjected to one-way analysis of variance (ANOVA) [21]. The differences among treatments was compared by Tukey's Honesty Significant Difference Test and considered significant at $P<0.05$ [22]. CoStat Computer Package (version 6.303, PMB 320, Monterey, CA, 93940 USA) was used for statistical analysis.

### Contamination control of MPs

As described by [23], the contamination control of MPs is necessary because of their adverse effects on organisms due to their small size and bioavailability. Throughout the entire study, contamination was avoided by cleaning all the petri dishes and equipment with acetone and distilled water. Tissue culture hood was also used to prevent the contamination of MPs.

## RESULTS

### Growth study of grass carp fed canola meal based test diets supplemented with MPs

Growth performance of grass carp fed MPs based diets in terms of final weight, weight gain and weight gain (%) is displayed in Figure 1 (a,b). Fish were fed different levels of MPs based diets such as 0% (control), 1%, 3%, 5%, 7% and 9%. During the whole experiment, weight gain and final weight were significantly ($P$<0.05) decreased in fish fed 9% MPs based diets. Lowest values of final weight (6.65±0.02g) and weight gain (0.58±0.03g) were observed when fish given 9% MPs based diet. Highest values of final weight and weight gain were recorded in fish fed control diet. It was noted that, there was a decreasing trend in final weight and weight gain when fish fed 9% MPs based diets while there was an increasing tendency in final weight and weight gain when fish were fed 0% inclusion of MPs in the diets of fish. It was observed that final weight and weight gain were significantly different from control diet and all other test diets. As compared to control group grass carp fed with 9% MPs had higher FCR value and low value of SGR as shown in Figure 1 (c,d), respectively.

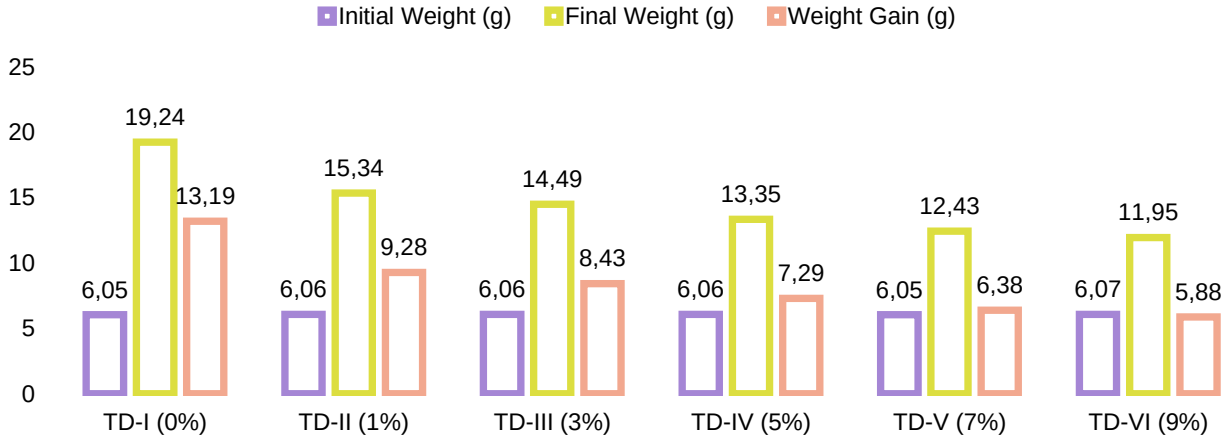



**Figure 1a.** Analysis of growth performance against varying levels of MPs concentration; Weight parameters.

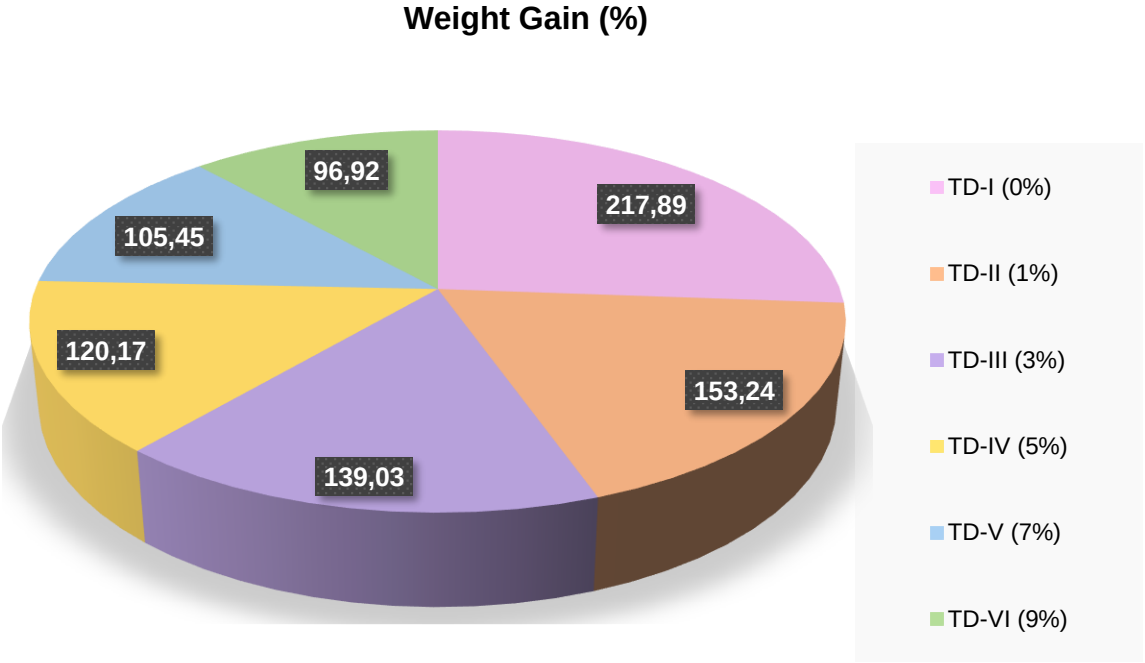



**Figure 1b.** Analysis of growth performance against varying levels of MPs concentration; Weight gain (%).

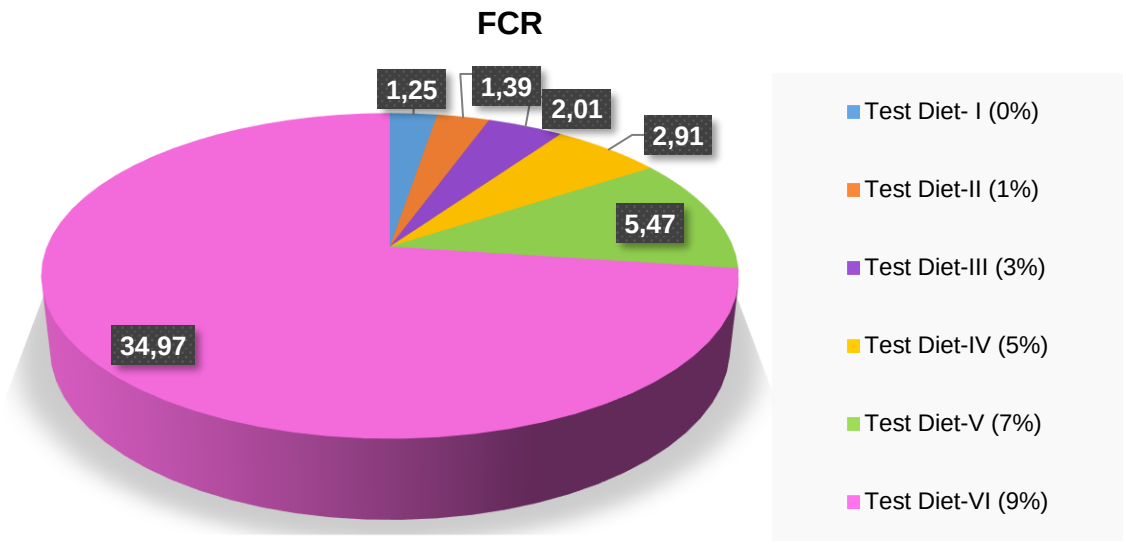



**Figure 1c.** Analysis of growth performance against varying levels of MPs concentration; FCR.

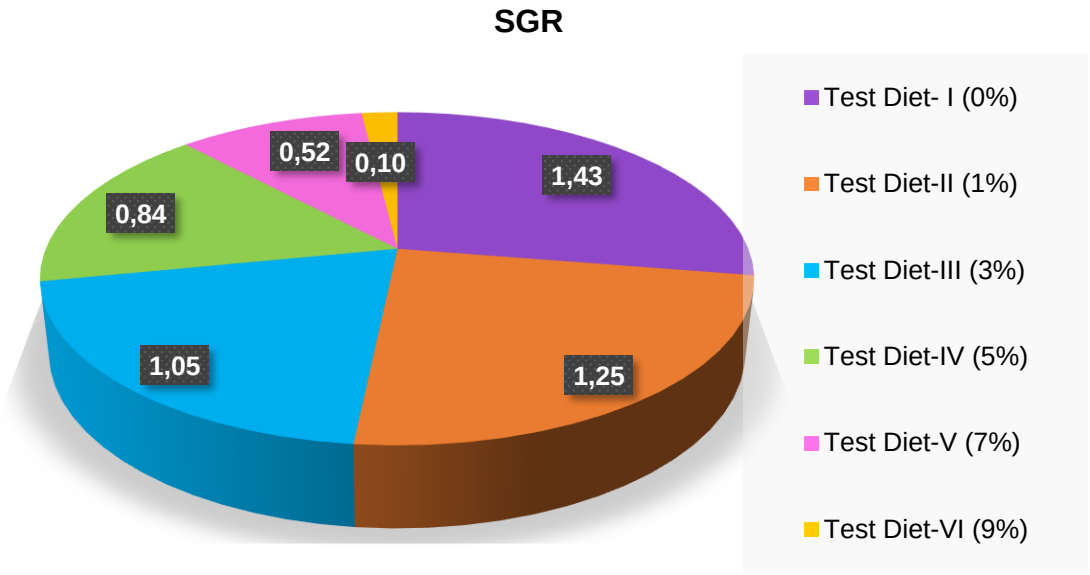



**Figure 1d.** Analysis of growth performance against varying levels of MPs concentration; SGR.

## Nutrient digestibility of grass carp

The nutrient digestibility in grass carp was studied by the analysis of feed and feces (Table 2 and 3). Fish fed with control diet had the lowest value of nutrients in their feces. It shows greatest nutrient digestibility values for protein (8.31±0.44%), fat (1.90±0.02%) and gross energy (1.28±0.01%), considerably different ($P$<0.05) from all other groups. The lowest crude protein percentage (30.86±0.04%) and crude fat (8.03±0.02%) of feed was observed at 9% MPs level. Maximum crude protein (30.95±0.02%) and crude fat (8.04±0.02%) was observed in control diet. The results explained that a diet with 9% MPs had lowest gross energy value. The study of apparent nutrient digestibility (ADC%) showed that MPs added canola meal based diet significantly lowered nutritional digestibility of grass carp fingerlings. The lowest crude protein digestibility percentage (42.03±0.77%) and crude fat digestibility (50.25±0.23%) of feed was observed at 9% MPs level. Maximum crude protein digestibility (77.63±0.34%) and crude fat digestibility (80.73±0.48%) was observed in control diet. The lowest value of gross energy was (32.28±0.66%), which had significant difference ($P$<0.05) from all other groups. The control group had the highest gross energy (68.54±0.35%) (Figure 2).

**Table 2.** Analyzed composition (%) of apparent crude protein (CP), crude fat (CF) and gross energy (GE) of feed of grass carp fed on biodegradable-MPs

| Test diets | Biodegradable-MPs % | CP (%) | CF (%) | GE (kcalg$^{-1}$) |
|---|---|---|---|---|
| Test Diet- I | 0% | 30.95±0.02$^{a}$ | 8.04±0.02$^{a}$ | 3.40±0.02$^{a}$ |
| Test Diet-II | 1% | 30.81±0.10$^{a}$ | 8.07±0.04$^{a}$ | 3.42±0.03$^{a}$ |
| Test Diet-III | 3% | 30.81±0.10$^{a}$ | 8.04±0.03$^{a}$ | 3.4±0.01$^{a}$ |
| Test Diet-IV | 5% | 30.81±0.10$^{a}$ | 8.04±0.03$^{a}$ | 3.41±0.02$^{a}$ |
| Test Diet-V | 7% | 30.82±0.17$^{a}$ | 8.06±0.02$^{a}$ | 3.41±0.03$^{a}$ |
| Test Diet-VI | 9% | 30.86±0.04$^{a}$ | 8.03±0.02$^{a}$ | 3.43±0.01$^{a}$ |

**Table 3.** Analyzed composition (%) of apparent crude protein (CP), crude fat (CF) and gross energy (GE) of feces of grass carp fed on biodegradable-MPs

| Test diets | Biodegradable-MPs % | CP (%) | CF (%) | GE (kcalg$^{-1}$) |
|---|---|---|---|---|
| Test Diet-I | 0% | 8.31±0.44$^{f}$ | 1.90±0.02$^{f}$ | 1.28±0.01$^{f}$ |
| Test Diet-II | 1% | 11.35±0.10$^{e}$ | 2.32±0.04$^{e}$ | 1.57±0.06$^{e}$ |
| Test Diet-III | 3% | 14.26±0.09$^{d}$ | 2.87±0.03$^{d}$ | 1.78±0.09$^{d}$ |
| Test Diet-IV | 5% | 16.43±0.13$^{c}$ | 3.62±0.04$^{c}$ | 2.23±0.02$^{c}$ |
| Test Diet-V | 7% | 18.94±0.04$^{b}$ | 4.23±0.02$^{b}$ | 2.42±0.01$^{b}$ |
| Test Diet-VI | 9% | 22.58±0.32$^{a}$ | 4.78±0.03$^{a}$ | 2.74±0.08$^{a}$ |

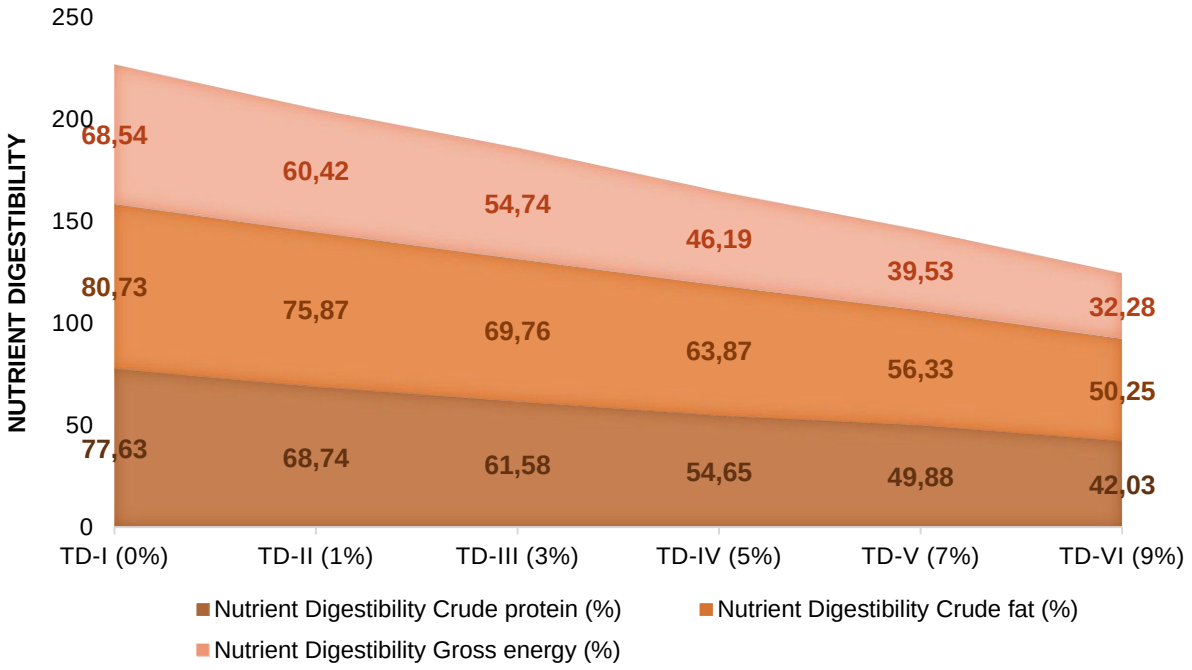



**Figure 2.** Representation of analyzed body composition (ADC %) against varying levels of MPs concentration.

## Body composition of grass carp fed on MPs based diets

The body composition of fingerlings treated with various concentrations of MPs added canola meal based diet over the course of 60 days is displayed in Figure 3. The crude protein, crude fat, ash and moisture content of fish was significantly different ($P$<0.05) in all experimental groups as shown in Figure 3 (a,b). The lowest value of CP (7.12±0.01%) and greatest value of CF (11.41±0.02%) were at tank six having 9% MPs added diet. The results in table showed that best values of CP (22.25±0.02%) and minimum values of CF (3.57±0.07%) were found in fish fed control diets. Lowest values of ash (2.13±0.02%) were observed in fingerlings fed 9% MPs added diet. Maximum value of ash (3.32±0.10%) content was noticed in fish fed 0%

MPs added diet. It was observed that minimal values were highly significant ($P$<0.05) from fish fed 0%, 1% and 3% MPs added diets. It was determined that protein and ash contents in fish were lowest in fish fed 9% MPs added diet as compared to other diets such as 0%, 1%, 3% and 5% levels of MPs. The lowest value of moisture (70.85±0.02%) was observed in fish fed control diet. While highest value of moisture (79.33±0.03%) was found in fish fed 9% MPs added diet.

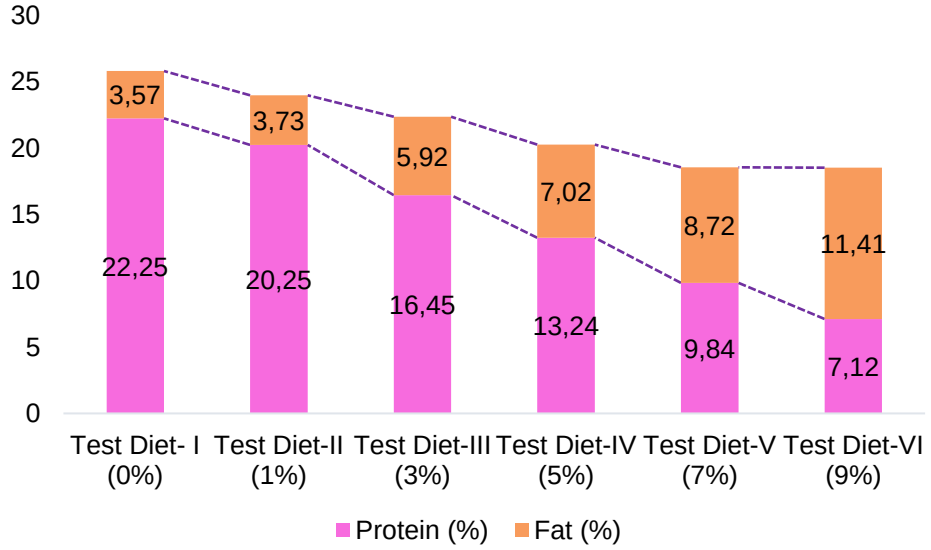



**Figure 3a.** Analysis of body composition against varying levels of MPs concentration; Protein and fat

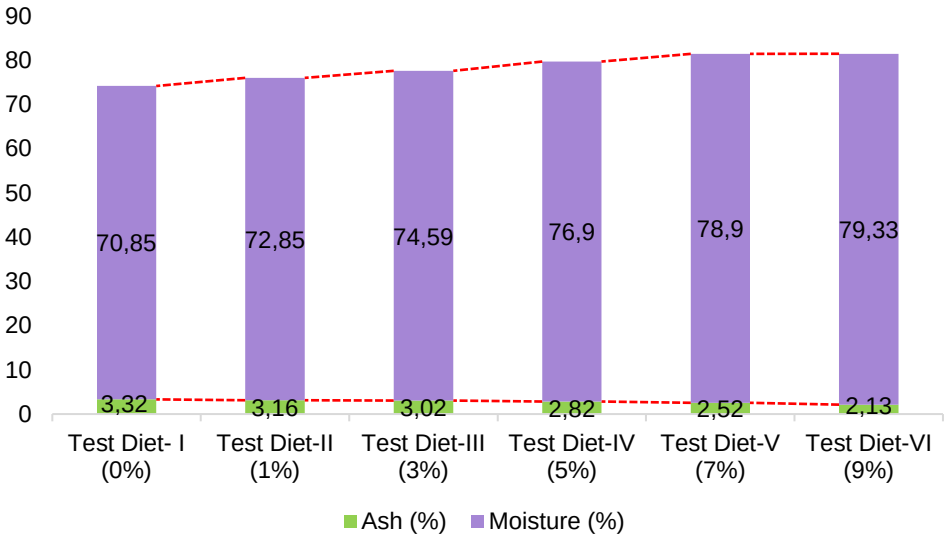



**Figure 3b.** Analysis of body composition against varying levels of MPs concentration; (b) Ash and moisture.

## Study of gut content of grass carp

The damage of MPs was observed in gut histology of grass carp (Figure 4). Slides were made by section cutting of gut then observed under light micro-scope. Our results showed that fish gut was damaged after the exposure to MPs. No damage to gut was showed by fish fed control diets because there were no MPs present in the gut. Fish fed 1% MPs added diet showed very less damage which was negligible. Fish fed 3% MPs added diet showed moderate damage to cells and tissues with a small hint of vacuolization. 5% MPs added diet fed to fish caused more damage to gut tissues. Vacuolization process was increased, cilia defects started to rise and moderate necrosis of enterocytes was observed in gut of fish fed 5% MPs added diet. A very small infiltration of inflammatory cells was observed in gut. The damage to gut tissues was more severe in fish fed 7% MPs added diet. There was high infiltration of inflammatory cells. Cilia defects were also increased. High necrosis of enterocytes was observed in fish. At the end in fish fed 9% MPs added diet there was severe

damage to intestinal tract. There was severe vacuolization in gut. Cilia were damaged severely. Severe necrosis of enterocytes was observed in gut of fish. The infiltration of inflammatory cells was also severe in gut of fish fed 9% MPs added diet.

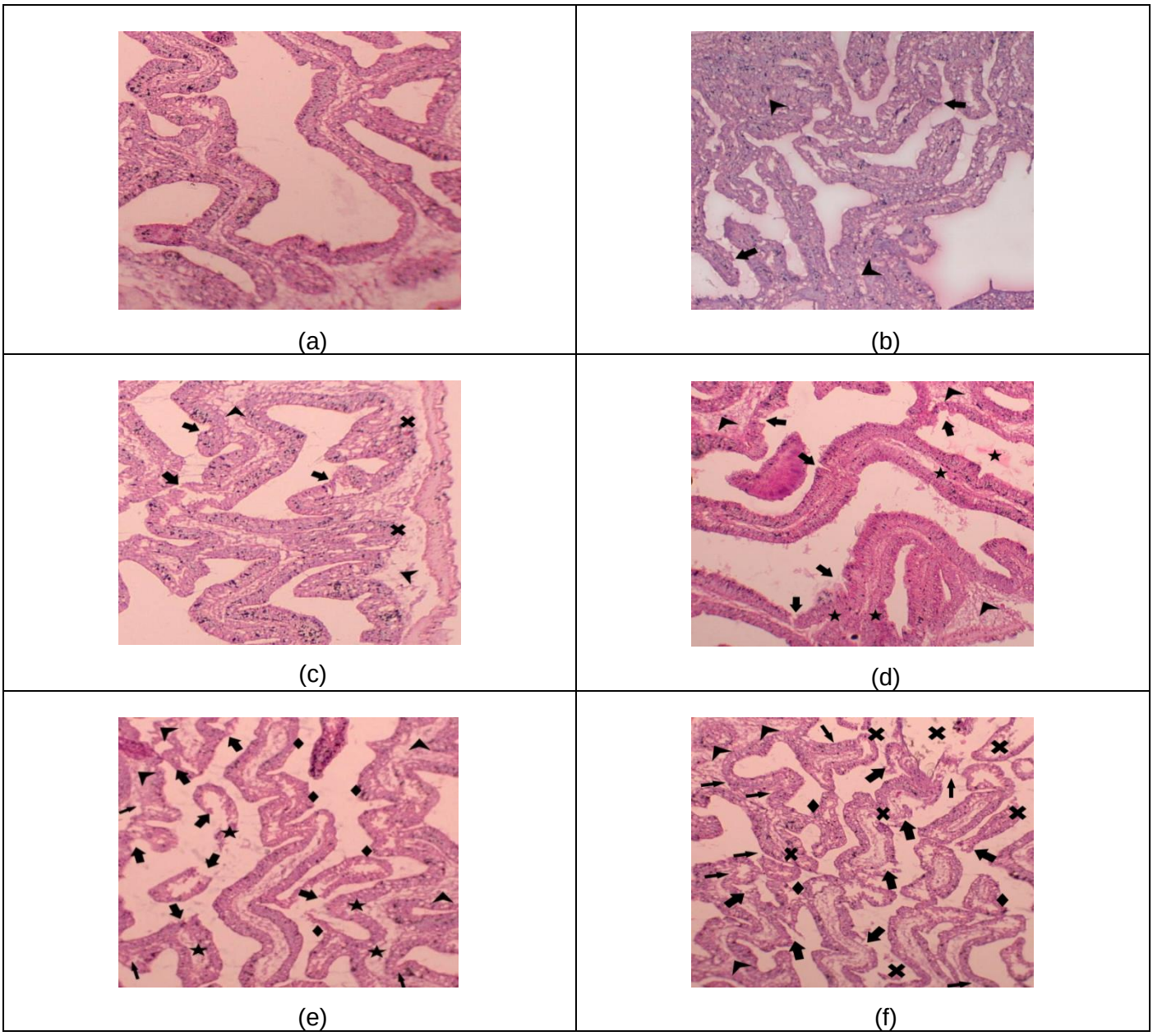



**Figure 4.** Effects of MPs on intestinal track of grass carp (a) control diet (b) 1% MPs added diet (c) 3% MPs added diet (d) 5% MPs added diet (e) 7% MPs added diet (f) 9% MPs added diet. Thin arrow= infiltration of inflammatory cells, thick arrow= necrosis of enterocytes, star= vacuolization, pointed arrow= cell sloughing, diamond= cilia defects.

## DISCUSSION

Over the past few years, concerns about the negative effects of microplastics on aquatic organisms have increased. Since, MPs may cause nerve damage, tissue disruption, free radical damage, behavioral impairment as well as growth retardation in fish [5,12]. Thus, our study was conducted to evaluate the effects of MPs added canola meal based diets on growth performance, body composition, nutrient digestibility and gut health of grass carp raised in fish tanks in experimental conditions.

Fish species are often used as bio-indicators of environmental contamination, because they are extremely sensitive to the existence of water-borne toxins [24]. Ingesting microplastics can cause malabsorption, which affects energy intake and processing [25]. This was in accordance with our results, which revealed that the treated fish's body weight and growth were considerably reduced. [26] studied that addition of microplastics to diet reduced the growth of fish as compared to the control group. This was in

accordance with our results as final weight and weight gain were significantly ($P$<0.05) decreased in fish fed 9% MPs based diets. [27] studied that satiated common carp were shown to grow less after being exposed to 32-40 µm polystyrene MPs (100 and 1000mg/L) for 60 days. But in starved fish less growth was observed only after 30 days indicating an interaction between food restriction and MPs on growth. It was again in accordance with our results because in our study lowest weight gain (0.58±0.03g) of fish was recorded when fish fed at 9% of MPs based diet while highest weight gain (15.94±0.09g) was observed when fish fed at 0% inclusion of MPs in the diet of fish. These results indicate that MPs had negative effect on growth performance of fish. Fish performance and weight gain are directly affected by the consumption of MPs since their tiny size increases their bioavailability.

Energy reserves are necessary to keep organisms alive during times when food is limited, environmental conditions are poor or energy is diverted to proper growth and development. It has been shown that the consumption of MPs causes an increase in the energy demand on organisms and a loss in their energy stores through the catalysis of lipids [28]. The body composition of grass carp treated with various concentrations of MPs added canola meal based diet over the course of 60 days was studied in our work. The crude protein, crude fat, ash and moisture content of fish showed that there was significant difference ($P$<0.05) between them. [29] reported that after exposure to polystyrene microplastics, crude protein and lipid levels in fish decreased slightly (df = 18, $P$<0.05). This is in accordance to our results that show the lowest value of CP (7.12±0.01%) and greatest value of CF (11.41±0.02%) were at 6$^{th}$ group having 9% MPs in grass carp's diet. It is recorded that these values were highly significant ($P$<0.05) from the group that fed control diet. [30,28] reported that ingesting polystyrene nanoparticles changed the lipid metabolism of fish. This is also in accordance with our results that lipid metabolism was changed with microplastics. It was observed that minimal values of ash were highly significant ($P$<0.05) from fingerlings fed 0%, 1% and 3% MPs added diets. [31] studied that protein and ash contents of juvenile yellow perch were decreased when microplastics were given with diet. It resembles with our results because for grass carp it was determined that protein and ash contents were lowest in fingerlings fed 9% MPs added diet. [29] in their experiment also studied that between the control and polystyrene microplastics treatments, the moisture showed no significant variations for the whole body. It is in contrast with our results, because in our study for grass carp, the lowest value of moisture (70.85±0.02%) was observed in fish fed 0% MPs added diet. While highest value of moisture (79.33±0.03%) was found in fish fed 9% MPs added diet which is highly significant. This variation in results may be due to different factors like different experimental conditions and amount of MPs added to diet.

The study of nutrient digestibility showed that MPs added diet significantly lowered nutritional digestibility of grass carp fingerlings. Grass carp is an herbivorous and agastric fish species that has a long gut to absorb the nutrients in blood. MPs consumption induces dysbiosis of the gut (imbalance of microbes), so this inflammation in the intestines reduces nutrient absorption in fish [32]. For grass carp, the lowest values for crude protein digestibility (42.03±0.77%), crude fat digestibility (50.25±0.23%) and gross energy (32.28±0.66%) were observed at 9% MPs level. By collating literature, it has come to know that the limited work has been done on effects of MPs to reduce absorption of nutrients in fish. However, [33] investigated and found that the digestion and gut microbiota of guppy fish after the exposure to microplastics, had become reduced. As a result, the digestion of nutrients was blocked. Another study showed that the zebra fish, when exposed to MPs in water, caused intestinal damage which ultimately reduced the digestion of nutrients [34].

The gut histology of grass carp was studied by section cutting of gut and then observing it under light microscope. Our results showed that damage to gut was increased as we increased the percentage of microplastics in diet. The most severe damage to gut was done by 9% MPs added diet followed by 7% MPs added diet. [35] reported that histology changes caused by MPs exposure have been observed in *Cyprinus carpio*. It is according to our results because in grass carp, gut histology was affected severely due to high incorporation of MPs in diet. [36] observed that when polyvinyl chloride MPs (PVC-MPs) were consumed continuously by European sea bass, it caused tissue damage that led to histological changes such as broadening of the lamina propria, vacuolization of enterocytes, shortening and thickening of the villi, and increased rodlet cells. These results show similarity to the results that we observed because in grass carp necrosis of enterocytes and damage to villi was observed when fed with 9% MPs added diet. It shows that MPs have severe effects on gut histology of fishes. [37] studied that zebra fish exposed to various MPs, including polyethylene (PE), had histological intestinal damage. It is again in accordance with our results that gut was damaged severely due to MPs. [38] stated that no discernible variations were seen at the histo-pathological level in gilthead sea bream treated with various kinds of virgin-MPs for 45 days. This is in contrast to our results because in our study there was severe damage to gut of fish. This difference in results may be due to type or size of MPs used. The time of exposure can also be a factor due to which in our study effect was severe. The fish species used may also be a major cause of difference in results.

## CONCLUSION

Conclusively, this study showed that dietary MPs exposure up to 9% in canola meal based diet decreased the growth performance, whole-body composition, nutrient digestibility and gut histology of grass carp. However, control diet did not affect these parameters. Further study on the effects of microplastics in other cultured carps is recommended because the whole food web ultimately disturbs.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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