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Short Communication

Growth Performance, Antioxidants, Intestinal Microbial Composition and Histological Effect of Grass Carp (*Ctenopharyngodon idella*) through diets Enriched to Pomegranate Peel (*Punica granatum*) Extracts

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Abstract

The pomegranate plant is considered one of the medicinal herbs rich in antioxidants that can be used in aquaculture to support immunity and health. The objective of this investigation was to ascertain the impact of pomegranate peel (*Punica granatum*) extract (PPE) on growth performance, the intestinal bacteria count, total number of fungi and histological analysis of the grass carp. One hundred five fingerlings (11.04±0.22g) were distributed among 21 aquariums (60-L) in a completely randomized design (triplicated) and fed diets containing 0, 0.5, and 1% PPE for 70 days at a feeding rate of 3% body weight. A spectrophotometry assessment indicated for PPE that the total phenol content was 151.09 and 175.95 GAE/100 g, and flavonoid content was 36.04 and 42.89 RE/100 g, respectively. The results of the present work revealed that growth indicators (final weight, weight gain, specific growth rate (SGR), and feed conversion ratio (FCR)) increased ($P<0.05$) in the PPE group in comparison to the control group. PPE had an impact on the total count of aerobic bacteria or lactic acid bacteria (LAB), but the experimental treatments dramatically decreased the amount of enteric Gram-negative bacteria ($P<0.05$). The total fungi count showed a significant increase in all treated fish ($P<0.05$). Also, histological examination showed improvement and elongation of the villi, as well as an increase in goblet cells for the treatments supplemented with PPE. Therefore, it is recommended to use PPE (0.5%) as a diet additive for grass carp to improve their growth performance and health.

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1. Introduction

Protecting the fish from infections while obtaining the most product per farm unit was one of the most important concerns in aquaculture about the development of intensive aquaculture systems; such a rearing intensification is stressful for fish that negatively affects the fish health (Liu *et al.,* 2016). The most effective way to address this problem, according to researchers, maybe to improve the quality of fish feed, and high-performance feeds have long been the subject of study. As a result, several synthetic feed additives, particularly antibiotics, have been added to fish feeds to preserve the quality of the meal and safeguard fish against potential illnesses and environmental issues (Ogunkalu *et al.,* 2019). Antibiotic-resistant bacteria, however, are known to arise as a result of the prolonged use of antibiotics and related synthetics (Cabello, 2006). Because of its harmful side effects on fish, humans, and animals, the use of antibiotics and other chemotherapeutics to treat diseases has come under fire. Therefore, the goal is to identify substitute natural promoters that are acceptable, affordable, safe, and safe. Various edible plants and their extracts, along with herbs, spices, and vegetables, have been proposed as unconventional feed additives, growth boosters, or immunostimulants (Badawi and Gomaa, 2016). Phytogenics, defined as ecologically benign plant-derived bioactive chemicals utilized as functional feed additives that exhibit favorable impacts on animal growth and health, have come to light as a secure substitute (Christaki *et al.,* 2020).

Aquaculture is interested in plant materials because the addition of plant-derived materials because they contain phenolics and flavonoids with diverse effects such as enhancing feed efficiency and digestion, reducing nitrogen excretion, and enhancing the gut microbial community and animal health status (Encarnaço, 2013). However, several studies have been conducted on several plants and the effects of their extracts on different fish, such as orange peel and Dayak Onion (*Eleutherine bulbosa*) on the growth performance of Nile tilapia (Salem and Abdel-Ghany, 2018; Fauzi *et al.,* 2024) and *Canarium indicium* on the growth and health of Asian seabass (*Lates calcalifer*) (Abdullah *et al.,* 2024), and other derived plants in feed fish. Nevertheless, one of the most prominent of these plants is pomegranate (*Punica granatum*), which has a long history in medicine and is rich in biologically active substances such as anthocyanidins, hydroxybenzoic acids, and flavonoids is one of these plant additives. Due to its high phenolic content, it has demonstrated various therapeutic benefits (Akhtar *et al.,* 2015).

The fruit of the pomegranate is frequently consumed both raw and in processed forms such as juice, jam, and wine. Pomegranate peel, a byproduct of the pomegranate juice business, is hence a low-cost commodity. Pomegranate peel makes up around 50% of the weight of the fruit and is known to contain proanthocyanidins, ellagitannins, flavonoids, complex polysaccharides, and high molecular weight phenolics. It also contains significant amounts of microelements. Strong apoptotic, antibacterial, antioxidant, and anti-mutagenic effects have been observed for these substances (Seeram *et al.,* 2005; Öztürk *et al.,* 2018). Along with other polyphenolic components, pomegranate peel also has significant concentrations of flavonoids, catechins, ellagic acid, flavonones, flavones, and anthocyanidins (Naveena *et al.,* 2008).

Because of this, it has frequently been used as a natural antioxidant in a range of nutritional supplements. However, several studies have shown beneficial results of pomegranate peels in feed in the fish tested in terms of immune parameters, antioxidants, and hematological and tissue profiles, such as Hussein *et al.* (2022) on Nile tilapia (*Oreochromis niloticus*); Sönmez *et al.* (2022) on Rainbow trout (*Oncorhynchus mykiss*); Rahbarian *et al.* (2022); Mohammadian *et al.* (2022) and Yousefi *et al.* (2023) on common carp (*Cyprinus carpio*), and Gupta *et al.* (2023) on rohu (*Labeo rohita*). Given its economic importance, the grass carp (*Ctenopharyngodon idella*) is one of the most extensively farmed freshwater fish species worldwide. Its significant reproductive powers, quick development rates, and high nutritional content make it an exceptional fish species. (FAO, 2016; Shehata *et al.*, 2018). Grass carp are preferential browsers of plants, and species will be consumed in order of preference; the access that grass carp have to them, however, contributes positively to the environment and public health by enhancing it through the removal of refuge vegetation that conceals snails (Hofstra, 2014; Mohammed *et al.*, 2024). Thus, according to Hamouda and Moustafa (2020), grass carp have the remarkable capacity to break down a large number of aquatic plants and release and recycle the nutrients that are present in them. This stimulation of preferred plankton communities leads to increased fish production and a reduction in freshwater wastage. Given the economic significance of grass carp and the potential benefits of PPE for enhancing immune and health system function, the goal of this study was to determine its effects on oxidative status, bacterial count, and histological changes of the intestine in

grass carp.

2. Materials and Methods

2.1 Materials

The materials that have been used are fish fingerlings, pomegranate peel, alcohol (ethanol), Folin-Ciocalteu reagent, sodium carbonate, gallic acid, NaNO2 solution, AlCl3, sodium hydroxide, agar (Plate Count, MRS, and Potato dextrose), commercial kits, formaldehyde solution, xylene, paraffin wax, and pigments.

The tools that have been used are a plastic pool, pestle, mortar, distilled water, layers of gauze, electric oven, Soxhlet apparatus, filter papers (Whatmann No. 2), rotating vacuum evaporator, water bath, spectrophotometer, homogenizer, Microtome, glass slides, and light microscopy.

2.1.1 Ethical approval

The ethical committee at the aquaculture unit laboratories, Collage of Agriculture, granted the necessary ethical approval for the study (UOB/COA.0001-IQ-2024-9-22). This work adheres to all the rules and regulations of the Heilongjiang River Fisheries Research Institute (CAFS) regarding the care and use of laboratory animals.

2.1.2 Preparation of grass carp

Grass carp (*C. idella*) fingerlings were brought from the Marine Science Center station to the Aquaculture Unit laboratory. They were acclimated to laboratory conditions for two weeks on a control diet in a 500-liter plastic pool. During the acclimation period, fingerlings were fed 5% of body weight twice daily (8 a.m. and 4 p.m.) on comparison diets. Feces and residual feed were removed from the rectangular aquariums, and ventilation was provided throughout the acclimation period to avoid stress.

2.1.3 Preparation of extracts

Pomegranate peels were separated, cleaned with tap water, and dried to a dryness of 700 mmHg in a vacuum oven at 50°C. The dried peels were processed into a coarse, 1 mm-sized powder using a pestle and mortar before being kept at 4°C in an incubator. 25 g of powdered pomegranate peel and 250 ml of distilled water (1:10) were combined, shaken for 30 minutes at 150 rpm, and allowed to soak for 24 hours in the refrigerator to make the aqueous extract (Handa *et al*., 2008). A concentrated extract was found at the bottom of the drying jar after the mixture was filtered through several layers of gauze to remove the insoluble plant materials and again using filter papers (Whatmann No. 2). After that, the filter was removed and placed in an electric oven set to 40°C to dry entirely. The Soxhlet extraction method, which involves enclosing 50 g of powdered materials in filter paper and placing them in the thimble chamber of the Soxhlet apparatus, was used to carry out the extraction process. The round bottom flask was filled with 95% ethanol as the extraction solvent, and the electronic hot plate was calibrated to the boiling point. After the extraction was finished and the refluxing solvent turned clear, the procedure was continued for eight to nine hours. A rotating vacuum evaporator operating at 60°C in a water bath was used to extract the ethanol. After that, the pure extract was frozen until needed.

2.1.4 Total phenolic compounds and total flavonoids assay

The Folin Ciocalteu reagent was used to analyze the compounds' phenolic content according to Chun *et al.* (2003) method. The Folin-Ciocalteu reagent and 0.5 ml of the extract were mixed together, to put it. The solution was kept at 25°C for five to eight minutes before adding 2 ml of sodium carbonate solution 7.5% and adjusting the volume to eight ml with water. After two hours, the absorbance was measured at 725 nm. Gallic acid served as the standard for the calibration curve. The total phenolic content was stated as $mg.g^{-1}$, or gallic acid equivalents per gram of sample. On the other hand, according to Zhishen *et al.* (1999) a colorimetric test was used to determine the total flavonoid concentration. Briefly, 100 ml of the extract was combined with four ml of distilled water. After that, 0.3 cc of 5% $NaNO_2$ solution was added. After five minutes, 0.3 cc of 10% $AlCl_3$ was added. After six minutes, 2 ml of 1 M sodium hydroxide was added to the mixture. After the liquid had been diluted, 3.3 cc of distilled water was added right away and thoroughly mixed. The absorbance was measured at 510 nm relative to a blank. Rutin was the reference point for the calibration curve. The total amount of flavonoids in the extract was calculated using rutin equivalents per gram of sample ($mg.g^{-1}$).

2.2 Methods

2.2.1 Experimental procedure

After adding PPE powder (0.5-1%), in addition to the control diet (C), a quantity of commercial ration was used. Then, using 60-liter rectangular glass aquariums, a feeding experiment was carried out indoors to assess the growth performance of grass

carp fingerlings for a 70-day raising period. With 10 cm of float, 50 liters of fresh water were placed within rectangular glass aquariums, which were then netted off. The tap was used to get fresh water. A completely randomized design was used to set up the experimental ponds, with three replicates of each treatment. After recording the initial weight, grass carp fingerlings (45 days old) were supplied at a random rate of five fingerlings in each experimental tub. The fingerlings were fed two times daily, at eight hours and 16 hours, with various experimental diets at a rate of 3% of body weight from Fingerlings were fed twice daily, every 8 and 16 hours, with different experimental diets at 3% of body weight of control (0%) and raw pomegranate peel powder T 1 and T 2 (0.5-1%). The same ratio is for the aqueous extract powder (T3 and T4) and the alcoholic extract (T5 and T6). Generally, to keep the experimental animals from feeling stressed, aeration was delivered in each tub. Thus, every day at 7:00 a.m., unfinished food and waste were removed, and each tub's water was drained and then refilled with the same amount of freshwater.

2.2.2 Growth performance parameters

At the end of the trial period, the following indicators shown by fingerlings fed on the experimental diets were calculated using the following mathematical formulas:

WG = Wf-Wi.....(i)
Where :
WG = Weight gain
Wf = final weight
Wi = initial weight

SGR %/Day) = 100×[(lnWf-lnWi)/days].....(ii)
Where :
SGR = Specific Growth Rate
lnWf = Natural logarithm of final weight
lnWi = Natural logarithm of initial weight

(FCR) = FI / WG.....(iii)
Where :
FCR = Feed Conversion Rate
FI = Feed intake
WG = Weight gain

2.2.3 Intestinal bacteria count

For the microbiological analyses, three fish were randomly selected from each aquarium. In short, 70% alcohol was used to clean the fish's body surface, and the Motlagh *et al.* (2019) advised approach was used to remove the fish's intestines. The intestinal samples were placed in two-centiliter cryovials containing fifteen glass beads. Using a homogenizer (Bioprep-24, China), the materials were homogenized for 20 seconds at 4,000 rpm. Serial dilutions between 108 and 1010 were made using sterile saline solution. The total number of aerobic bacteria, lactic acid bacteria (LAB), enteric Gram-negative bacteria, and fungi were counted using the Plate Count Agar (Merck, Germany), MRS Agar (Merck, Germany), Potato dextrose agar (Ibersco, Switzerland), and MacConkey agar (Merck, Germany) media, in that order. The incubation was carried out under aerobic conditions for 24 hours at 37°C.

2.2.4 Antioxidant activity

Antioxidant enzyme activities of fish were determined in liver tissues at period end using commercial kits according to the instructions of the manufacturers: Catalase (CAT) (CAYMAN Catalase Assay Kit, Item no: 707002) and Glutathione peroxidase (GPx) (CAYMAN Glutathione Peroxidase Assay Kit, Item no: 703102).

2.2.5 Histology analysis

The digestion of broken-down food into the stomach starts in the midgut, which is the forward section of the gut. It's interesting to note that the midgut, which is the longest part of the gut, varies greatly in length and is typically associated with the feeding patterns of the fish species (De Marco *et al.*, 2023). However, samples were collected and put in vials containing 10% neutral buffered formaldehyde solution (pH 7). They were then cleaned in xylene, rinsed in tap water, dried in a graduated series of ethanol concentrations, embedded in paraffin wax with a melting point of 54–56°C, sectioned using a Cambridge Rocking Microtome to a thickness of 5 m, and placed on glass slides sterile. Following deparaffinization, sections were ready for light microscopy inspection and histological investigations by being stained with hematoxylin and eosin (H&E) (Romeis, 1989).

2.2.6 Analysis data

The data was shown as mean ± SD. A one-way analysis of variance (ANOVA) was performed on the data to ascertain the effect of treatment inclusion on fish performance. Data were examined with the IBM SPSS (2022) program, Version 26. The multiple range test of LSD was employed to examine mean differences at the significance level ($P<0.05$).

3. Results and Discussion

Water quality parameters such as temperature (24.33°C), pH (8.77), salinity (1.29 ppt), and dissolved oxygen (9.44 mg/l) were assessed weekly following American Public Health Association standard methods (APHA, 1998).

The present findings in Figure 1 show that the alcoholic extract had the highest phenol and flavonoid concentration, followed by the aqueous extract. According to a study by Badawi and Gomaa (2016), the alcoholic extract contained 185 GAE.100 g^{-1} of phenols and 32 RE.100 g^{-1} of flavonoids, whereas a study by Al-Jandal (2021) revealed that the phenols in the alcoholic and aqueous extracts, respectively, were 154.75 and 127.32 GAE.100 g^{-1} of phenols. However, the present findings are in agreement with those Aqil *et al.* (2006), who found flavonoids and phenols in the alcoholic extracts of several medicinal plants, including pomegranate. In addition, the study of Mashkor and Muhson (2014) which showed that the number of phenols ranged from 84.15 to 168.26 GAE.100 g^{-1}.

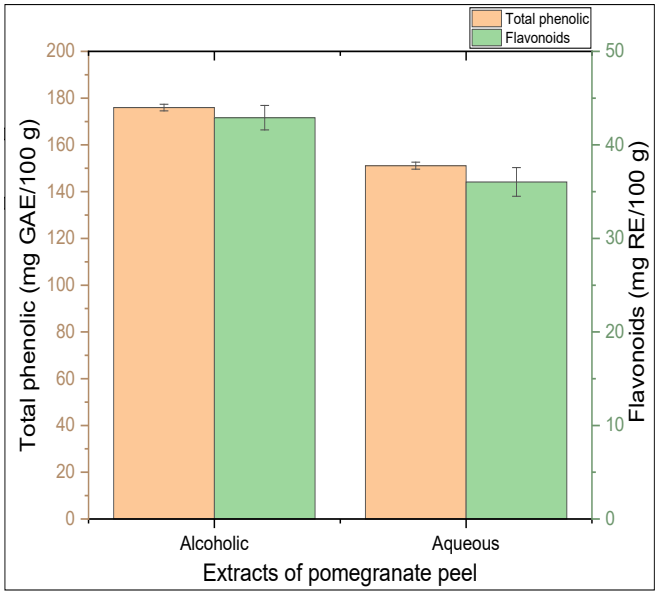

Figure 1. PPE's total flavonoid and phenolic content (n = 3). RE/100 g = mg rutin equivalents, and GAE/100 g = mg gallic acid equivalents.

Overall, Wang *et al.* (2011) and Malviya *et al.* (2014) reported pomegranate peels contain more phenols and flavonoids when solubilized in ethanol than when solubilized in water, methanol, or ether. The capacity to extract phenols from plants is generally significantly influenced by the solvent (Turkmen *et al.*, 2006). Therefore, the significance of phenolic compounds is underlined by the fact that they are potent natural antioxidants with numerous culinary and medical applications, such as recycling these wastes and employing them as substitutes for industrial antioxidants and food additives.

The present results are consistent with several studies, including El-Sayed *et al.* (2014) on Nile tilapia (*O. niloticus*), in which 5% PPE was used with the enzyme *Allzyme* SSF without negative effects on growth parameters, and the study of Avazeh *et al.* (2021) on Rainbow trout (*O. mykiss*) fed on PPE, as 1% achieved the best value for weight gain, while the specific growth improved significantly ($P<0.05$). Additionally, the findings of our study agreed with those of Toutou *et al.* (2019), who included PPE in the diet of monosex Nile tilapia (*O. niloticus*). The ratio of 1000 mg/kg of PPE was found to be superior to all treatments in the study by Sönmez *et al.* (2022).

The result showed that the SGR is superior in PPE compared to the control treatment (C) except for T1 (Figure 2). This may be due to the reduction of stress, as well as the increase in vital reactions and, thus the increase in building muscle tissue in fish. Phenolic compounds may be the reason for improving the digestion process (Lovkova, 2001; Schultz *et al.*, 2004).

Antioxidants generally have two main functions: they either quench free radicals or chelate redox metals to prevent or mitigate oxidative stress (Ayyat *et al.*, 2020). For instance, it has been observed that adding purple coneflower (*Echinacea purpurea*) polyphenols as a nutritional supplement to the feeding regimen has antioxidant benefits that shield fish against disorders linked to oxidative stress (Oniszczuk *et al.*, 2019).

The present results showed that the treatments in which PPE were added significantly superior ($P>0.05$), as an increase in the Cat and GPx enzymes was observed (Figure 3). However, some phenolic compounds found in PPE, such as protocatechuic acid, calcium acid, pyrogallol, p-coumaric acid, catechins, rosmarinic acid, rutin, naringenin, myricetin, scopoletin, and hesperidin, may be the cause of the increase in CAT and GPx. These compounds have antioxidant properties by lowering stress brought on by the production of free radicals and lipid peroxidation (Moskaug *et al.*, 2005; Mashkor and Muhson, 2014). By enhancing the elimination of free radicals by CAT and GPx, PPE may play a significant role in shielding tissues from oxidative damage (Abdel Moneim, 2012).

The trend to employ natural antimicrobials in aquaculture feeds has developed since controlling the microbial population promotes better health management and higher productivity in aquaculture (Motlagh *et al.*, 2019). It is commonly known that polyphenols, tannins, and flavonoids have antibacterial properties. Studies have shown a connection between microbial inhibition and PPE's total phenolic content (Naz *et al.*, 2007; Prashanth *et al.*, 2001).

The impact of PPE on the intestinal bacterial count in grass carp is seen in Table 1. PPE-treated treatments did not significantly enhance the number of lactic acid bacteria or total aerobic bacteria. The PPE treatments considerably reduced the number of enteric Gram-negative bacteria compared to the control ($P<0.05$). Additionally, according to the data, there were considerably more total fungi in the 1, 2, and 4% treatments than in the control and 0.1 PPE-treated fish ($P<0.05$).

Figure 2. Growth parameters for grass carp (WG = weight gain; RGR = relative growth rate and SGR = specific growth rate). The data are shown as mean ± SD for each replication (n = 3). Letters denote statistical differences ($P<0.05$) between treatments.

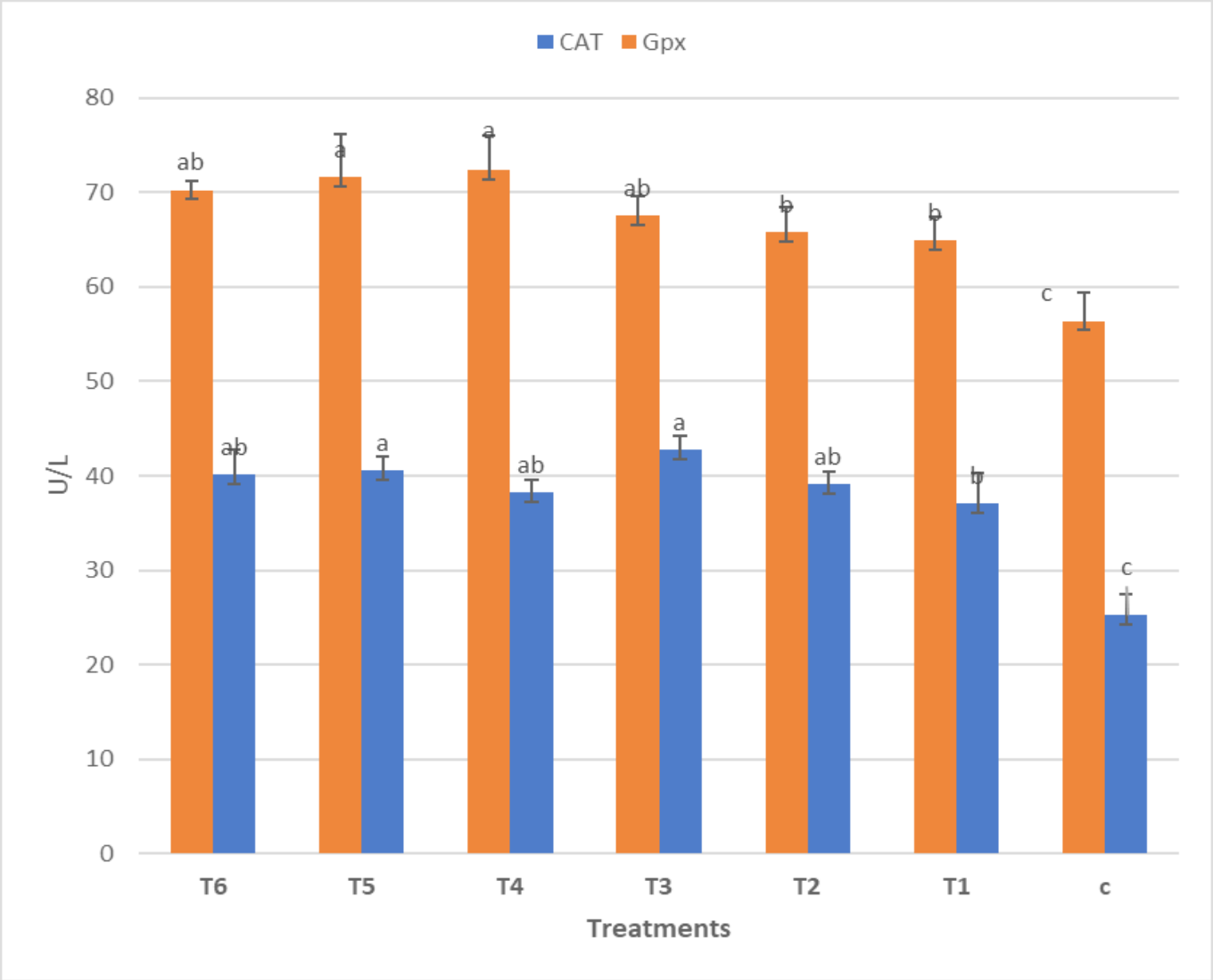

Figure 3. CAT and GPx activity of common carp in groups fed supplementation with PPE. (See Fig. 2 for statistical information).

Table 1. Total aerobic bacteria (10^5), LAB\lactic acid bacteria (10^3), enteric gram negative (10^3) and Fungi (10^5) bacteria in the gut of grass carp for 70 days (mean ± SD, n=3).

Treatments	Total Aerobic Bacteria	LAB	Enteric Gram Negative	Fungi
C	63.12 ± 9.43^{a}	17.07 ± 1.72^{b}	147.05 ± 5.80^{c}	30.41 ± 3.12^{b}
T1	69.23 ± 4.99^{a}	26.40 ± 1.56^{a}	122.85 ± 2.56^{b}	55.20 ± 5.50^{a}
T2	55.39 ± 8.91^{a}	25.90 ± 2.63^{a}	121.16 ± 4.68^{ab}	61.09 ± 4.71^{a}
T3	78.45 ± 7.60^{a}	28.82 ±2 .66^{a}	100.67 ± 2.43^{a}	80.12 ± 8.21^{a}
T4	72.86 ± 6.69^{a}	26.69 ± 2.86^{a}	101.08 ± 2.65^{a}	69.45 ± 5.20^{a}
T5	80.23 ± 12.13^{a}	28.30 ± 2.21^{a}	111.78 ± 5.83^{ab}	77.12 ± 9.19^{a}
T6	70.93 ± 9.67^{a}	27.39 ± 1.61^{a}	114.52 ± 4.33^{ab}	71.78 ± 7.07^{a}

Different superscripts in the same column show that there are significant differences ($p < 0.05$).

Generally, controlling microorganisms supports enhanced production in aquaculture and helps to improve health management. Therefore, antimicrobials play a part in controlling development and reproduction, and their use in aquaculture feed has become more crucial (Motlagh *et al.,* 2019; Wang *et al.,* 2019). By taking part in the release of digestive enzymes (amylase, protease, and lipase), microbial communities in the digestive tract improve the digestion of proteins, lipids, and carbohydrates in the feed; more than 50 kinds of LAB are helpful species, lactic acid is produced by these bacteria, and they consume carbohydrates as fuel (Motlagh *et al.,* 2012; 2017). According to Charalampopoulos *et al.* (2002) indigestible feed components like fiber can encourage LAB proliferation and have symbiotic effects.

Adding PPE to the diet boosted LAB in the intestines and decreased Gram-negative bacteria, according to the present results. However, the antibacterial properties of phenolic substances including punicalin, ellagic acid, cholic acid, and anthocyanins found in pomegranate peels are the cause

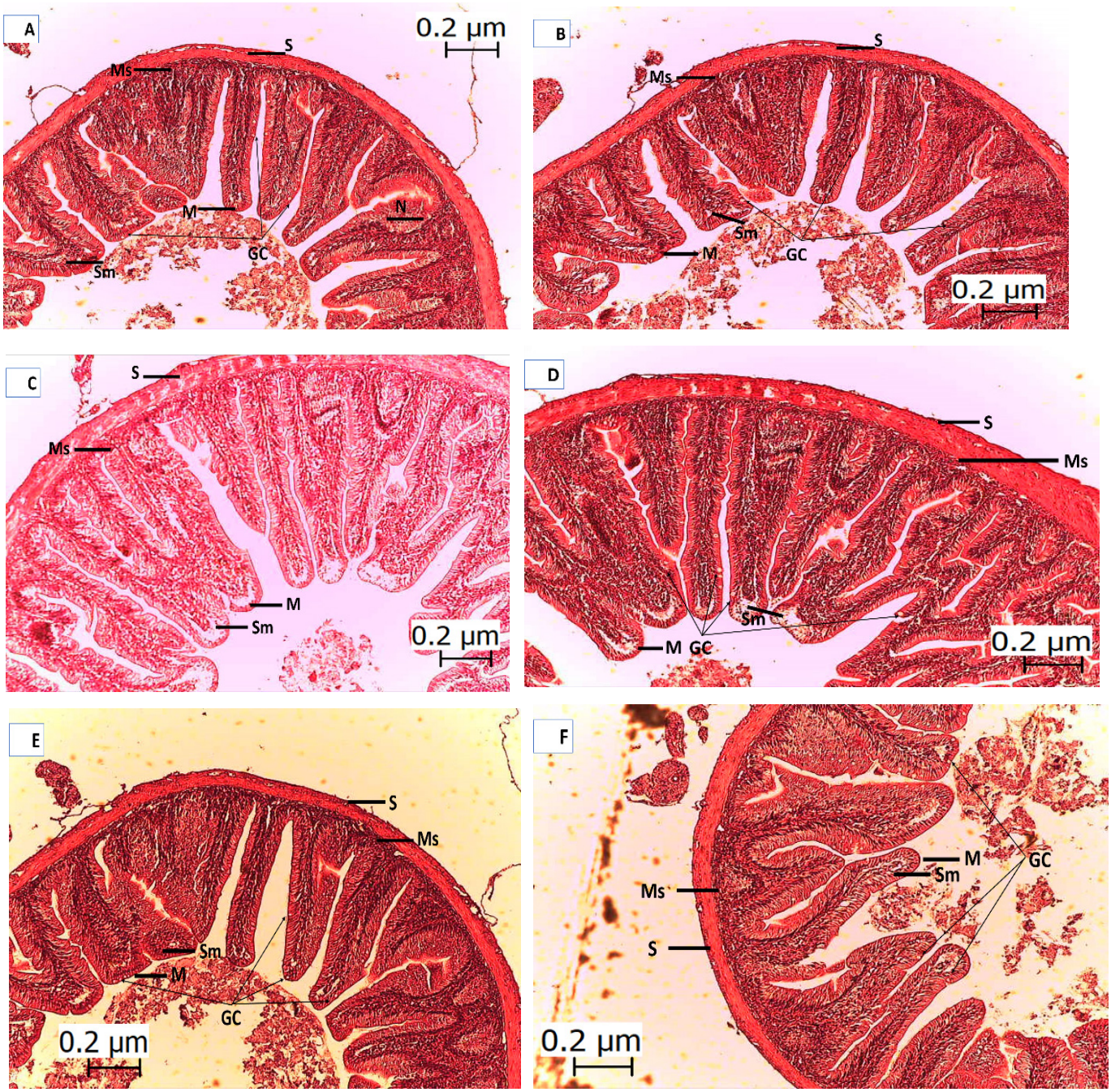

Figure 4. Cross-section of fish intestine showing normal structures and volume variation in fold size. (A) Control, (B-F) fish fed on PPE, where the villi elongated. M, mucosa; SM, submucosal; Mr, musellaris; S, serosa; GC, goblet cells and N, necrosis (HE, 400X).

of this. This activity is caused by punocalin and gallic acid (Abdollahzadeh *et al*., 2011; Naz *et al*., 2007; Reddy *et al*., 2007). In general, phenolic compounds are described as antibacterial by suppressing harmful bacteria in the mucous layer of the intestine and adhering to pathogenic microbes via the "lectin-receptor" mechanism (Vidanarachchi *et al*., 2005).

On the other hand, the present results of histologic analysis in the treatments fed PPE showed there was a considerable lengthening and depth of the crypts, which provides a greater region for absorption, in contrast to the control treatment (C), which had necrosis and villi that were shorter. However, the mechanisms and processes that take place in the digestive system are included, and the term "gut health" is currently receiving significant interest in the field of animal production (Laudadio *et al*., 2012). In order for the intestinal mucosa to play its intended job, the structure and functionality of its surface are essential for maintaining intestinal health.

Histological analysis of the intestines of fish fed a diet consisting of PPE showed effects on the intestine, including fusion and elongation of villi and the rise of goblet cells (Figure 4). However, when analyzing food additives and commercial feeds, the lengthening of the villi is a helpful histological sign that may be monitored; this is related to the current findings are consistent with studies by Toutou *et al.* (2019) and Hussein *et al.* (2022) by expanding the absorption area for nutrients (Aanyu *et al*., 2014). As opposed to the control treatment (C), the results showed that PPE increased the number of goblet cells. Thus, these cells function as lubricators through the mucus they secrete and are connected to fish's immunological state, which may increase in response to irritation (Da Silva *et al*., 2012).

4. Conclusion

The current study shows that dietary supplements for grass carp, whether aqueous or alcoholic, will not have a negative impact on growth. Additionally, gut microbial tests showed that PPE had an effect on total fungal counts and Gram-negative enteric bacteria, which both decreased and increased, respectively. With no adverse effects on fish growth, giving a diet containing PPE will alter the gut flora. The intestines also had a striking look that suggested effective absorption in this aspect. It seems that adding this extract, especially 0.5%, to aqua-feeds may be useful for creating functional diets. Finally, the current findings and related studies support the usefulness and effectiveness of PPE in aquaculture.

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Authors' Contributions

All authors contributed to the drafting of the manuscript. Fazaa; carried out the analytical statistics for the experiment. Sayed-Lafi; collected the pomegranate peel, extracted it, and measured the antioxidant and other parameters of the blood. Sultan; prepared the first draft of the manuscript in addition to the methodology.

Conflict of Interest

The authors declare that they have no competing interests.

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