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APPLIED STUDIES

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Growth performance and intestinal microbial changes of *Carassius auratus* in response to pomegranate (*Punica granatum*) peel extract-supplemented diets

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Abstract

This study aimed to investigate the effect of pomegranate (*Punica granatum*) peel extract (PPE) on growth indices, intestinal bacteria, and total fungi count of *Carassius auratus* intestine. Ninety *C. auratus* fries (11.04 ± 0.22 g) were distributed among 15 aquariums (100-L) in a completely randomized design (triplicated), and were fed diets containing 0.1, 1, 2, and 4% PPE for 60 days at a feeding rate of 2% body weight during the experiment. A spectrophotometry assessment indicated that the total phenol and flavonoid content of PPE was 5.44 ± 0.03 and 49.80 ± 1.00 mg/g, respectively. According to the results, the extract significantly reduced the growth indices (final weight, weight gain, specific growth rate (SGR), and feed conversion ratio (FCR)) at 4% compared to the control ($p < .05$). PPE did not affect the total aerobic bacteria or total lactic acid bacteria count, but the enteric gram-negative bacteria count was significantly reduced in the experimental treatments ($p < .05$). The total fungi count showed a significant increase in all treated fish ($p < .05$). According to the results, because of the negative effect of the high dose of PPE on

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growth, there is a recommendation for it to be used at 2% of the diet to reduce intestinal gram-negative bacteria and increase the total fungi count.

KEYWORDS
aquaculture, flavonoid, intestinal microbiota, phenol, pomegranate

1 | INTRODUCTION

Aquaculture is experiencing the fastest growth in food production industries (Ladisa, Bruni, & Lovatelli, 2017), especially in terms of species diversity, density, and breeding to maximize the production efficiency and profitability. However, the industry also has limitations, such as the spread of various diseases, where antibiotics are widely used to control them. The overuse of antibiotics has caused particular problems, including drug resistance, environmental issues, and bioaccumulation of antibiotics in aquatic tissues (Lee & Gao, 2012). Researchers have been testing antibacterial and immunostimulant substances such as organic acids and their salts, probiotics, prebiotics, synbiotics, and herbal substances to overcome the restrictions regarding the antibiotics (Ahmadniaye Motlagh, Sarkheil, Safari, & Paolucci, 2019). Herbal extracts have been the subject of great attention because of their high efficiency, fewer side effects, low price, and affordability (Ahmadniaye Motlagh, Sarkheil, Safari, & Paolucci, 2019).

Pomegranate (*Punica granatum*) is an important fruit crop adaptable to a wide range of agroclimatic conditions; it is native to Iran but is grown in many countries (Kahramanoglu & Usanmaz, 2016). Pomegranate peel, a juice byproduct (comprises portion, carbohydrates, and Vitamin C), is often considered waste. Phenolic compounds, including flavonoids, tannins, and phenolic acids, are primarily concentrated in the peel portion of the pomegranate fruit (Singh, Singh, Kaur, & Singh, 2018). Pomegranate peel extract (PPE) is known for its antibacterial (Abdollahzadeh et al., 2011), antioxidant (Li et al., 2006; Mushtaq, Sultana, Anwar, Adnan, & Rizvi, 2015), anticarcinogenic, wound-healing (Chidambara Murthy, Reddy, Veigas, & Murthy, 2004), cytotoxic, hypoglycemic (Rajput, Sagar, & Adiga, 2011), hypolipidemic, hepatoprotective (Belal, Abdel-Rahman, Mohamed, Osman, & Hassan, 2009), and anti-inflammatory (Jurenka, 2008) properties. It is also used in the food industry as a preservative agent in foods prone to oxidative corruption (Al-Zoreky, 2009; Tarkhasi, 2016; Zarei, Ramezani, Ein-Tavasoly, & Chadorbaf, 2015). A study examined the effects of edible PPE coating on the quality and shelf life of silver carp fillet during refrigerated storage. It was observed that the addition of PPE considerably delayed lipid oxidation and prevented microbial spoilage in silver carp fish fillet (Tarkhasi, 2016). Phenolic antioxidant compounds such as Ellagic Acid in PPE have also shown significant antimicrobial activity against pathogenic bacteria such as *Vibrio*, *Salmonella*, and *Escherichia coli* (Siri, Wadbua, Wongphathanakul, Kitancharoen, & Chantaranothai, 2008). Although the studies on the use of this substance in aquaculture have been limited, the positive effects of PPE have been proven on the nonspecific immune system (Oliveira et al., 2010), resistance to viruses (Ismail, Sestili, & Akhtar, 2012), wound healing (Chidambara Murthy et al., 2004; Rajput et al., 2011), and growth performance (Kishawy, Omar, & Gomaa, 2016) of terrestrial animals. Recently, researchers studied the effect of PPE in the diet of *Oreochromis niloticus* (Badawi & Gomaa, 2016) and *Cyprinus carpio* (Shafiei, Soofiani, Ebrahimi, Nematollahi, & Mohebbi, 2016). Based on the results, PPE could improve the efficiency of nonspecific immunity and blood parameters (Alanine aminotransferase (ALT), aspartate aminotransferase (AST), cholesterol, triglyceride, low-density lipoprotein cholesterol (LDL), hematocrit (Hct), and WBC) in both fishes, but it had no significant effect on *O. niloticus* growth. Another study investigated the protective effects of pomegranate against a parasitic Ciliophora, *Philasterides dicentrarchi* in olive flounder (*Paralichythys olivaceus*). According to results, pomegranate-fortified diets improved hematological and blood biochemical parameters and boosted the innate immune system in olive flounder against the parasite (Harikrishnan, Kim, Kim, Balasundaram, & Heo, 2012).

Carassius auratus is a freshwater ornamental fish belonging to the Cyprinidae family. This fish is a good model for biological research because of some special advantages such as ease of cultivation and reproduction in laboratory conditions (Ahmadniaye Motlagh et al., 2017). *C. auratus* has been subjected to various investigations, but to date, no study has reported the effects of dietary PPE on the gut microbiota of this fish or other aquatics. Accordingly, this study was designed to investigate changes in *Carassius auratus* intestinal bacteria and fungi in response to *Punica granatum* peel extract-supplemented diets.

2 | MATERIALS AND METHODS

2.1 | Preparation of methanolic extract of pomegranate peel

The pomegranate peels were oven-dried (at 40°C for 10 days), milled, and well mixed with methanol (96%) at the ratio of 1:10 and then stirred for 48 hr for complete extraction. The solvent was smoothed using a vacuum pump, and the initial extract of the powder was obtained. To measure the dry extract, the samples were placed on a rotary shaker at 40°C at 180 rpm, where the concentrated sample was poured into a Petri dish and dried at 40°C for 48 hr. The extract, at a concentration of 20 mg/ml was used to create the experimental diets.

2.2 | The concentration of total phenols and total flavonoids in PPE

The total content of phenol in PPE was assayed through spectrophotometry. According to the standard method (Capannesi, Palchetti, Mascini, & Parenti, 2000), 0.5 ml of the sample was mixed with 0.5 ml of Folin, after which (1 min later) 1-ml sodium carbonate was added. The solution volume was diluted to 10 ml with distilled water. After 2 hr of exposure to darkness, the absorbance was read at 765 nm.

The spectrophotometric method was also used to determine the total flavonoid content based on the formation of a complex flavonoid– aluminum (Roshanak, Rahimmalek, & Goli, 2016). first, 0.5 ml of the previously prepared PPE was mixed with 2 ml of distilled water and 0.15 ml of NaNO2 (5%). Then, 0.15 ml of AlCl3 (10%) was added after 6 min, with the solution being left for another 6 min. Subsequently, 2 ml of NaOH (4%) was added to the mixture; then, by adding distilled water, the volume of the solution was diluted to 5 ml. After 15 min, the absorbance was read at 510 nm.

2.3 | Experimental design and experimental diets

A total of 90 healthy *Carassius auratus* fries (11.04 ± 0.22 g) were randomly distributed between 15 glass aquariums (100 L) at a density of 6 fish per tank. After 10 days of adaptation to laboratory conditions, the experiment was carried out in the form of a completely randomized design (triplicated). Commercial ornamental fish feed (Energy Iran) was used during the 60 days of the experimental period. The nutrient compositions of feed were as follows (%): dry matter 69.74 ± 2.25, crude protein 40.92 ± 1.37, crude lipid 3.72 ± 0.70, ash 2.72 ± 0.59, crude fiber 3.15 ± 0.95, and nitrogen-free extract 19.23 ± 1.12. To prepare the experimental diets, PPE was sprayed on the basal diet at levels of 0% (control), 0.1%, 1%, 2%, and 4% according to the literature and pretest. Based on the protocol (Ahmadniaye Motlagh, Sarkheil, Safari, & Paolucci, 2019), gelatin (8 g/L) was mixed with PPE to prevent extract leaching into the water. The prepared diets were air-dried and stored at 4°C until use. The physicochemical parameters of the water, such as temperature (28.2 ± 3°C), dissolved oxygen (6.7 ± 0.5 ppm) and pH (7.8 ± 0.5), were maintained in accordance with the standard culture conditions. Feeding was performed at 2% body weight, and all experiments were carried out according to Ferdowsi University of Mshhad animal ethics.

2.4 | Sample collection, growth performance, and gut microbial analyses

At the end of the trial, all fish were weighed on a digital scale individually (0.01 g) to evaluate the final weight. Weight gain, SGR, and FCR were calculated as follows:

$$\text{Weight gain}(\text{g}) = \text{Final weight} - \text{Initial weight}$$

$$\text{SGR} = [(\text{Ln final weight}(\text{g}) - \text{Ln initial weight}(\text{g}))/\text{experiment days}] \times 100$$

$$\text{FCR} = \text{Feed consumed}(\text{g})/\text{Weight gain}(\text{g})$$

After anesthetizing the fish using clove powder (0.5 g/L), three fish per each aquarium were randomly selected for microbial analyses. The fish's body surface was disinfected with 70% alcohol, with the fish intestine removed according to the standard method of Ahmadnia and co-workers (Ahmadniaye Motlagh, Sarkheil, Safari, & Paolucci, 2019). The intestinal samples were transferred into cryovials (2 cc) containing 15 pieces of glass beads. The samples were homogenized by a homogenizer (Bioprep-24, china) for 20 s at 4,000 rpm. Serial dilutions within the range of 10^{-8} to 10^{-10} were prepared using sterile saline solution. Plate Count Agar (Merck, Germany), MRS Agar (Merck, Germany), Potato Dextrose Agar (ibersco, Switzerland), and MacConkey Agar (Merck, Germany) media were used to count the total aerobic bacteria, lactic acid bacteria, fungi, and enteric gram-negative bacteria, respectively. The incubation was performed for 24 hr at 37°C under aerobic conditions.

Determining the normality of data using the Kolmogorov–Smirnov test and homogeneity of variance using Levene's test, the data were analyzed using one-way ANOVA. Tukey's test was used to compare means ($p < .05$). Polynomial regression models were applied to determine regression relations between PPE levels, SGR as well as FCR, and enteric gram-negative bacteria count in *Carassius auratus* fries.

3 | RESULTS

Spectrophotometry assessment indicated that total phenol and flavonoid contents of PPE were 5.44 ± 0.03 and 49.80 ± 1.00 mg/g, respectively. The results of different levels of PPE on the growth parameters of *C. auratus* during 60 days of the experiment are presented in Table 1. While 0.1, 1, and 2% PPE showed no significant difference compared to the control, the final weight in the 4% treatment was significantly lower than in the control and other groups ($p < .05$). The SGR and weight gain were also significantly lower in 4% PPE-treated fish than the control ($p < .05$). The highest FCR was observed in 4% treatment ($p < .05$).

Table 2 reports the effect of PPE on *C. auratus* gut microbiota. Treatments receiving PPE had no significant increase in terms of total aerobic bacteria and lactic acid bacteria counts. Enteric gram-negative bacteria count in the PPE treatments was significantly lower than the control ($p < .05$). The results also indicated that the total fungi count in 1, 2, and 4% treatments were significantly higher than in the control and 0.1 PPE treated fish ($p < .05$).

Polynomial models in Figure 1 indicated that there were significant linear regression relations ($p < .05$) between PPE levels, SGR value ($r^2 = .83$) (a), and FCR value ($r^2 = 0.88$) (b). There was a significant inverse logarithm model between PPE levels and enteric gram-negative bacteria count ($r^2 = 0.80$; $p < .05$) (Figure 2).

4 | DISCUSSION

Various portions of pomegranate are rich in vitamins and appetizing substances. They can also serve as a source of rich anthocyanins and antioxidants, thereby supporting growth and health. These features have led to the use of

TABLE 1 Growth performance parameters of *C. auratus* fed pomegranate peel extract (PPE)-supplemented diets for 60 days (mean ± SD, *n* = 3)

Dietary pomegranate peel extract (PPE) levels (%)						
	0	0.1	1	2	4	*p* Value
Initial weight (g)	10.92 ± 0.24	10.87 ± 0.14	11.23 ± 0.12	10.90 ± 0.16	11.29 ± 0.06	.115
Final weight (g)	14.45 ± 0.12^{b}	13.76 ± 0.06^{ab}	14.21 ± 0.40^{b}	13.67 ± 0.73^{ab}	13.01 ± 0.50^{a}	.019
Weight gain (g)	3.53 ± 0.28^{b}	2.88 ± 0.04^{b}	3.00 ± 0.30 ^{b}	2.76 ± 0.45a^{b}	1.72 ± 0.4^{a}	.003
SGR (% BW/day)	0.56 ± 0.04 ^{b}	0.47 ± 0.01 ^{b}	0.47 ± 0.04 ^{b}	0.45 ± 0.85 ^{b}	0.28 ± 0.06^{a}	.002
FCR	2.10 ± 0.00^{a}	2.56 ± 0.02^{ab}	2.65 ± 0.28^{ab}	2.94 ± 0.66^{ab}	3.34 ± 0.25 ^{b}	.006

Note: Means with different letters in the same row are significantly different (ANOVA, $p < .05$).

TABLE 2 Total aerobic bacteria (10^5), lactic acid bacteria (10^3), Fungi (10^5), and enteric gram-negative (10^3) bacteria in the gut of *C. auratus* fed pomegranate peel extract (PPE)-supplemented diets for 60 days (mean ± SD, *n* = 3)

Dietary pomegranate peel extract (PPE) levels (%)						
	0	0.1	1	2	4	*p* Value
Total aerobic bacteria	53.00 ± 10.21^{a}	38.20 ± 17.00^{a}	50.00 ± 9.50^{a}	67.00 ± 12.47^{a}	71.66 ± 15.73^{a}	.068
Enteric gram negative	168.37 ± 18.51^{c}	102.16 ± 16.04^{b}	73.00 ± 9.40^{ab}	60.11 ± 12.00^{a}	68.09 ± 7.11^{a}	.000
Lactic acid bacteria	10.14 ± 6.86^{a}	20.16 ± 8.53 ^{a}	18.96 ± 10.22^{a}	33.65 ± 13.59^{a}	23.47 ± 12.00^{a}	.177
Fungi	27.03 ± 4.00^{a}	85.47 ± 10.44^{b}	46.77 ± 9.87^{a}	79.11 ± 7.32^{b}	99.86 ± 10.84^{b}	.000

Note: Means with different letters in the same row are significantly different (ANOVA, $p < .05$).

pomegranate in the food industry, but few studies have examined the impact of pomegranate peel on various aspects of aquaculture (Tehranifar, Selahvarzi, Kharrazi, & Bakhsh, 2011).

The results of this study showed that the administration of 4% PPE in *C. auratus* diet significantly reduced the final weight and SGR, while FCR increased. A review of the literature revealed that dietary administration of PPE resulted in increased food digestibility, improved beneficial microbial flora, and boosted immunity in broilers without any adverse effect on FCR and weight gain (Rezvani & Rahimi, 2017). Researchers suggested that weight gain in the extract-treated animals could be attributed to increased palatability, improved digestibility, and enhanced antioxidant activity. Furthermore, changes in the intestinal microbial flora can also inhibit the pathogenic bacteria and neutralize their toxins, which can reduce the digestibility of the protein (Rezvani & Rahimi, 2017). The use of diets containing 1, 2, 3, and 4% pomegranate seed powder showed that a maximum of 3% pomegranate seed powder could increase growth in rainbow trout (*Oncorhynchus mykiss*). Phenolic and tannin compounds present in pomegranate seed powder can stimulate appetite and yield a better growth rate to a certain extent (Emadi, Negarestan, & Heidari, 2017).

Indeed, the increased or decreased fish growth in response to feed additives is the result of a cascade of pathways (Ahmadniaye Motlagh, Sarkheil, Safari, & Paolucci, 2019). Studies have shown that high levels of tannins may slow down the absorption of nutrients, digestibility of protein and carbohydrates, palatability, and bowel movements (Reed, 1995). Elsewhere, the effect of pomegranate extract on Holstein calf nutrition showed that digestion of dry matter, organic matter, and starch was not affected, but protein and fat digestion was significantly reduced (Oliveira et al., 2010). In the current research, it seems that, because of to the high amounts of tannins and polyphenols found in PPE and their anti-nutritional properties, intestinal movements, digestion, and absorption of proteins and fats diminished in the 4% treatment.

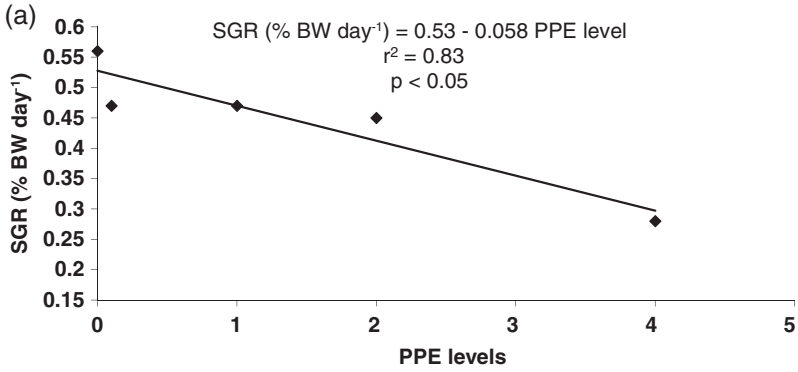

FIGURE 1 Polynomial model fitting specific growth rate (SGR; %BW/day) (a) and feed conversion ratio (FCR) (b) to pomegranate peel extract (PPE) level (%) in *C. auratus* fed PPE-supplemented diets for 60 days (mean ± SD, *n* = 3)

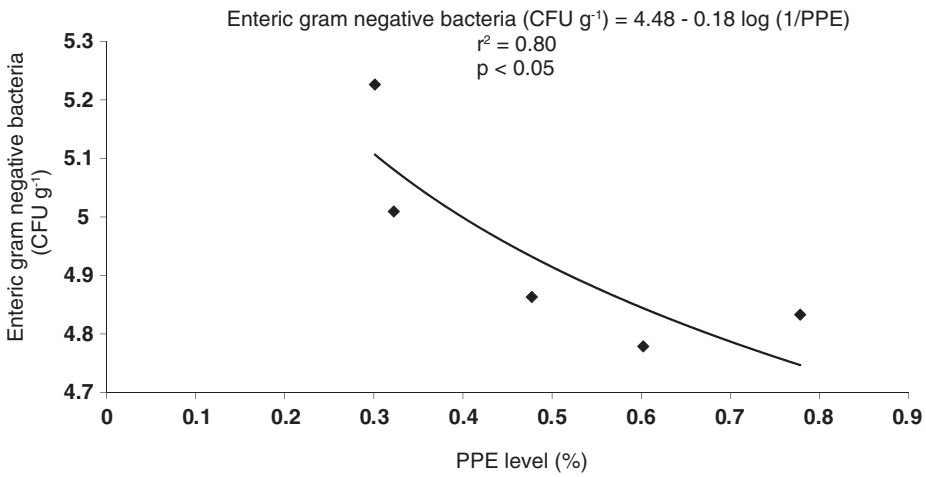

FIGURE 2 Polynomial model fitting enteric gram-negative bacteria count (CFU/g) to pomegranate peel extract (PPE) level (%) in *C. auratus* fed PPE-supplemented diets for 60 days (mean ± SD, *n* = 3)

Controlling the microbial population helps improve health management and supports higher production in aquaculture; hence, today, the tendency to use natural antimicrobials in aquaculture feeds has increased (Ahmadniaye Motlagh, Safari, & Paolucci, 2019). Among the various components of pomegranate, the peel extract has the greatest

in-vitro antimicrobial activity because of high levels of phenolic compounds (such as punicalin, ellagic acid, gallic acid, and anthocyanins) (Abdollahzadeh et al., 2011; Naz, Siddiqi, Ahmad, Rasool, & Sayeed, 2007; Reddy, Gupta, Jacob, Khan, & Ferreira, 2007). The antimicrobial activity of polyphenols, tannins, and flavonoids has been well documented (Naz et al., 2007; Prashanth, Asha, & Amit, 2001). Researchers have indicated the relationship between the total phenolic content of PPE and microbial inhibition. According to the literature reviewed, this study is the first to investigate the effect of PPE on the intestinal microbiota.

Based on the results, the PPE effectively reduced enteric gram-negative bacteria, possibly even the antibacterial activity of hydrolyzable tannins and polyphenolic compounds; in particular, punicalin and gallic acid, are responsible for this (Reddy et al., 2007). The antimicrobial effect of tannins is related to their toxicity and molecular structure. Tannins can adversely influence the bacteria by precipitating proteins throughout the cell membrane (Vasconcelos et al., 2006); they may also suppress the activity of many enzymes such as glycosyltransferases (Abdollahzadeh et al., 2011; Vasconcelos et al., 2006). Thus, the antimicrobial properties of pomegranate extract can directly reduce gastrointestinal infections (Oliveira et al., 2010). Researchers have also examined the antimicrobial activity of aqueous and ethanolic extracts of pomegranate peel against some pathogenic bacteria and concluded that the ethanolic extract had a superior effect on *Salmonella*, *Shigella*, and *E. coli* compared to other pomegranate products (Pai et al., 2011).

However, because of the reduction in competition with enteric gram-negative bacteria, the count of lactic acid bacteria was expected to rise. According to the literature, antimicrobial compounds in PPE are also likely to inhibit gram-positive bacteria such as Bacillus.

PPE was reported to include active antifungal compounds such as punicalagin, castagalagin, granatin, catechin, gallocatechin, kaempferol, and quercetin (Perez & Anesini, 1994). In this regard, a study examining different parts of pomegranate for phenolic contents and their antifungal activity demonstrated that the peel has the highest phenol content, and the methanolic extract of pomegranate peel effectively prevented the growth of fungal mycelium (Tehranifar et al., 2011). Similarly, the formation of biofilms by *Candida albicans* was efficiently inhibited by the methanolic extract of pomegranate (Bakkiyaraj, Nandhini, Malathy, & Pandian, 2013). Dahham and coworkers explored the antifungal activities of PPE against five fungal strains and found that the highest antifungal activity was recorded against *Aspergillus niger* (Dahham, Ali, Tabassum, & Khan, 2010). They also reported that the fungistatic activity of pomegranate peel varied with test organisms, and it did not affect the growth of some species such as *Aspergillus flavus* and *Aspergillus parasiticus*. Thus, it is assumed that, in the current study, the PPE-resistant strains could have proliferated in the low-competitive environment and increased the fungal count in the treated fish intestine. Further studies are required to determine which fungal species are affected by PPE.

5 | CONCLUSIONS

The current study showed that supplementation of *C. auratus* diets by lower than 4% would not adversely affect growth. Furthermore, gut microbial analyses indicated that enteric gram-negative bacteria and total fungi count decreased and increased, respectively, under the influence of PPE (2 and 4%). Thus, dietary administration with 2% of PPE will modify gut bacteria without any adverse effect on fish growth. It appears that the supplementation of aquatic feed with this extract may be effective in producing functional diets. Finally, there is a suggestion to evaluate the other effects of PPE on the health of other tissues such as intestine and liver.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Hamidreza Ahmadniaye Motlagh contributed to the project administration, writing, and data analysis; Zahra Rokhnareh dealt with data acquisition; and Omid Safari and Yahya Selahvarzi contributed to financial support and data analysis.

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