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Residual pomegranate affecting the nonspecific immunity of juvenile Darkbarbel catfish

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ABSTRACT

The improvement of resistance diseases, antioxidant and nonspecific immunity responses for Darkbarbel catfish by dietary residual pomegranate was investigated. The number of pathogenic bacteria and cumulative mortality of Darkbarbel catfish under 15–45% groups were decreased compared with control group (CK). Bioactive substances (polyphenols and vitamin) in residual pomegranate enhanced AKP, ACP, phagocytic, SOD, CAT activities through up-regulating *AKP, ACP, SOD, CAT* genes expression levels. Theoretical analysis showed bioactive substances regulated these genes expressions and enzyme activities as stimulus signal, component, active center. Moreover, residual pomegranate improved mTOR and NF-kB signaling pathway responses. Thus, residual pomegranate inhibited *Aeromonas hydrophila* that increased resistance to diseases. This technology completed the solid waste recovery and the Darkbarbel catfish growth performance simultaneously.

1. Introduction

Pelteobagrus vachelli, namely Darkbarbel catfish meat is fine and tender, and is rich in the protein, fat, carbohydrate, vitamin, nicotinic acid and inorganic components such as calcium, phosphorus, iron [1]. Therefore, it is one of the most important and popular aquaculture species in China. But, the large-scale aquaculture for Darkbarbel catfish cause the pollution of aquaculture water and the frequent occurrence of disease by viruses, bacteria, fungi, parasites, and other pathogens (*Hemorrhagic edema*, *Saprolegniasis*) [2]. Among, *Aeromonas hydrophila* (*A. hydrophila*) is the main pathogen for Darkbarbel catfish. These directly reduce the production performance of aquatic animals [3,4]. Thus, the rapid growth of aquaculture and intensive production requires to adopt new strategies in breeding to improve the product quality. In recent years, non-synthetic dietary supplements (natural substances) have attracted much attention in aquaculture to replace chemical substances in order to improve the growth performance and disease resistance of fish [5,6].

Pomegranate is rich in anthocyanins, polyphenols, 1,1-diphenyl-2-picrylhydrazyl, flavonoids, polysaccharides, tannins, alkaloids, organic acids, with anti-aging and reducing blood lipid and blood pressure functions [7,8]. As a result, pomegranate is widely grown and eaten. With the increase of demand, a large number of residual pomegranate (peel, core, even flesh) are produced as solid waste, which cause the environmental problems of solid waste pollution. Moreover, this also lead to the resource waste because pomegranate residue as nature resource has the potential of recycling to replace fish dietary.

Therefore, a novel strategy of the improvement of disease resistance for using residual pomegranate is proposed in this work. The residual pomegranate is directly re-utilized. The new strategy is conducive to solid waste reduction, chemical feed reduction, resource recovery, aquaculture at the same time.

To our best knowledge, the enhancement of disease resistance for Darkbarbel catfish by residual pomegranate was not researched. Moreover, the mechanism also is not clear that residual pomegranate regulate the immunity, antioxidant and disease resistance of Darkbarbel catfish. Therefore, the purpose of the work is to investigate the feasibility of the residual pomegranate enhancing disease resistance; to explain the mechanism of the residual pomegranate affecting disease resistance in terms of nonspecific immunity, antioxidant and mTOR and NF-kB signaling pathways responses.

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Table 1
The ingredients and inclusion levels under CK, 15%, 35%, 45% groups.

Composition (g/kg)	Control group	15% group	35% group	45% group
Crude protein	424	424	424	424
Crude fat	80	80	80	80
Vitamin	5	7	11	14
Mineral	20	23	25	27
Flavonoids	0	0.05	0.08	0.1
Polyphenols	0	75	170	240

2. Materials and methods

2.1. Fish rearing by the residual pomegranate and challenge test

The experiments were carried out at May to June 2018. Darkbarbel catfish (30 ± 5 g and 11 ± 3 cm) were bought from the local fish farming plant. Pomegranate purchased from local market. Residual pomegranate (peel and core) was crushed to puree. A total of 150 fish were acclimated in tank at least 7 days. During the acclimatization, the fish were fed every day with commercial fish feed.

After acclimatization, 120 fish was selected from 150 Darkbarbel catfish and assigned to triplicate four groups with 12 tanks (10 fish per 80 L tank containing 60 L water) randomly. Full-ration formula feed was partially replaced by residual pomegranate. The ingredients and inclusion levels of each feedstuff was presented in Table 1. These inclusion (protein and lipid) levels were chose based on the requirements of Darkbarbel catfish as per the method [1,2] and the ingredients and inclusion levels of pomegranate. These experimental diets was isonitrogenous and isolipidic (Table 1). Four processing groups were set and as follows: control group without substitution supplement; 15% substitution supplement group; 35% substitution supplement group; 45% substitution supplement group. Each processing group (CK, 15%, 35%, 45%) was repeated three times. The original water was renewed daily and the feces was removed. Darkbarbel catfish were fed once daily at a rate of 10%–15% of body weight during the experiment based on Lin et al., 2017 [9]. Fish feces was removed with a siphon once daily during culturing. Water temperature (25.0 ± 1.0 °C), dissolved oxygen (6.0 ± 1.0 mg/L), and pH (7.0 ± 1.0) were respectively determined daily using a thermometer, DO meter, and pH meter. Afterward, in this work, all fish were administered in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (China).

After 30 days, the challenge test was carried out for all fishes according to Lin et al., 2017; Kong et al., 2017; Chiu et al., 2015; Yuan et al., 2019 [9–12]. *Aeromonas hydrophila* (*A. hydrophila*, strain number: ATCC7966, presentation from Dalian Ocean University), selected as the pathogen, was cultured on tryptic soy agar for 24 h at 28 °C and transferred to 50 mL of tryptic soy broth for 24 h at 28 °C as the stock test culture. Broth cultures were centrifuged at 7000*g* for 10 min at 4 °C. The supernatant was discarded, and bacterial pellets were re-suspended in sterilized saline solution (0.85% NaCl) as the stock bacterial solution. The stock bacterial solution was not washed before injection. The challenge test was carried out in triplicate by an intraperitoneal injection of 60 μL of the stock bacterial solution, resulting in 4×10^6 cfu/g body weight.

2.2. Analysis and measurement

After 45 days feeding (after 15 days of challenge), three individuals per tank were collected in each processing group. Darkbarbel catfish were anesthetized in ice water prior to euthanasia. The fish were then decontaminated with 70% ethanol and dissected immediately with sterile scissors. Small pieces of liver and head kidney were immersed in TRIzol reagent and stored in −80 °C until RNA extraction. Freshly dissected intestine were placed into filter-sterilized PBS and frozen at −20 °C until DNA extraction.

2.2.1. Cumulative mortality and the populations of A. hydrophila

The mortality was observed after 15 days of challenge. The cumulative mortality was calculated. The concentration of *Aeromonas hydrophila (A. hydrophila)* was also measured using selective media. Each dissected intestine sample (5 g) was put into 50 ml of sterile distilled water and incubated in a rotary shaker (160 rpm) at 28 °C for 30 min. To assess the populations of *A. hydrophila*, the suspensions (200 μl) were smeared on Rimler-shotts and AHM culture mediums according to the National standard law of China GBT18652-2002 (Methods for detection of pathogenic *A. hydrophila*).

2.2.2. Determination of various enzyme activities

The superoxide dismutase (SOD) and catalase (CAT) activities in liver; the alkaline phosphatase (AKP), acid phosphatase (ACP) in head kidney were measured at 550 nm, 240 nm, 510 nm, 440 nm, 700 nm using the assay kit (Nanjing Jiancheng Bioengineering Institute, China) by a UV/Vis spectrophotometer (Pharmacia Biotech Ultrospec 2000) respectively and according to Lin et al., 2017; Kong et al., 2017; Chiu et al., 2015; Yuan et al., 2019 [9–12]. The head kidney macrophages were isolated and prepared according to Secombes 1990 [13]. The phagocytic activity of macrophages was determined by the following Sakai et al., 1995 and Houwen, 2002 [14,15].

2.2.3. Immune enzymes-related genes and antioxidant enzymes-related genes expression

According to Kong et al., 2017; Qi et al., 2017 [10,16], total RNA was extracted from tissue samples by TRIzol Reagent (Cwbio, Beijing, China) and treated with 4 × gDNA wiper Mix to minimize the contamination of genomic DNA. The quality and purity of RNA were verified by electrophoresis on ethidium bromide staining 1.0% agarose gels and by A260 nm/A280 nm ratio. Complementary DNA was then synthesized using the HiScript® Reverse Transcriptase Kit (Vazyme, Jiangsu, China) following the instructions. The real-time quantitative PCR (RT-qPCR) was performed using AceQ™ qPCR SYBR ® Green Master Mix kit and CFX96 Real-Time PCR Detection System (Bio-Rad, USA). The β-actin gene was used as a house keeping gene. The PCR primer sequences and the reaction conditions used for real-time quantitative PCR are listed in Table S1, and the cycleindex was 30. The PCR efficiency of each primer was between 95.6% and 99.2%. RNA extracted from the head kidney was performed to detect the expression of immune enzymes-related genes (*AKP, ACP*) genes and *TOR gene*, *4E-BP gene* in mTOR and *NF-kB p65*, *IkB* in NF-kB signaling pathway genes. RNA extracted from the livers was performed to detect the expression of antioxidant enzymes-related (*SOD, CAT*) genes. Each individual sample was run in triplicate wells. The RT-qPCR data were analyzed by the $2^{-\triangle\triangle Ct}$ method [17].

2.3. Statistical analyses

Data were subjected to one-way analysis of variance (ANOVA) in SPSS 18.0; mean differences among treatments were compared using Duncan's multiple-range test. The significant level was set at $P < 0.05$. Before statistical analyses, data were checked for normality of distribution and homogeneity of variance with the KolmogorovSmirnov test and Levene's test, respectively. If the data did not conform to normality of distribution and homogeneity of variance, the data were transformed by square root transformation or logarithmic transformation or arcsine transformation. Data were expressed as means ± SE.

Table 2
The number of pathogenic bacteria and cumulative mortality of Darkbarbel catfish under CK, 15%, 35%, 45% groups Values (mean ± S.E.) with different superscript letters significantly differ from each other ($P < 0.05$).

Group	Number of *A.hydrophila* (CFU/g)	Cumulative mortality (%)
CK	9.8×10^{11a}	60.43 ± 4.41^{a}
15%	9.4×10^{8b}	50.87 ± 3.49^{b}
35%	4.4×10^{4c}	25.41 ± 0.57^{c}
45%	4.3×10^{4c}	25.65 ± 0.53^{c}

3. Results

3.1. The feasibility of increasing resistance diseases ability for Darkbarbel catfish with residual pomegranate

Aeromonas hydrophila (A. hydrophila). The mortality was observed after 15 days of *Aeromonas hydrophila (A. hydrophila)* challenge. The cumulative mortality was calculated (Table 2). Table 2 indicated that residual pomegranate inhibited *A. hydrophila*. The number of pathogenic bacteria and cumulative mortality in 15%, 35%, 45% groups were the lower than CK group, and presented significant difference ($P < 0.05$). Among, 35%, 45% groups was the best for the number of pathogenic bacteria and cumulative mortality. Meanwhile, the cumulative mortality (4–6%) was showed in non-challenged control group (Table S2). Further, Figs. S1–S2 showed the pathological changes of spleen and intestine were observed. These indicated that the cumulative mortality of challenge were mainly caused by *A. hydrophila* in CK, 15%, 35%, 45% groups. Darkbarbel catfish was infected by *A. hydrophila*. In addition, the growth of Darkbarbel catfish after feeding CK, 15%, 35% and 45% substitution supplement group were very well and its yield was significantly increased (Table S2).

3.2. Improving non-specific immune and antioxidant response to fish diseases

Table 2 showed that the resistance diseases were improved by residual pomegranate. These findings might be related to the nonspecific immunity and antioxidant responses. Therefore, superoxide dismutase (SOD) and catalase (CAT) activities in liver; the alkaline phosphatase (AKP), acid phosphatase (ACP) in head kidney were measured (Fig. 1).

The AKP, ACP, phagocytic, SOD, CAT activities of Darkbarbel catfish in 15–45% groups were the better than the control group, and presented significant difference for CK group ($P < 0.05$). Among, 35%, 45% groups was the best and showed significant difference for other groups ($P < 0.05$).

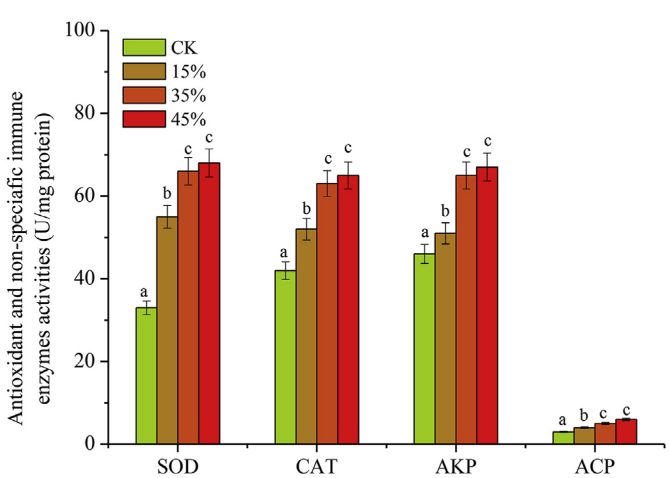

Fig. 1. The nonspecific immune related enzyme and antioxidant related enzyme activities of Darkbarbel catfish under CK, 15%, 35%, 45% groups Values (mean ± S.E.) with different superscript letters significantly differ from each other ($P < 0.05$).

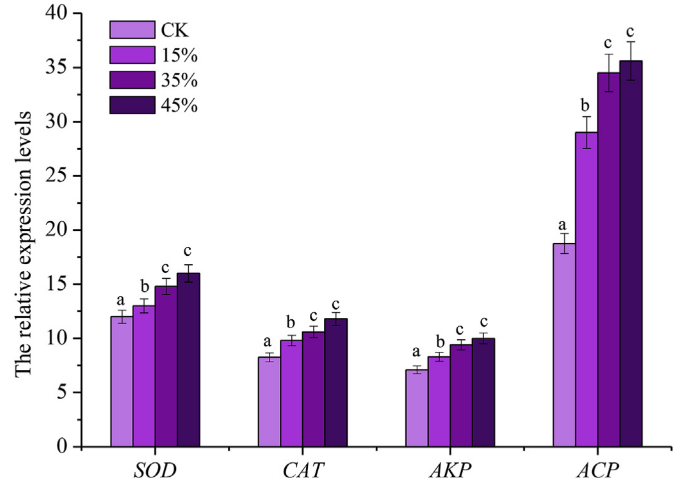

Fig. 2. The relative expression levels of *AKP, ACP, SOD, CAT* genes under CK, 15%, 35%, 45% groups Values (mean ± S.E.) with different superscript letters significantly differ from each other ($P < 0.05$).

Meanwhile, to further investigate the molecular biological mechanism of the residual pomegranate regulating nonspecific immunity and antioxidant, *AKP, ACP, SOD, CAT* gene expression levels were determined. These results were showed in Fig. 2. The *AKP, ACP, SOD, CAT* gene expression levels in other three given groups were the better than the control group, and 15%, 35%, 45% groups presented significant difference for the control group ($P < 0.05$). Among, 35%, 45% groups had the best *AKP, ACP, SOD, CAT* gene expression levels, and showed significant difference for other groups ($P < 0.05$).

3.3. Regulating mTOR and NF-kB signal transduction pathways response to fish diseases

Table 2 showed that the resistance diseases were improved by residual pomegranate. These findings might be related to the mTOR and NF-kB signaling pathway responses. RNA extracted from the head kidney was performed to detect the expression of *TOR gene*, *4E-BP gene* in mTOR and *NF-kB p65*, *IkB* in NF-kB signaling pathway genes (Fig. 3).

Compared with the control group, the relative expression level of

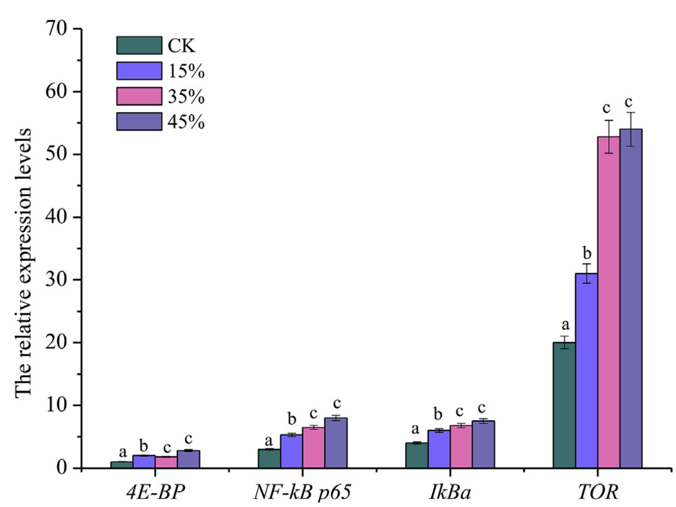

Fig. 3. The relative expression levels of *TOR*, *4E-BP*, *NF-kB p65*, *IkBa* genes in mTOR and NF-kB signaling pathway under CK, 15%, 35%, 45% groups Values (mean ± S.E.) with different superscript letters significantly differ from each other ($P < 0.05$).

TOR gene, *4E-BP gene* in mTOR and *NF-kB p65*, *IkBa* in NF-kB was increased in 15%, 35%, 45% groups, and 35%, 45% groups presented significant difference for CK group ($P < 0.05$). Among, 35%, 45% groups was the best, and showed significant difference for other groups ($P < 0.05$).

4. Discussion

Current research showed that 15–45% groups had better promoting effect on the resistance diseases than control group (CK) (Table 2). The main reason was the replacement of dietary residual pomegranate. It could be found form Table 1 that these diets are isonitrogenous and isolipidic. But, compared with CK group, the supplement of dietary residual pomegranate provided more rich and multiplex nutrients such as (flavonoids and polyphenols) for Darkbarbel catfish (Table 1). Costa et al., 2019; Khodabakhshian, 2017 [18,19] showed that residual pomegranate contained bioactive substances (flavonoids, vitamin, polyphenols) and metal ions. Further, Hoskin et al., 2018; Yi et al., 2017 [20,21] observed the effects of polyphenol on enzyme activities of antioxidant and immune systems in vitro. Li et al., 2019 [22] used flavonoids to improve antioxidant and immune-related enzyme activities in juvenile northern snakehead fish. Ruiz et al., 2019; Wu et al., 2016 [23,24] observed that vitamin C, E, K increased the antioxidant and non-specific immune enzyme activities in various aquatics. Table 2 indicated that it was feasible to promote resistance diseases by residual pomegranate.

In this work, these findings in Figs. 1–2 indicated that the residual pomegranate improved the nonspecific immunity and antioxidant responses by regulating the expression level of related genes. For nonspecific immunity systems, both AKP and ACP were the marker enzyme of macrophage lysosome in organism, and were also the important hydrolytic enzyme in nonspecific immunity [25,26]. They could kill invading pathogens, and also accelerate the phagocytosis of phagocytes and the degradation rate of foreign bodies. Moreover, AKP was closely related to the growth of aquatic animals, and played an important role in the absorption and utilization of nutrition, even the synthesis of protein. Thus, higher AKP and ACP activities had a positive effect on defense against external pathogens and microbial invasions. Meanwhile, it was also observed from Fig. 1 that phagocytic activity was significantly increased. Leukocyte had the function of the phagocytosis for pathogenic bacteria and bactericidal, which was an important aspect of non-specific immunization [9,10]. As Fig. 1 shown, 15–45% groups significantly increased the AKP, ACP, phagocytic activities of Darkbarbel catfish, which improved the nonspecific immunity response ability, disease resistance (Table 2).

As for antioxidant systems, the SOD, CAT were the vital enzymes in antioxidant defense system [10,16]. They were able to scavenge reactive oxygen species (Ros) and alleviate its damage to cells. Moreover, SOD could enhance the defense function of macrophages, and was closely related to the immune system. As Fig. 2 shown, 15–45% groups significantly increased SOD, CAT activities. This finding indicated that residual pomegranate could enhance the antioxidant ability of Darkbarbel catfish, protected cells from damage, improved the survival rate and promoted the growth of Darkbarbel catfish (Table 2). Thus, Table 2 indicated that residual pomegranate inhibited *A. hydrophila* because that it enhanced the antioxidant and non-specific immune ability of Darkbarbel catfish (Figs. 1–2).

Concerning the mechanism of bioactive substances and metal ions regulating these enzymes activities, there might be two reasons. Firstly, the bioactive substances and metal ions constituted enzymes or regulated enzyme synthesis pathway. Residual pomegranate contained a variety of amino acids, which were the basic components of enzymatic proteins [27]. At the same time, they contained a large number of vitamins, which constituted a variety of co-enzymes (flavin mononucleotide, Coenzyme A, transmethylase) [27]. Iron, magnesium and zinc were also the active center of the AKP, SOD, CAT in this work.

Secondly, residual pomegranate also might induce or stimulate the expression of *AKP, ACP, SOD, CAT* gene as stimulation signal or activation factor (Fig. 2). The supplement of dietary residual pomegranate provided flavonoids and polyphenols for Darkbarbel catfish (Table 1). The substitution of residual pomegranate in diet affected the expression of *AKP, ACP, SOD, CAT* gene. This view was explained by some researches. Residual pomegranate were rich in vitamin, polyphenol, flavonoids, organic acid [18,19]. Zhao et al., 2019 [28] observed that polyphenol modulated the gene expression in NRF2/HO-1 MAPK signaling. Rahman et al., 2019 [29] found that effect of vitamin C on the gene expression of the *Nile tilapia*. He et al., 2017 [30] observed that the organic acids increased the gene expression levels of TNF-α, LITAF and RAB6A in shrimp. Tian et al., 2019 [31] observed that flavonoids increased the gene expression in NF-κB and MAPK signaling pathways.

As a signal transduction pathway, the mTOR signaling pathway, which plays a vital role in nutrition regulation and has complex impact on cell growth [32], widely exists in eukaryotes [33], food intake and environmental stresses [34]. TOR pathway is a key regulator of the balance between protein synthesis and degradation in response to nutrition quality and quantity [35,36], and the protein synthesis is essential for cell growth, proliferation, apoptosis, and autophagy [37]. Moreover, immune protein synthesis and nutrient transport are also each related to mTOR [38]. In this study, higher genes expression in mTOR signaling pathway was induced in resistance diseases, immune-related enzymes were enhanced in 15–45% groups.

As for NF-kB signaling pathway, it regulated the congenital and acquired immunity, inflammation, stress response and the formation of B cell and lymphoid organ [39,40]. It was closely related to the differentiation of immune cells [41]. In this study, higher genes expression in NF-kB signaling pathway was induced in 15–45% groups, and then immune-related enzymes activities were enhanced in 15–45% groups. Therefore, phagocytic activity was enhanced under 15–45% groups in this work. These results suggested that residual pomegranate promoted the activities of immune-related enzymes by regulating NF-kB signaling pathway. Thus, the number of pathogenic bacteria and cumulative mortality of Darkbarbel catfish was decreased under 15–45% groups, and thus resistance diseases was increased (Table 2).

To the best of researchers' knowledge, the present study is the first one addressing to enhancing resistance diseases for Darkbarbel catfish with the residual pomegranate. Table 2 indicated that residual pomegranate could be reused directly to enhance resistance diseases. Residual pomegranate improved the technology reduced the use of chemical feeds in aquaculture, and completed the recycle and reuse of residual pomegranate.

5. Conclusion

Improvement of disease resistance for Darkbarbel catfish by residual pomegranate was feasible. Bioactive substances in residual pomegranate improved nonspecific immunity, antioxidant, mTOR and NF-kB signaling pathway responses through up-regulating related genes expression levels. Theoretical analysis showed residual pomegranate including bioactive substances regulated these genes expressions and enzyme activities as stimulus signal, component, active center. Thus, residual pomegranate inhibited the number of pathogenic bacteria and cumulative mortality of Darkbarbel catfish. This technology increased the disease resistance of Darkbarbel catfish.

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Appendix A. Supplementary data

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