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Effluent containing *Rubrivivax gelatinosus* promoting the yield, digestion system, disease resistance, mTOR and NF-kB signaling pathway, intestinal microbiota and aquaculture water quality of crucian carp

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ABSTRACT

The employment of traditional bait and medicament in freshwater aquaculture causes the frequent occurrence of environmental pollution and disease. Effluent collected after *Rubrivivax gelatinosus*-mediated wastewater treatment could be re-utilized as microbial feeds, medicament, and aquaculture water to culture Crucian carp. Therefore, a novel integrated system of wastewater treatment using effluent containing *R. gelatinosus* that improves yield, increases disease resistance, and enhances the quality of aquaculture water for Crucian carp culture was proposed and investigated. Crucian carp can grow well in effluent containing *R. gelatinosus* (ER). The survival rate, yield, and whole body composition of the ER group were all increased compared to the control group (CK). The biochemical (B vitamin) and other substances in the effluent of *R. gelatinosus* enhanced the activity of proteases, amylases, lipases, AKP, ACP, phagocytic, SOD, CAT by up-regulating the expression of *akp, acp, sod,* and *cat* genes. Theoretical analysis showed that biochemicals regulate the expression of these gene and enzyme activities by acting as a signal that stimulates the active center. Moreover, *R. gelatinosus* and biochemical substances improved mTOR and NF-kB signaling pathway and intestinal microbiota.

Furthermore, *R. gelatinosus* inhibited *Aeromonas hydrophila* that increases resistance against fish disease and promotes the growth of intestinal bacteria. Meanwhile, *R. gelatinosus* in the effluent also improved the aquaculture water quality. This technology would save the aquaculture water, reduce water pollution and wastewater discharge, and increase the output and disease resistance of Crucian carp, simultaneously.

1. Introduction

Crucian carp meat is fine and tender, and is rich in the protein, fat, carbohydrate, vitamin, nicotinic acid and inorganic components such as calcium, phosphorus, iron [1,2]. Therefore, it is one of the most important and popular freshwater aquaculture species in China. The total output is over three million tons per year. But, the large-scale freshwater aquaculture and the application of commercial bait and medicament (antibiotics and chemotherapeutics) cause the pollution of water environment and the frequent occurrence of disease [3]. These directly reduce the production performance of aquatic animals [4]. Thus, it has become necessary to exploit the natural bait and fish disease drugs instead of chemical substances methods, like probiotic. Safari and Paolucci et al., 2017 used *galactooligosaccharide* and *Enterococcus faecalis* to improve the growth and survival of juvenile crayfish feed [5]. Nguyen et al., 2017 used probiotic *Lactococcus lactis* to enhance growth rate in olive flounder [6]. Rahimnejad et al., 2017 found the improvement of growth performance in rock fish with *Pediococcus acidilactici* [7]. Now, the probiotics are garnering increasing scientific and commercial interest, and are quite common in health-promoting functional feeds as well as therapeutic, prophylactic, and growth supplements [8].

Rubrivivax gelatinosus (R. gelatinosus) is a kind of photosynthetic bacteria (PSB) and probiotic, widely distributing in rivers, lakes, seas [9]. Like other PSB, *R. gelatinosus* are rich in high value biochemicals such as single cell proteins, carotenoids, vitamins, folic acid, antiviral

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substances, antigens [10]. Moreover, PSB *(R. gelatinosus)* are non-toxic and harmless, and do not secrete toxic or harmful substances. Thus, PSB *(R. gelatinosus)* is very suitable for the improvement of Crucian carp yield as microbial feeds and medicament. Similarly, Chumpol et al., 2018 demonstrated the single cell protein in photosynthetic bacteria to enhance growth and survival in white shrimp cultivation [11]. Chiu and Liu, 2014 found the extract of photosynthetic bacteria enhanced the growth performance and innate immune responses of seawater red tilapia. Meanwhile, they are capable of handling a variety of highly concentrated organic wastewater [12]. Idi et al., 2015 described photosynthetic bacteria removed high amount of nitrate and ammonia without nitrite accumulation [13]. Ponsano et al., 2008 used photosynthetic bacteria to treat the poultry slaughterhouse wastewater [14]. Wu et al., 2014 treated the soybean protein wastewater with photosynthetic bacteria [15]. Hülsen et al., 2018 described photosynthetic bacteria treated poultry processing wastewater [16]. To sum up, PSB *(R. gelatinosus)* has such potential. Firstly, PSB *(R. gelatinosus)* are used to treat organic wastewater. Afterward, the effluent without the separation of PSB-water is reused directly to culture Crucian carp as aquaculture water and feeds, and to purify aquaculture water quality simultaneously.

Therefore, a novel integrated system of the wastewater treatment, the improvement of yield, disease resistance and aquaculture water quality of Crucian carp by effluent containing *R. gelatinosus* is proposed in this work. The effluent is directly re-utilized to culture Crucian carp as microbial feeds, medicament and aquaculture water. The new strategy owns the advantage of wastewater treatment, the reduction of aquaculture water consumption, the improvement of yield, disease resistance and aquaculture water quality at the same time.

Soybean processing wastewater is non-toxic and harmless, do not contain heavy metals and is rich in nutrients that was required by the growth of Crucian carp and *R. gelatinosus* [17]. Thus, soybean protein wastewater is used in the above strategy. The strategy will not cause two pollution for aquaculture water as both *R. gelatinosus* and wastewater were non-toxic harmless.

To our the best knowledge, the enhancement of yield, disease resistance and aquaculture water quality of Crucian carp by effluent containing *R. gelatinosus* was not researched. Studies on the increased of yield in aquaculture by photosynthetic bacteria had focused on their extracts and nutrients [11,12]. Moreover, the mechanism also is not clear that *R. gelatinosus* regulate the growth, digestion, immunity, antioxidation and disease resistance of Crucian carp. Therefore, the purpose of the work is to investigate the feasibility of the effluent culturing Crucian carp and enhancing its yield and disease resistance; to explain the mechanism of the effluent affecting Crucian carp yield and disease resistance in terms of digestion, nonspecific immunity, antioxidation, intestinal flora and mTOR and NF-kB signaling pathways.

2. Materials and methods

2.1. Wastewater treatment

Soybean processing wastewater (SPW) was employed for *R. gelatinosus* treatment and Crucian carp growth due to it being non-toxic and harmless. It was obtained using the soybean soak process from the Dalian Soybean Products Machining Factory (Dalian, China). The basic characteristics of the diluted SPW are shown in Table S1. The pH of SPW was adjusted to 7 before treatment. The *R. gelatinosus* strain was maintained at 4 °C in a fridge and grown in the improved medium in a thermostat shaker (120 × *g*, 32 °C ± 2 °C) for approximately 48 h before the experiment [15].

The photo-bioreactor used is shown in Fig. S1. Both the SPW and reactor were sterilized for 30 min by a sterilizer at 121 °C before the addition of *R. gelatinosus*. The wastewater/bacteria mixtures were placed in a 30 °C ± 2 °C thermostat shaker under a rotating speed of 120 × *g*. Light-aerobic conditions were used for 1–3 days, and light-anaerobic conditions were used for 4–6 days.

After six days of *R. gelatinosus* treatment, the quality of the SPW effluent for aquaculture water was inspected. The results are shown in Table S1. The *R. gelatinosus* biomass reached 4000 mg/L, the residual COD, ammonia nitrogen, and metal ions in the effluent were 200, 1, and 0.1 mg/L. Ammonia nitrogen levels were high for aquaculture water. The effluent was diluted by a factor of six. After dilution, ammonia nitrogen levels were below 0.2 mg/L, which reaches the aquaculture water quality standards of China. In addition, 2 mL 1% sodium alginate was added to 10 mL effluent to immobilize *R. gelatinosus* for Crucian carp ingestion.

2.2. Fish rearing by the effluent

The experiments were carried out from May to August 2018. Crucian carp (30 ± 5 g and 11 ± 3 cm) were bought from the local fish farming plant. After acclimatization, 120 fish were selected from 150 Crucian carp and assigned to four triplicate groups in 12 tanks (10 fish per 80 L tank containing 60 L water) randomly. Four processing groups were set as follows: CK represents the control group of water; WR represents the water and *R. gelatinosus*; EO represents the effluent without *R. gelatinosus*; ER represents the effluent containing *R. gelatinosus* used to breed Crucian carp. Each processing group (CK, WR, ER, and EO) was repeated three times.

The commercial fish basal diet was used as food in control group (Table S2). The ER processing group contained *R. gelatinosus* and residual (COD, TOC) organic matter as food (Table S1). The WR processing group contained *R. gelatinosus* as food. EO processing group contained the residual organic matter as food. In this work, *R. gelatinosus* and residual (COD, TOC) organic matter in effluent were immobilized and used as food in WR, ER, EO groups, respectively. The *R. gelatinosus* biomass was 40 g in 60 L water (Table S1). The food were sufficient for Crucian carp in the WR and ER groups.

The original water (effluent) was renewed daily, and the feces were removed. With the renewal of water, Crucian carp were fed once daily. Fish feces were removed with a siphon once daily during culturing. Crucian carp were fed once daily at a rate of 10%–15% of body weight during the experiment. In addition, the water quality experiments were conducted separately. In water quality experiments, the original water (effluent) was not renewed during the last week. The water temperature (25.0 °C ± 1.0 °C), dissolved oxygen (6.0 ± 1.0 mg/L), and pH (7.0 ± 1.0) were determined daily using a thermometer, a DO meter, and a pH meter, respectively. Afterward, all fish were administered in strict accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals (China).

2.3. Analysis and measurement

Wastewater from the Crucian carp aquaculture were collected from tanks at the last week. Triplicate samples were used to detect the chemical oxygen demand (COD), total organic carbon (TOC), total nitrogen (TN), and ammonium (NH_4^+) in Crucian carp aquaculture water at the last week.

The COD and TOC were determined using a COD analyzer (JY-203, Tianjin, China) and TOC analyzer (multi N/C 3100, Analytik Jena), respectively. The ammonium (NH_4^+) levels and TN were determined at 420 nm, 538 nm, 220 nm, 220 nm, and 270 nm wavelengths using a visible-ultraviolet spectrophotometer (UV-7500, Shanghai, China) according to the American Public Health Association (APHA) Standard Methods for the Examination of Water and Wastewater and the National Standard Methods of China.

2.4. Determination of yield and quality of Crucian carp

After 123 days feeding, each Crucian carp was individually weighed (± 0.01) on an electronic scale (AND, Japan). The survival was

calculated as follows:

Survival (%) = (final number of fish/initial number of fish) × 100

At the end of the feeding, three individuals per tank were collected in each processing group. Crucian carp 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.

Meanwhile, the dorsal muscle were evenly removed. The analysis of crude protein, crude lipids, and ash were analyzed by standard procedures as per the Association of Official Analytical Chemists (AOAC). Moisture content was estimated by gravimetric analysis after oven drying at 105 °C for 12 h. Crude protein was determined by Kjeldahl method (Kjeltec 2100, FOSS, Tecator, Sweden) after acid hydrolysis [18]. Crude lipids were extracted based on the method of Folch et al., 1957. Total ash was determined gravimetrically by ignition at 600 °C for 6 h in muffle furnace [19].

2.5. Determination of various enzyme activities

After stop feeding one day, the freshly dissected intestine were placed into filter-sterilized PBS and frozen at −80 °C until digestive enzyme determination. The protease, amylase and lipase activities in intestine were determined respectively at 366 nm, 540 nm and 550 nm with a UV/Vis spectrophotometer (Pharmacia Biotech Ultrospec 2000) [20–22]. In the present study, specific enzyme activity was defined as enzyme units (U) per milligram of total protein.

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 Kong et al., 2017; Lin et al., 2017; Cooper et al., 2002; Yuan et al., 2019 [8,23–25]. The head kidney macrophages (HKM) were isolated and prepared according to Secombes, 1990 [26]. The phagocytic activity of macrophages was determined by the following Sakai et al., 1995 and Houwen, 2002 [27].

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

According to Kong et al., 2017; Qi et al., 2017, 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 [23,28]. 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 S3, 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 [29] and the control group was calibration.

2.7. DNA extraction and 16S rDNA sequencing and bioinformatic analysis

According to Qi et al., 2017, the V3–V4 region of the bacteria 16S ribosomal RNA gene were amplified by PCR (95 °C for 2min, followed by 27 cycles at 95 °C for 30s, 55 °C for 30s, and 72 °C for 30s and a final extension at 72 °C for 5min) using primers 341F 5' - CCTAYGGGRBG-CASCAG -3′ and 806R 5' - GGACTACNNGGGTATCTAAT - 3', where barcode is an eight-base sequence unique to each sample [28]. The PCR reactions were performed in triplicate 20 μL mixture containing 4 μL of 5 × FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase, and 10 ng of template DNA.

Then amplicons were extracted from 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.) according to the manufacturer's instructions and quantified using QuantiFluor -ST (Promega, U.S.). Purified amplicons were pooled in equimolar and paired-end sequenced (2 × 250) on an Illumina platform according to the standard protocols. The data were denoised by removing the sequences containing sequencing errors using the Mothur platform [30]. PCR chimeras were filtered out using the "chimeraslayer" command in Mothur software [31]. Finally, the filtered sequences were assigned to a taxon by the RDP classifier (version 2.6).

2.8. Challenge test

The challenge test was carried out separately according to Lin et al., 2017; Chiu et al., 2015 [8,32]. After the 60 days, fish were randomly selected for pathogen injection. *Aeromonas hydrophila (A. hydrophila)*, 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 saline solution (0.85% NaCl) as the stock bacterial solution. 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. The mortality was observed after 14 days of challenge. The cumulative mortality was calculated.

In addition, the concentration of *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 *g*) 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 *Aeromonas hydrophila*).

2.9. Statistical analyses

All data in this study were analyzed by Statistical Product and Service Solutions (SPSS 18.0) and were expressed as mean ± SE. Statistical analyses were performed using one-way ANOVA. Tukey's multiple-comparisons test (SAS Institute, Cary, NC, USA) was conducted to examine differences among four groups. Significant difference is set at the level of $P < 0.05$.

3. Results

3.1. The feasibility of culturing Crucian carp with effluent containing R. gelatinosus

To research the effect of effluent containing *R. gelatinosus* on Crucian carp growth, the survival rate, yield and whole fish body composition were determined. The results were showed in Table 1.

It was observed from Table 1 that Crucian carp could survive and grow well under all given processing groups (CK, WR, ER, EO). The survival rate and yield in EO group were the lowest due to lack of food. The yield did not show significant difference between CK, WR, ER groups ($P > 0.05$). The survival rate was higher under WR, ER group

Table 1
The survival rate, yield and whole fish body composition of Crucian carp after three months under CK, WR, ER, EO groups Values (mean ± S.E.) in the same column with different superscript letters significantly differ from each other ($P < 0.05$).

Group	Yield (g)	Survival rate	Moisture	Ash	Crude protein	Crude fat (%)
CK	48.32 ± 1.99^{a}	96.87 ± 0.33^{a}	78.03 ± 1.15	2.80 ± 1.11	14.87 ± 1.49^{a}	2.16 ± 0.03^{a}
EO	43.10 ± 2.20^{b}	95.33 ± 0.75^{c}	78.18 ± 1.49	2.80 ± 1.09	14.37 ± 1.18^{a}	2.13 ± 0.53^{a}
WR	47.80 ± 2.85^{a}	98.13 ± 0.68^{b}	78.13 ± 0.75	2.85 ± 1.06	16.48 ± 1.41^{b}	2.64 ± 0.19^{b}
ER	49.72 ± 3.85^{a}	98.67 ± 0.88^{b}	78.43 ± 0.87	2.97 ± 1.28	16.58 ± 1.43^{b}	2.87 ± 0.13^{b}

than CK group, and showed significant difference ($P < 0.05$). The ER group was the best for the survival rate and yield under all given groups. The increased of survival rate and yield in ER group might be associated with *R. gelatinosus* and residual organic matter in effluent through the analysis for the composition of the aquaculture water and bait. Table 1 indicated that it had very good feasibility to culture Crucian carp using the effluent containing *R. gelatinosus*.

Meanwhile, the moisture and ash content in whole fish body composition did not show significant difference between all given groups ($P > 0.05$). Compared with CK, EO groups, the crude protein and crude fat of Crucian carp were improved under ER, WR groups, and presented significant difference ($P < 0.05$).

3.2. Enhancing the intestinal digestive enzyme activity of Crucian carp

Table 1 showed that the growth, crude protein and crude fat contents were improved by effluent containing *R. gelatinosus*. This finding indicated the nutrients (*R. gelatinosus* and residual organic compounds) were digested and absorbed by intestinal digestive system of Crucian carp. To investigate the mechanism of the effluent improving Crucian carp growth from the perspective of digestion and absorption, the protease, amylase and lipase activities in intestine were determined. The results were showed in Table 2.

Compared with the control group, the protease, amylase activities were improved under ER, WR groups. The protease, amylase activities of ER group were the highest, and presented significant difference for CK, EO groups ($P < 0.05$). The protease, amylase activities were similar in CK, EO groups. The lipase activity did not present significant difference between all given groups ($P > 0.05$). The digestive enzyme activity determined the amount and speed of Crucian carp digesting and absorbing nutrients, and then affected the growth and development. The improvement of the intestinal digestive system had been reported with different probiotics and biochemicals as diet. Zhang et al., 2018 used soybean meal to improve the digestive enzymes activity of *Lateolabrax japonicus* [33]. Wang et al., 2015 found the amylase activity, cellulase activity and alginase activity of juvenile sea cucumber were increased by yeast *R. benthica* D30 [34]. Zhou et al., 2009 found the probiotic *B. coagulans SC8168* improved digestive enzyme activities of larvae shrimp [35]. Liu et al., 2017 *Bacillus subtilis* HAINUP40 enhanced the protease and amylase activity in the digestive tract of tilapia [36].

3.3. Affecting the disease resistance of Crucian carp

Table 1 showed that the survival rate were improved by the effluent containing *R. gelatinosus*. These findings might be related to the nonspecific immunity and antioxidant systems. To investigate the mechanism by which the effluent affected Crucian carp growth from the point of nonspecific immunity and antioxidant, the alkaline phosphatase (AKP), acid phosphatase (ACP), phagocytic, superoxide dismutase (SOD) and catalase (CAT) activities of Crucian carp were determined. The results were showed in Fig. 1.

Table 2
The digestive enzymes activities of Crucian carp under CK, WR, ER, EO groups Values (mean ± S.E.) in the same column with different superscript letters significantly differ from each other ($P < 0.05$).

Group	Amylase	Protease	Lipase (U/mg)
CK	164.8 ± 1.3^{a}	52.97 ± 4.69^{a}	13.57 ± 0.51
EO	168.3 ± 1.6^{a}	55.78 ± 4.69^{a}	13.49 ± 0.51
WR	178.5 ± 1.8^{b}	68.13 ± 1.60^{b}	13.74 ± 0.37
ER	180.9 ± 2.5^{b}	71.40 ± 3.63^{b}	14.01 ± 0.93

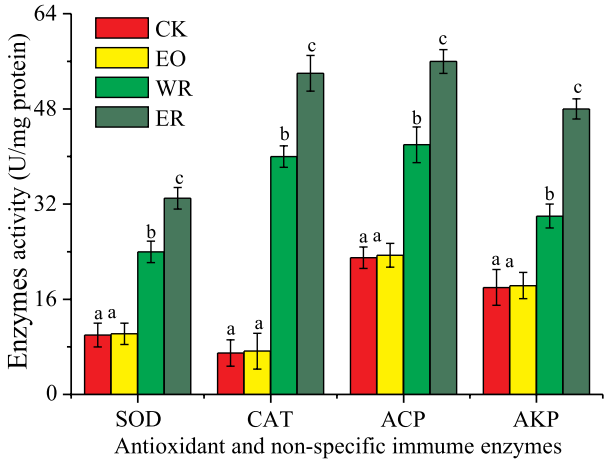

Fig. 1. The nonspecific immune related enzyme and antioxidant related enzyme activities of Crucian carp under CK, WR, ER, EO groups Values (mean ± S.E.) in the same column with different superscript letters significantly differ from each other ($P < 0.05$).

The AKP, ACP, phagocytic, SOD, CAT activities of Crucian carp in other three groups were the better than the control group, and ER, WR groups presented significant difference for CK group ($P < 0.05$). Among, the ER group was the best and showed significant difference for other three groups ($P < 0.05$). Phagocytic activity was 14.9% (CK), 19.7% (WR), 20.3% (ER), 15.3% (EO) respectively. The improvement of the nonspecific immunity and antioxidant systems of aquatic animal had been reported with different probiotics and biochemicals. Lin et al., 2017 found that the probiotics mixture could increase respiratory bursts and phagocytic activity [8]. Meidong et al., 2017 reported the dietary administration of strain B_{81e} increased the serum lysozyme, bactericidal, phagocytic and respiratory burst activities of *P. bocourti* significantly [37]. Park et al., 2017 observed the superoxide dismutase (SOD), nitro blue tetrazolium (NBT) and lysozyme activities in starry flounder were increased significantly with multi-probiotics [38]. Zhang et al., 2018 used soybean meal (protein, isoflavone) to improve the nonspecific immunity and antioxidant systems of Japanese seabass [33]. Zhou et al., 2015 enhanced immune response of juvenile yellow catfish with different arginine levels [39].

Meanwhile, Lin et al., 2017 and Kong et al., 2017 also observed that the probiotics and some biochemicals could regulate the expression level of gene (*Mx* mRNA expression) [8,23]. To further investigate the molecular biological mechanism of the effluent regulating nonspecific immunity and antioxidant, *akp, acp, sod,* and *cat* gene expression levels were determined. These results were showed in Fig. 2. The *akp, acp, sod,* and *cat* gene expression levels in other three given groups were the better than the control group, and ER, WR groups presented significant

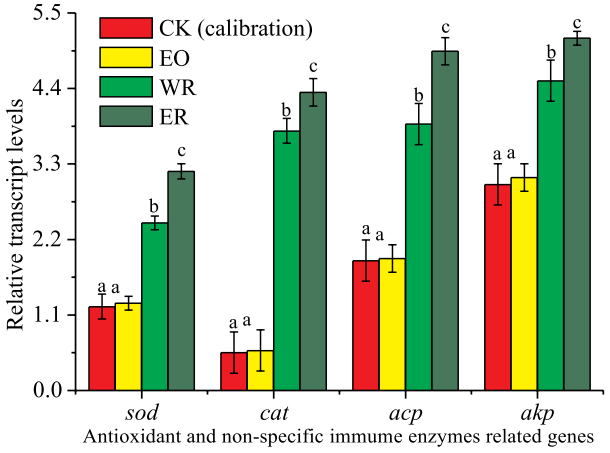

Fig. 2. The relative expression levels of *akp, acp, sod,* and *cat* genes under CK, WR, ER, EO groups Values (mean ± S.E.) in the same column with different superscript letters significantly differ from each other ($P < 0.05$).

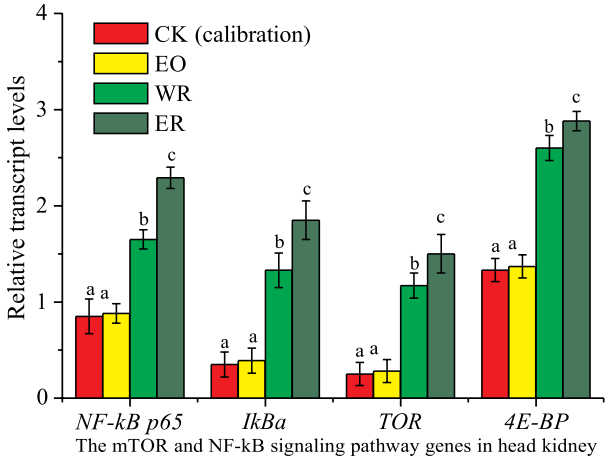

Fig. 3. The relative expression levels of *TOR*, *4E-BP, NF-kB p65*, *IkBa* genes in mTOR and NF-kB signaling pathway under CK, WR, ER, EO groups Values (mean ± S.E.) in the same column with different superscript letters significantly differ from each other ($P < 0.05$).

difference for the control group ($P < 0.05$). Among, ER group had the best *akp, acp, sod,* and *cat* gene expression levels, and showed significant difference for other three groups ($P < 0.05$). Figs. 1–2 indicated that *R. gelatinosus* and remainder biochemicals in effluent improved AKP, ACP, SOD, CAT activities through up-regulating *akp, acp, sod,* and *cat* gene expression levels.

Furthermore, the *Aeromonas hydrophila (A. hydrophila)* challenge test was also conducted. Table 3 showed the number of pathogenic bacteria and cumulative mortality of Crucian carp in ER, WR groups were the lower than CK, EO groups, and presented significant difference ($P < 0.05$). Among, ER group was the best. These findings indicated that effluent with *R. gelatinosus* could inhibit *A.hydrophila.*

3.4. Regulation of mTOR and NF-kB signal transduction pathways

The relative expression levels of mTOR and NF-kB signaling pathway genes in head kidney were exhibited in Fig. 3. Compared with the control group, the relative expression level of *TOR gene*, *4E-BP gene* in mTOR and *NF-kB p65*, *IkBa* in NF-kB was increased in ER, WR, EO groups, and ER, WR groups presented significant difference for CK, EO groups ($P < 0.05$). Among, ER group was the best, and showed significant difference for other three groups ($P < 0.05$). Fig. 3 indicated that *R. gelatinosus* promoted mTOR and NF-kB signaling pathway through up-regulating *TOR gene*, *4E-BP gene* in mTOR and *NF-kB p65*, *IkBa* in NF-kB gene expression levels.

3.5. Effect on the intestinal microbiota and the aquaculture water quality

Table 1 showed that the growth, the crude protein and fat of Crucian carp were improved, which was also inseparable from the support of intestinal microbiota. This was because both intestinal microbiota could promote the nutrient absorption even disease resistance for Crucian carp. To investigate the mechanism of effluent containing *R. gelatinosus* improving Crucian carp growth from the aspect of intestinal

Table 3
The number of pathogenic bacteria and cumulative mortality of Crucian carp under CK, WR, ER, EO groups Values (mean ± S.E.) in the same column with different superscript letters significantly differ from each other (P < 0.05).

Group	Number of *A.hydrophila* (CFU/g)	Cumulative mortality (%)
CK	5.5×10^{11a}	79.43 ± 3.41^{a}
EO	5.0×10^{11a}	75.67 ± 4.56^{a}
WR	4.3×10^{4b}	15.87 ± 5.49^{b}
ER	4.1×10^{4b}	13.41 ± 6.75^{b}

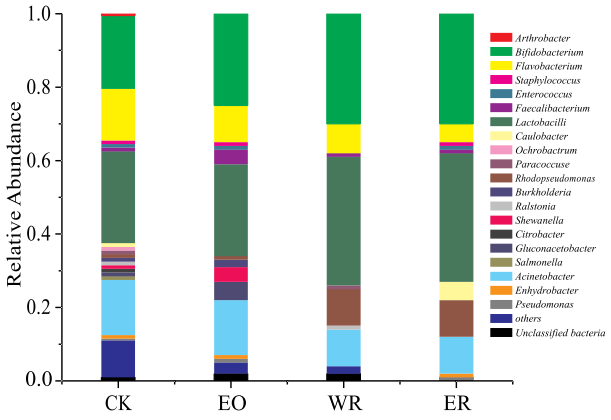

Fig. 4. The change of intestinal microbiota of Crucian carp under CK, WR, ER, EO groups.

microbiota, the change of intestinal microbiota of Crucian carp were determined. The results were showed in Fig. 4.

The change of intestinal microbiota of Crucian carp was observed from Fig. 4. Compared with CK group, the *Flavobacterium* and *Acinetobacter* were reduced extremely significantly in WR, ER groups. The *bifidobacteria* and *lactobacillus* in WR, ER groups were significantly higher than the CK group. *Flavobacterium* and *Acinetobacter* were the pathogen that seriously threatened the life and health of aquatic animals. *Bifidobacteria, lactobacilli* were probiotics, which played an important role in the promotion of absorption, nonspecific immunity and intestinal environment. The change of intestinal microbiota of aquatic animals had been reported with addition of probiotics and biochemicals. Miao et al., 2017 found that probiotics enhanced the proportion of *Bacillus subtilis and Lactococcus* in intestinal microbiota of giant freshwater prawn [40]. González-Félix et al., 2018 reported that probiotic supplements changed intestinal microbiota and histology of *Totoaba macdonaldi* [41].

In addition, to clarify the effect of *R. gelatinosus* in effluent on Crucian carp aquaculture water quality, the ammonia, COD were determined in Crucian carp aquaculture water. It was found from Table 4 that the Crucian carp aquaculture water quality in WR, ER groups were significantly improved comparing with CK, EO groups. The ammonia and COD contents were reduced under WR and ER groups.

Table 4
The ammonia nitrogen, COD contents in Crucian carp aquaculture water after three months under CK, WR, ER, EO groups Values (mean ± S.E.) in the same column with different superscript letters significantly differ from each other ($P < 0.05$).

Group	Ammonium	COD (mg/L)
CK	5.88 ± 0.56[a]	149 ± 14.31[a]
EO	5.41 ± 0.81[a]	140 ± 13.15[a]
WR	3.53 ± 0.31[b]	58.17 ± 5.89[b]
ER	3.34 ± 0.71[b]	54.65 ± 5.78[b]

4. Discussion

Current research showed that effluent containing *R. gelatinosus* (ER group) had better promoting effect on the growth and the crude protein, crude fat contents than other three groups under the same breeding conditions (Table 1). The main reason was that the effluent, as aquaculture water, contained *R. gelatinosus* and residual organic matter. Compared with chemical bait or water (CK), *R. gelatinosus* or effluent provided more abundant and diverse nutrients for Crucian carp. *R. gelatinosus* were rich in and secreted diverse biochemicals such as amino acid (arginine), B vitamins, lipids, pigments, trace element (copper) [9,10]. These biochemicals contributed to the growth, metabolism and the synthesis of substances in Crucian carp. Pereira et al., 2017 found that arginine affected on growth and whole-body composition for Nile tilapia [42]. Yuan et al., 2019 studied that the copper supplementation enhanced the growth for juvenile *Litopenaeus vannamei* [25].

Meanwhile, an interesting phenomenon was observed from Table 1. Crucian carp could also grow well and the yield also was enhanced under EO group compared with initial weight. Moreover, the yield and the crude protein, crude fat contents were also different between ER and WR groups under the same amount of *R. gelatinosus*. It was found by analysis and comparison that the residual organic components in effluent was only different between ER and WR groups. SPW also was rich in some biochemicals (isoflavone, saponin, sterols and oligosaccharides) and nutrient substance [17].

These residual organic compounds could serve as food (protein, amino acid) even healthcare substance (isoflavone) [17]. For example, soy isoflavones could enhance the non-specific immunity and antioxidant capacities of cells according to Cao et al., 2019 [43]. Thus, Zhang et al., 2018 used soybean meal (protein, isoflavone) to improve the growth of Japanese seabass (*Lateolabrax japonicus*) [33]. Crucian carp could directly absorb and utilize these residual substances in effluent. Moreover, *R. gelatinosus* could degrade unceasingly the residual organic matter into monosaccharide, amino acids and fatty acids. They also could secrete continually biochemicals using the residual organic matter as substrate.

Table 1 indicated that it was feasible to promote Crucian carp production, survival rate and whole fish body composition by effluent containing *R. gelatinosus* and residual biochemicals. Similar studies had been reported on probiotics and biochemicals. Safari and Paolucci et al., 2017 observed the maximum specific growth rate (2.32% day^{-1}) and survival rate (93.67%) in the juvenile crayfish fed with *galactooligosaccharide + Enterococcus faecalis* diet [5]. Nguyen et al., 2017 used the probiotic *Lactococcus lactis* WFLU12 to improve growth rate in olive flounder [6]. Although numerous studies have examined the effects of probiotics in aquatic animals, currently there was no consensus regarding the usefulness of probiotics, and the individual probiotic strains greatly differed in their effects on many aspects of host functions (Lin et al., 2017). Moreover, there was no studies on Crucian carp aquaculture and the improvement of yield using the effluent containing *R. gelatinosus* and residual biochemicals [8].

Hidalgo et al., 1999 and Zhang et al., 2018 found the food (bait) stimulated and influenced the secretion of fish intestinal digestive enzymes [33,44]. In artificial breeding conditions, the composition and content of nutrients in diet directly determined the secretion and activity of digestive enzymes. In this work, under ER group, *R. gelatinosus* and residual organic matter in effluent provided more diverse and abundant nutrients such as protein, amylase, polysaccharides than other groups. Moreover, *R. gelatinosus* also secreted some digestive enzymes. Thus, the protease, amylase activities were the highest in ER group (Table 2). But, the lipase activity did not present significant difference for all given groups (Table 2). These were consistent with the reference reports. Suzer et al., 2008; Lu et al., 2018 thought the protease, amylase activities were closely related to protein and starch content in diets and is positively correlated [45,46]. The relationship between lipase of fish and the lipid content in diet was complex, and there was no obvious correlation between them.

In this work, these findings in Figs. 1–2 indicated that the effluent with *R. gelatinosus* and biochemicals improved the nonspecific immunity and antioxidant capabilities 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 [47,48]. 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 [8,23]. As Fig. 1 shown, ER group significantly increased the AKP, ACP, phagocytic activities of Crucian carp, which improved the nonspecific immunity ability, disease resistance, survival rate and growth (Table 1).

As for antioxidant systems, the SOD, CAT were the vital enzymes in antioxidant defense system [23,28]. 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. 1 shown, ER group significantly increased SOD, CAT activities. This finding indicated that *R. gelatinosus* could enhance the antioxidant ability of Crucian carp, protected cells from damage, improved the survival rate and promoted the growth of Crucian carp (Table 1).

Furthermore, Table 3 indicated that effluent with *R. gelatinosus* could inhibit *A.hydrophila*. For one thing, the remainder biochemicals and *R. gelatinosus* in effluent enhanced the antioxidant and non-specific immune ability of Crucian carp (Fig. 1), which inhibited or killed *A.hydrophila*. For another, *R. gelatinosus* owned or secreted some bioactive substances as inhibitors to inhibit or kill directly pathogenic bacteria *A.hydrophila*. Jagielo et al., 1998 described that *R. gelatinosus* cell wall contained 3-hydroxy-sebacic acid and diphosphonic acid lipoid A [49]. Baker et al., 1990 observed that *R. gelatinosus* contained antiviral, antigen Ag1, Ag2, Ag3 and immune factors, even secreted antibiotics [50]. Above bio-active substances could inhibit or kill pathogenic bacteria [49,50]. Moreover, *R. gelatinosus* with absolute dominance also competed the nutrition and space with pathogenic microbes. Besides, *R. gelatinosus* also might destroy the pathogenic bacteria by activating the specific immune response of Crucian carp. Fečkaninová et al., 2017 summaried the use of probiotic bacteria against *Aeromonas* infections in salmonid aquaculture [51]. Yi et al., 2017 studied the antimicrobial activity against fish pathogenic bacteria of probiotic *Bacillus velezensis JW* [52]. Zhou et al., 2015 resisted to *Aeromonas hydrophila* of juvenile yellow catfish with different arginine levels [39].

Meanwhile, these findings of Table 2 and Fig. 1 showed the effluent enhanced digestive enzymes, AKP, ACP, SOD, CAT activities simultaneously. Wu et al., 2019; Yu et al., 1998; Mujahid et al., 2011 [9,17,53] showed that both *R. gelatinosus* and SPW effluent contained the

biochemical substances (carotenoids, PHB, pantothenic acid, riboflavin, citric and sorbic acid) and a few metal ions. Further, Liu et al., 2019 observed the effects of carotenoids on the immune related enzymes of yellow catfish [54]. Duan et al., 2017 studied the effect of dietary poly-β-hydroxybutyrate (PHB) on digestive enzymes of *Litopenaeus vannamei* [55]. Li et al., 2015 found that dietary pantothenic acid deficiency and excess depressed the digestive enzymes activities of grass carp [56]. Chen et al., 2015 found that dietary riboflavin deficiency decreases antioxidant related enzymes activities in the gills of Ctenopharyngodon idella [57]. He et al., 2017 observed that the citric and sorbic acid increased the alkaline phosphatase, phenoloxidase, glutathione peroxidase activities in shrimp [58]. To sum up, *R. gelatinosus* and SPW effluent regulated digestive enzymes, AKP, ACP, SOD, CAT activities by containing biochemical substances and metal ions.

With regard to the mechanism of biochemical substances and metal ions regulating these enzymes activities, there might be two reasons. Firstly, the biochemical substances and metal ions constituted enzymes or regulated enzyme synthesis pathway. *R. gelatinosus* contained a variety of amino acids, which were the basic components of enzymatic proteins [10].

Secondly, *R. gelatinosus* and the remainder biochemicals also might induce or stimulate the expression of *akp, acp, sod,* and *cat* gene as stimulation signal or activation factor (Fig. 2). This view was explained by some researches. *R. gelatinosus* were rich in carotenoid [10]. Chiu and Liu, 2014 observed that the carotenoid product enhanced the gene expression levels of GHR1 and IGF-1 and ACH_{50} in tilapia [12]. Meanwhile, the composition of SPW and effluent was consistent with that of soybean meal [17]. Zhang et al., 2018 used soybean meal (protein, isoflavone) to improve the expression of gut transporter genes of *Lateolabrax japonicus* [33].

As a signal transduction pathway, the mTOR signaling pathway, which plays a vital role in nutrition regulation and has complex impact on cell growth [59], widely exists in eukaryotes [60], food intake and environmental stresses [61,62]. TOR pathway is a key regulator of the balance between protein synthesis and degradation in response to nutrition quality and quantity [63,64], and the protein synthesis is essential for cell growth, proliferation, apoptosis, and autophagy [65]. Moreover, immune protein synthesis and nutrient transport are also each related to mTOR [66]. In this study, higher genes expression in mTOR signaling pathway was induced in ER, WR groups. The yield, digestive enzymes, immune-related enzymes were enhanced in ER, WR 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 [56,67]. It was closely related to the differentiation of immune cells [68]. In this study, higher genes expression in NF-kB signaling pathway was induced in ER, WR groups, and then immune-related enzymes activities were enhanced in ER, WR groups. Jagielo et al., 1998; Baker et al., 1990 thought that *R. gelatinosus* contained the antigen Ag1, Ag2, Ag3 and the non-methylation dinucleotide sequence (CpG-DNA) [49,50]. Further, Baker et al., 1990; Morales-Nebreda et al., 2019 observed that antigen Ag1, Ag2, Ag3 and CpG-DNA had very strong immune stimulation and induced the release of immune factors and differentiation of immune cells [50,69]. Therefore, phagocytic activity was enhanced under ER, WR groups in this work. These results suggested that *R. gelatinosus* promoted the differentiation of immune cells and the activities of immune-related enzymes by regulating NF-kB signaling pathway. Besides, *R. gelatinosus* as stimulation signal also might activate the specific immune response to destroy pathogenic bacteria by regulating signal transduction pathway. Thus, the number of pathogenic bacteria and cumulative mortality of Crucian carp was decreased under WR, ER groups, and thus survival rate was increased (Tables 1, 3).

Adeoye et al., 2016; Zhao et al., 2018 found that intestinal microbiota, especially probiotics, played an important role in non-specific immune system, antioxidant system, disease resistance and digestive system [70,71]. Figure. 5 and Jagielo et al., 1998; Baker et al., 1990 supported that *R. gelatinosus* in effluent inhibited or killed pathogenic bacteria (*Flavobacterium* and *Acinetobacter*), and promoted the growth of probiotics in Crucian carp intestine [49,50]. Moreover, *R. gelatinosus* themselves were also probiotics. As Fig. 4 shown, the *bifidobacterium*, *lactobacilli* became dominant bacteria. These dominant bacteria were able to secrete nutrients themselves, and promoted the secretion of digestive enzymes (protease, amylase and lipase) and the absorption of nutrients (Table 2). They also could improve the disease resistance of Crucian carp by the inhibition for pathogen and the improvement of non-specific immune (AKP, ACP) and antioxidant (SOD, CAT) systems (Figs. 1–2).

In addition, the ammonia and COD contents in aquaculture water were reduced under WR and ER groups (Table 4). According to Idi et al., 2015, *R. gelatinosus* had good removal effect on ammonia nitrogen due to they could use them as substrates nitrogen source [13]. Similarly, Luo et al., 2012 found that purple non-sulfur bacteria cleaned up aquaculture water quality during shrimp cultivation [72]. Table 4 and Idi et al., 2015; Luo et al., 2012 expressed that *R. gelatinosus* can removed ammonia nitrogen and promoted fish growth as feed at the same time [13,72]. A closed loop was formed that *R. gelatinosus*-crucian carp-feces-*R. gelatinosus*.

To the best of researchers' knowledge, the present study is the first one addressing to culturing Crucian carp with the effluent containing *R. gelatinosus*. Tables 1, 4 indicated that effluent containing *R. gelatinosus* could be reused directly to culture Crucian carp as aquaculture water, microbial feeds and medicament, and to purify aquaculture water quality simultaneously. *R. gelatinosus* improved the yield and survival rate, the digestion, nonspecific immunity, antioxidant and disease resistance capacities, the mTOR, NF-kB signaling pathway, the intestinal microbiota, and purified aquaculture water quality. The technology reduced the use of chemical feeds, medicament and aquaculture water in freshwater aquaculture, and completed the recycle and reuse of wastewater effluent and *R. gelatinosus*. It also simplified the subsequent treatment process, burden, cost and energy consumption of Crucian carp aquaculture wastewater.

5. Conclusion

Improvement of yield, disease resistance and aquaculture water quality of Crucian carp by effluent containing *R. gelatinosus* was feasible. Survival rate, yield, whole fish body composition were increased under ER group. Biochemical and remainder substances in effluent and *R. gelatinosus* improved digestion capacity, nonspecific immunity, antioxidant, mTOR and NF-kB signaling pathway through up-regulating related genes expression levels. Theoretical analysis showed biochemical substances regulated these genes expressions and enzyme activities as stimulus signal, component, active center. Moreover, *R. gelatinosus* and biochemical substances improved intestinal microbiota and inhibited *Aeromonas hydrophila*. Meanwhile, *R. gelatinosus* in effluent purified aquaculture water quality. This technology would saved the water of aquaculture, reduced the water pollution and wastewater discharge, and increased the output and disease resistance of Crucian carp simultaneously.

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Appendix A. Supplementary data

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