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Feasibility of cultivation of Spinibarbus sinensis with coconut oil and its effect on disease resistance (nonspecific immunity, antioxidation and mTOR and NF-kB signaling pathways)

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ABSTRACT

Application of traditional bait in aquaculture caused environment pollution and disease frequent occurrence. Residual coconut could be re-utilized to culture Spinibarbus sinensis as dietary supplement. Therefore, a novel integrated system of the improvement of yield, antioxidant and nonspecific immunity of Spinibarbus sinensis by dietary residual coconut was proposed and investigated. Spinibarbus sinensis could grow well in all supplement residual coconut groups. Survival rate, yield, whole fish body composition under 15–45% groups were increased compared with control group (CK). Bioactive substances (polyphenols and vitamin) in residual coconut 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 coconut improved mTOR and NF-kB signaling pathway. Furthermore, residual coconut inhibited *Aeromonas hydrophila* that increased resistance to diseases. This technology completed the solid waste recovery and the Spinibarbus sinensis culture simultaneously.

1. Introduction

Spinibarbus sinensis meat is fine and tender, and is rich in the protein, fat, carbohydrate, vitamin, nicotinic acid and inorganic com ponents such as calcium, phosphorus, iron [1,2]. Therefore, it is one of the most important and popular aquaculture species in China. But, the large-scale aquaculture and the application of commercial bait cause the pollution of aquaculture water environment and the frequent occurrence of disease [3]. These directly reduce the production performance of aquatic animals [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 yield and disease resistance of fish [5,6].

Coconut is rich in polysaccharides, pectin, phenols, pigment, vitamins (thiamine, riboflavin, nicotinic acid, vitamin C), organic acids, amino acids (citrulline, glutamic acid, arginine), unsaturated fatty acids (linoleic acid) and trace elements. It has the function of diuresis and hypotension and treatment of nephritis [7,8]. With the increase of demand, a large number of residual coconut (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 coconut residue as nature resource has the potential of recycling to replace fish dietary.

Therefore, a novel strategy of the improvement of Spinibarbus sinensis production performance using residual coconut is proposed in this work. The residual coconut is directly re-utilized to culture Spinibarbus sinensis as dietary even medicament. The new strategy is conducive to solid waste reduction, chemical feed reduction, resource recovery, aquaculture at the same time.

To the best knowledge, the enhancement of production performance of Spinibarbus sinensis by residual coconut was not researched. Moreover, the mechanism also is not clear that residual coconut

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regulate the yield, immunity, antioxidation and disease resistance of Spinibarbus sinensis. Therefore, the purpose of the work is to investigate the feasibility of the residual coconut culturing Spinibarbus sinensis and enhancing its yield and disease resistance; to explain the mechanism of the residual coconut affecting Spinibarbus sinensis yield and disease resistance in terms of nonspecific immunity, antioxidation and mTOR and NF-kB signaling pathways.

2. Materials and methods

2.1. Fish rearing by the residual coconut

The experiments were carried out at May to August 2018. Juvenile Spinibarbus sinensis (30 ± 5 g and 11 ± 3 cm) were bought from the local fish farming plant. coconut purchased from local market. Residual coconut (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 Spinibarbus sinensis 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 crushed and mixed by water, and was partially replaced by residual coconut. 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. Spinibarbus sinensis were fed once daily at a rate of 10%–15% of body weight during the experiment. 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).

2.2. Analysis and measurement

2.2.1. Determination of yield and quality of spinibarbus sinensis

After 123 days feeding, each Spinibarbus sinensis was individually weighed (± 0.01) on an electronic scale (AND, Japan). The survival was calculated as follows:

2.3. 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. Spinibarbus sinensis were anesthetized in ice water prior to execution. 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) [9]. 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 [10]. Crude lipids were extracted based on the method of Folch et al., 1957 [11]. Total ash was determined gravimetrically by ignition at 600 °C for 6 h in muffle furnace.

2.3.1. 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 Kong et al., 2017; Lin et al., 2017; Cooper et al., 2002; Yuan et al., 2019 [12–15]. The head kidney macrophages (HKM) were isolated and prepared according to Secombes, 1990 [16]. The phagocytic activity of macrophages was determined by the following Sakai et al., 1995 and Houwen, 2002 [17,18].

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

According to Kong et al., 2017; Qi et al., 2017 [12,19], 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 [20].

2.3.3. Challenge test

The challenge test was carried out separately according to Lin et al., 2017; Chiu et al., 2015 [13,21]. 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 supplement, 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 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 *Aeromonas hydrophila*).

2.4. 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. Significant difference is set at the level of $P < 0.05$.

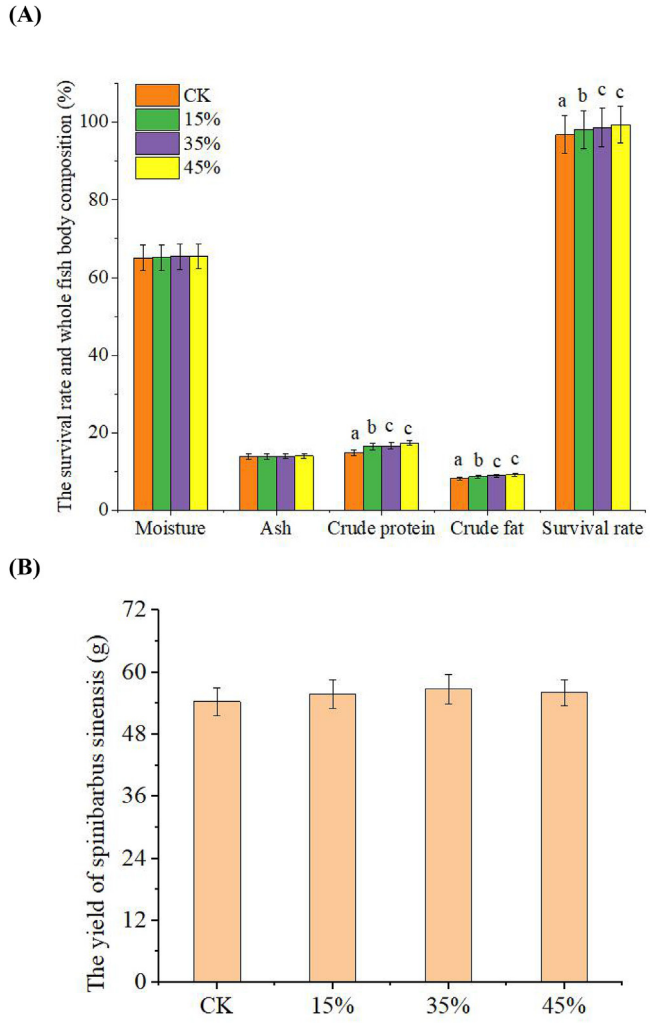

Fig. 1. The survival rate, yield and whole fish body composition of Spinibarbus sinensis after 123 days under CK, 15%, 35%, 45% groups (A) Survival rate and whole fish body composition; (B) Yield Values (mean ± S.E.) in the same column with different superscript letters significantly differ from each other ($P < 0.05$).

3. Results

3.1. The feasibility of culturing juvenile spinibarbus sinensis with residual coconut

To research the effect of dietary residual coconut supplement on Spinibarbus sinensis culture, the yield, survival rate and whole fish body composition were determined. The results were showed in Fig. 1.

It was observed from Fig. 1 that Spinibarbus sinensis could survive and grow well under all given processing groups (CK, 15%, 35%, 45%). The yield did not show significant difference between CK, 15%, 35%, 45% groups ($P > 0.05$) (Fig. 1B). The survival rate was higher under 15–45% groups than CK group, and showed significant difference ($P < 0.05$) (Fig. 1A). The 35%, 45% groups was the best for the survival rate and yield under all given groups. The increased of survival rate and yield in 15–45% groups might be associated with residual coconut (Fig. 1A). The survival rate and yield of 35%, 45% groups was almost the same, indicating that the nutritional requirements of Spinibarbus sinensis was fully satisfy under 35% group. More coconut supplements (45% group) also did not affect Spinibarbus sinensis growth. But, the nutritional requirements was not satisfy under 15% group. The survival rate and yield of 15% group was lower than 35%, 45% groups. Fig. 1 indicated that it had very good feasibility to culture Spinibarbus sinensis using residual coconut.

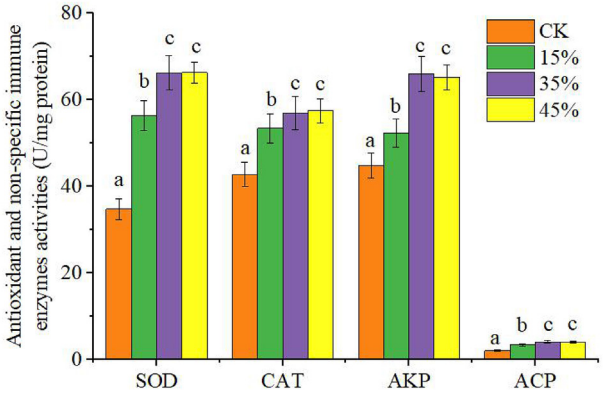

Fig. 2. The nonspecific immune related enzyme and antioxidant related enzyme activities of Spinibarbus sinensis under CK, 15%, 35%, 45% groups Values (mean ± S.E.) in the same column with different superscript letters significantly differ from each other ($P < 0.05$).

Meanwhile, the moisture and ash content in whole fish body composition did not show significant difference between all given groups ($P > 0.05$) (Fig. 1B). Compared with CK group, the crude protein and crude fat of Spinibarbus sinensis were improved under 15–45% groups, and presented significant difference ($P < 0.05$).

3.2. Affecting antioxidant, nonspecific immunity, disease resistance of spinibarbus sinensis

Fig. 1A showed that the survival rate were improved by residual coconut. These findings might be related to the nonspecific immunity and antioxidant systems. To investigate the mechanism by which the residual coconut affected Spinibarbus sinensis 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 Spinibarbus sinensis were determined. The results were showed in Fig. 2.

The AKP, ACP, phagocytic, SOD, CAT activities of Spinibarbus sinensis 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 ($P < 0.05$).

Meanwhile, to further investigate the molecular biological mechanism of the residual coconut regulating nonspecific immunity and antioxidant, *AKP, ACP, SOD, CAT* gene expression levels were determined. These results were showed in Fig. 3. 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 ($P < 0.05$). Figs. 2–3 indicated that residual coconut improved AKP, ACP, SOD, CAT activities through up-regulating *AKP, ACP, SOD, CAT* gene expression levels.

Furthermore, the *Aeromonas hydrophila (A. hydrophila)* challenge test was also conducted. Table 1 showed the number of pathogenic bacteria and cumulative mortality of Spinibarbus sinensis 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. These findings indicated that residual coconut could inhibit *A. hydrophila*.

3.3. 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. 4. Compared with the control group, the relative expression level of *TOR gene*, *4E-BP gene*

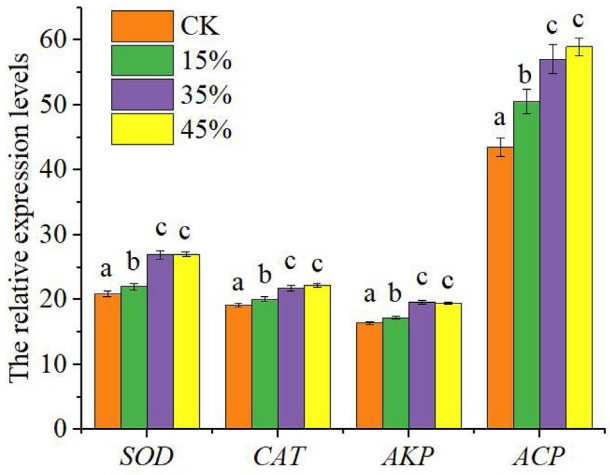

Fig. 3. The relative expression levels of *AKP, ACP, SOD, CAT* genes under CK, 15%, 35%, 45% groups Values (mean ± S.E.) in the same column with different superscript letters significantly differ from each other ($P < 0.05$).

Table 1
The number of pathogenic bacteria and cumulative mortality of Spinibarbus sinensis under CK, 15%, 35%, 45% 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	9.9 × 1011a	86.34 ± 3.42a
15%	8.7 × 1011b	66.78 ± 5.50b
35%	7.5 × 104c	21.32 ± 6.76c
45%	8.4 × 104c	23.58 ± 4.57c

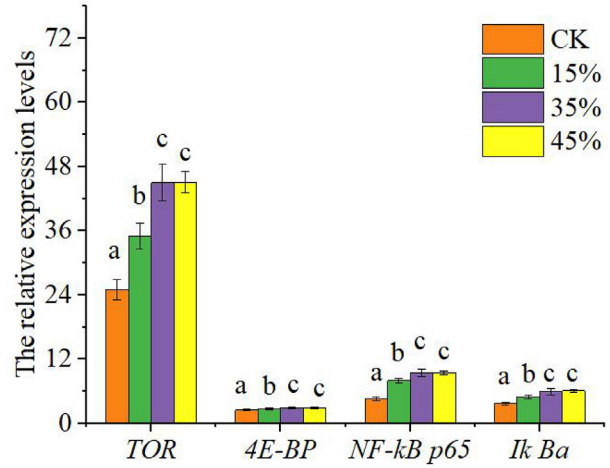

Fig. 4. The relative expression levels of *TOR*, *4E-BP*, *NF-kB p65*, *IkBa* genes in mTOR and NF-kB signaling pathway under CK, 15%, 35%, 45% groups Values (mean ± S.E.) in the same column with different superscript letters significantly differ from each other ($P < 0.05$).

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 ($P < 0.05$). Fig. 4 indicated that *residual coconut* 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.

4. Discussion

Current research showed that 15–45% groups had better promoting effect on the yield and the crude protein, crude fat contents than control group (CK) (Fig. 1A and B). The main reason was the replacement of dietary residual coconut. Compared with CK group, the supplement of dietary residual coconut provided more rich and multiplex nutrients for Spinibarbus sinensis. Residual coconut were still rich in diverse bioactive substances such as pentosan, pectin, ash, organic acid, vitamin, polyphenols, trace elements, lignin, hemicellulose, cellulose [7,8]. Among, cellulose content was 53.06% of dry weight, and contained α-cellulose, β-cellulose and γ-cellulose. These bioactive substances contributed to the yield and metabolism of Spinibarbus sinensis. Long et al., 2017 [22] researched the effects of dietary polyphenols on growth performance of *Megalobrama amblycephala*. Xun et al., 2019 [23] observed effects of dietary vitamin on growth performance of juvenile golden pompano. Yuan et al., 2019 [24] studied that the copper supplementation enhanced the growth for Litopenaeus vannamei.

Moreover, some in bioactive substances (amino acid) also were the raw materials of crude protein, crude fat. Thus, residual coconut supplement groups were better than CK group for crude protein, crude fat contents (Fig. 1A). Furthermore, these bioactive substances could serve as feed (protein, amino acid) even healthcare substance (polyphenols). For example, polyphenols and vitamin had antioxidant and non-specific immune capacities according to Xun et al., 2019; Li et al., 2018; Hoskin et al., 2019 [23,25,26]. Fig. 1 indicated that it was feasible to promote Spinibarbus sinensis yield, survival rate and whole fish body composition by residual coconut.

In this work, these findings in Figs. 2–3 indicated that the residual coconut 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 [27,28]. 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. 2 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 [12,13]. As Fig. 2 shown, 15–45% groups significantly increased the AKP, ACP, phagocytic activities of Spinibarbus sinensis, which improved the nonspecific immunity ability, disease resistance, survival rate and yield (Fig. 1).

As for antioxidant systems, the SOD, CAT were the vital enzymes in antioxidant defense system [12,19]. 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 coconut could enhance the antioxidant ability of Spinibarbus sinensis, protected cells from damage, improved the survival rate and promoted the yield of Spinibarbus sinensis (Fig. 1A and B). Furthermore, Table 1 indicated that residual coconut inhibited *A. hydrophila* because that it enhanced the antioxidant and non-specific immune ability of Spinibarbus sinensis (Fig. 2).

Meanwhile, these findings of Fig. 2 showed the residual coconut enhanced AKP, ACP, SOD, CAT activities simultaneously. Joshi et al., 2019; Al-Sayed et al., 2013 [7,8] showed that residual coconut contained bioactive substances (vitamin, polyphenols) and metal ions. Further, Hoskin et al., 2018; Yi et al., 2017 [26,29] observed the effects of polyphenol on enzyme activities of antioxidant and immune systems in vitro. Ruiz et al., 2019; Wu et al., 2016 [30,31] observed that vitamin C, E, K increased the antioxidant and non-specific immune enzyme activities in various aquatics. To sum up, residual coconut regulated AKP, ACP, SOD, CAT activities by containing bioactive substances and metal ions.

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 coconut contained a variety of amino acids, which were the basic components of enzymatic proteins [32]. At the same time, they contained a large number of vitamins, which constituted a variety of co-enzymes (flavin mononucleotide, Coenzyme A, transmethylase) [32]. Iron, magnesium and zinc were also the active center of the AKP, SOD, CAT in this work.

Secondly, residual coconut also might induce or stimulate the expression of *AKP, ACP, SOD, CAT* gene as stimulation signal or activation factor (Fig. 3). This view was explained by some researches. This view was explained by some researches. Residual coconut were rich in vitamin, polyphenol, organic acid [7,8]. Zhao et al., 2019 [33] observed that polyphenol modulated the gene expression in NRF2/HO-1 MAPK signaling. Rahman et al., 2019 [34] found that effect of vitamin C on the gene expression of the Nile tilapia. He et al., 2017 [35] observed that the organic acids increased the gene expression levels of TNF-α, LITAF and RAB6A in shrimp.

As a signal transduction pathway, the mTOR signaling pathway, which plays a vital role in nutrition regulation and has complex impact on cell growth [36], widely exists in eukaryotes [37], food intake and environmental stresses [25,38]. TOR pathway is a key regulator of the balance between protein synthesis and degradation in response to nutrition quality and quantity [39,40], and the protein synthesis is essential for cell growth, proliferation, apoptosis, and autophagy [41]. Moreover, immune protein synthesis and nutrient transport are also each related to mTOR [42]. In this study, higher genes expression in mTOR signaling pathway was induced in 15–45% groups. The yield, 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 [43,44]. It was closely related to the differentiation of immune cells [45]. 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 coconut promoted the activities of immune-related enzymes by regulating NF-kB signaling pathway. Thus, the number of pathogenic bacteria and cumulative mortality of Spinibarbus sinensis was decreased under 15–45% groups, and thus survival rate was increased (Fig. 1A).

To the best of researchers' knowledge, the present study is the first one addressing to culturing Spinibarbus sinensis with the residual coconut. Fig. 1 indicated that residual coconut could be reused directly to culture Spinibarbus sinensis. Residual coconut improved the yield and survival rate, the nonspecific immunity, antioxidant and disease resistance capacities, the mTOR, NF-kB signaling pathway. The technology reduced the use of chemical feeds in aquaculture, and completed the recycle and reuse of residual coconut.

5. Conclusion

Improvement of yield, disease resistance of Spinibarbus sinensis by residual coconut was feasible. Survival rate, yield, whole fish body composition were increased under 15–45% groups. Bioactive substances in residual coconut improved nonspecific immunity, antioxidant, mTOR and NF-kB signaling pathway through up-regulating related genes expression levels. Theoretical analysis showed residual coconut including bioactive substances regulated these genes expressions and enzyme activities as stimulus signal, component, active center. Moreover, residual coconut inhibited *Aeromonas hydrophila*. This technology increased the output and disease resistance of Spinibarbus sinensis simultaneously.

Conflicts of interest

The authors declare that they have no conflict of interest.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the authors.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.06.052.

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