Effects of Replacing Fish Meal with Soybean Meal on the Composition and Protease-Producing Capacity of Intestinal Protease-Producing Aerobic Bacteria in Snakehead (Channa argus) Postprint

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Abstract

This study evaluated the effects of replacement of fish meal with soybean meal on the composition of intestinal aerobic bacteria and their protease-producing capacity in Channa argus by isolating and cultivating protease-producing aerobic bacterial strains and determining their protease activity.

Full Text

Effects of Replacement of Fish Meal by Soybean Meal on Composition and Protease-Producing Activity of Aerobic Bacteria in the Intestine of Channa argus

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Abstract

This study evaluated the effects of replacing fish meal with soybean meal on the composition and protease-producing activity of aerobic bacteria in the intestine of Channa argus by isolating and culturing protease-producing bacterial strains and measuring their enzymatic activity. A control diet (D1 group) was formulated with fish meal as the main protein source at 55% inclusion level. Two experimental diets were prepared by replacing different proportions of fish meal with soybean meal, resulting in dietary soybean meal levels of 35% (D2 group) and 75% (D3 group). The three experimental diets were fed to juvenile Channa argus with an average body weight of (10.50$±$0.84) g for 8 weeks, with each diet assigned to three replicates of 28 fish each, in an indoor recirculating aquaculture system. The results showed that final body weight, weight gain rate, and specific growth rate of Channa argus decreased significantly as the proportion of fish meal replaced by soybean meal increased ($P < 0.05$).

A total of 21 protease-producing aerobic bacterial strains were isolated from the intestines of fish in the three groups, including 16 strains from D1 group, 19 from D2 group, and 20 from D3 group. Protease-producing activity was expressed as the ratio of hydrolysis zone diameter to colony diameter (R/r). Replacement of fish meal with soybean meal significantly decreased the R/r values of strains P1004 and P1018 ($P < 0.05$), but significantly increased the R/r values of strains P1009 and P1012 ($P < 0.05$). As the proportion of fish meal replaced by soybean meal increased, the R/r value of strain P1018 decreased significantly ($P < 0.05$).

Physiological and biochemical characterization and 16S rDNA sequence analysis of strain P1009, which exhibited the highest protease-producing activity, identified it as *Pseudomonas aeruginosa*. The results of this study indicate that the intestinal tract of Channa argus harbors a rich diversity of protease-producing aerobic bacteria, and that replacing fish meal with soybean meal affects both the quantity and protease-producing activity of these bacteria. Strain P1009, showing the strongest protease-producing capacity in the intestine of Channa argus, was identified as *Pseudomonas aeruginosa* and could be considered as a potential candidate for probiotic development.

Keywords: Channa argus; protein source; protease-producing aerobic bacteria; protease-producing activity; probiotics

Introduction

A complex and dynamic microbial community adheres to the mucosal surface of the fish digestive tract, with bacterial populations generally maintained at $10^{7\text{-}10}$8 cells per gram [1]. These bacteria reside in specific locations within the host' s digestive tract and participate in feed digestion, nutrient absorption, and energy metabolism [2-3]. Large-scale shotgun sequencing analysis of the human distal gut microbiome metagenome revealed that microbial genomes contain numerous genes related to the metabolism of carbohydrates, amino acids, xenobiotics, methane, vitamins, and cholesterol [4]. Bairagi et al. [5] found that the intestine of grass carp (*Ctenopharyngodon idella*) is rich in microorganisms capable of secreting amylase, protease, and cellulase. Purification and cultivation of digestive enzyme-producing microorganisms from fish intestines represents a primary source for aquaculture probiotics. Colonizing probiotic strains in the fish intestine through various pathways can synergize with digestive enzymes secreted by the fish intestine to degrade nutrients in feed, thereby improving feed utilization efficiency and growth performance. For example, dietary probiotic supplementation effectively enhanced growth rate and intestinal digestive enzyme activity in *Schizothorax davidi* [6] and *Oreochromis niloticus* × *O. aureus* [7].

Furthermore, the gut microbiota structure is plastic and susceptible to dietary nutrient composition [8-11]. Zhong et al. [12] investigated the intestinal microbiota structure and diversity of *Elopichthys bambusa* using PCR-DGGE fingerprinting combined with cloning and sequencing, and found significant differences in the microbiota structure of intestinal contents between fish fed formulated feed and fresh fish. The rapid development of aquaculture has led to a sharp increase in demand for fish meal. However, due to overfishing, environmental pollution, and adverse climate events such as El Niño, wild fish meal resources are dwindling, and global fish meal supply can no longer meet aquaculture demands. Reducing fish meal inclusion in aquafeeds to decrease reliance on fish meal resources represents an effective measure to alleviate pressure on fish meal supplies. In research on fish meal alternative protein sources, soybean meal is considered a suitable plant protein source for aquafeeds due to its high protein content, high digestibility, and balanced amino acid profile. This study evaluated the effects of different protein sources (fish meal only versus soybean meal partially replacing fish meal) on the composition and protease-producing activity of aerobic bacteria in the intestine of *Channa argus* by cultivating protease-producing aerobic bacterial strains and measuring their protease-producing capacity, providing data support for further development of microecological nutrition theory for *Channa argus*.

Experimental Diet Formulation and Preparation

The control diet (D1 group) used fish meal as the protein source at an inclusion level of 55%. Two experimental diets were formulated by replacing different proportions of fish meal with soybean meal based on the control diet, resulting in dietary soybean meal levels of 35% (D2 group) and 75% (D3 group). The composition and nutrient levels of the experimental diets are shown in Table 1 . Prior to feed preparation, all ingredients were passed through an 80-mesh sieve and mixed thoroughly. Fish oil and soybean lecithin were then added and mixed completely. Water was added to the mixture, which was then extruded into 3.0 mm × 4.0 mm pellets using a screw extruder. The pellets were dried in a 50 °C oven and stored at -20 °C.

Experimental Fish and Culture Management

Juvenile *Channa argus* were purchased from Gaoyou Dong' s Special Fish Farm in Yangzhou City, Jiangsu Province. The fish were from the same batch of artificially bred fingerlings in the current year, with uni-

form size and healthy condition. The feeding trial was conducted in a greenhouse aquaculture facility at Yangzhou University. The culture containers were 300 L fiberglass tanks with a water flow rate of 2 L/min. Prior to the experiment, fish were fed a commercial diet (45% crude protein) for a 2-week acclimation period. At the start of the experiment, fish were fasted for 24 h. Juvenile *Channa argus* with similar body weight [(10.50$±0.84)*g*]*androbusthealthwerethenselectedandrandomlydividedintothreegroups*, *withthreereplicatesp*
fedtheexperimentaldietstwicedaily(08 : 00*and*17 : 00).*Feedingbehaviorwasobservedandrecordeddaily*, *andfe*
°C, pH was 6.7-6.9, dissolved oxygen concentration was 5.5-6.5 mg/L, ammonia nitrogen concentration was 0.037-0.072 mg/L, and nitrite nitrogen concentration was 0.014-0.031 mg/L.

Sample Collection and Parameter Measurement

At the end of the feeding trial, experimental fish were fasted for 24 h before sampling. All fish were anesthetized with MS-222 (100 mg/kg) prior to sampling to reduce stress from handling. Individual fish were weighed to calculate weight gain rate (WGR) and specific growth rate (SGR). The number of fish in each tank was counted to calculate survival rate (SR). The formulas were as follows:

Weight gain rate (%) = 100 × (Wt -W0)/W0
Specific growth rate (%/d) = 100 × (ln Wt -ln W0)/t
Survival rate (%) = 100 × Nt/N0

Where: W0 is the initial total weight of fish in each group (g); Wt is the final total weight of fish in each group (g); W0 is the initial average body weight of fish in each group (g); Wt is the final average body weight of fish in each group (g); t is the experimental duration in days (d); N0 is the initial number of fish in each group; Nt is the final number of fish in each group.

Subsequently, 10 fish were randomly selected from each replicate. Their body surfaces were wiped with 75% ethanol cotton balls to reduce bacterial contamination, and they were dissected using sterile surgical scissors and scalpels. After removal, the intestines were tied at both ends with sterile cotton thread, placed in centrifuge tubes, and 2.5 mL of phosphate-buffered saline (PBS) was added. To ensure reliability of the experimental data, intestines from 10 fish were pooled in the same centrifuge tube, and three replicate samples were prepared.

Aerobic bacterial culture: Intestinal mucosa was homogenized using a sterile handheld homogenizer, and the homogenate was serially diluted with sterile PBS to 10^-1, 10^-2, 10^-3, 10^-4, and 10^-5. Aliquots (50 µL) of each dilution were spread-plated on freshwater fish culture medium (product of Qingdao Hi-Tech Park Hope Bio-Technology Co., Ltd.; composition: peptone 5.0 g, beef extract 2.5 g, yeast extract 2.5 g, glucose 1.0 g, NaCl 15.0 g, MgSO4 0.05 g, K2HPO4 0.2 g, agar 15.0 g, pH 7.2-7.4), with three parallels per sample. After incubation at 30 °C for 48 h, colonies were counted, and single colonies of different sizes, colors, and shapes were sequentially picked with an inoculation loop and purified on culture medium. Following 24 h incubation at 30 °C, purified colonies were selected, inoculated into liquid medium, and cultured at 30 °C on a shaker (200 r/min) for 18 h to expand the culture. One milliliter of each bacterial suspension was transferred to a sterile centrifuge tube, mixed 1:1 with 40% glycerol, and stored at -20 °C for subsequent DNA extraction and strain identification.

Screening of protease-producing aerobic bacteria and determination of protease-producing activity: Purified bacterial suspensions were diluted to 10^-7, and 50 µL aliquots were spread-plated on protease screening medium (composition: casein 10.0 g, Na2HPO4 2.0 g, MgSO4 0.5 g, NaCl 0.20 g, L-tyrosine 0.05 g, beef extract 3.0 g, agar 20 g, distilled water 1,000 mL, pH 8.0, sterilized at 121 °C for 20 min), with five parallels per sample. After incubation at 28 °C for 24 h, plates were examined for hydrolysis zones around colonies. The diameter of protease-producing colonies (r, cm) and the corresponding hydrolysis zone diameter (R, cm) were measured, and the ratio of hydrolysis zone diameter to colony diameter (R/r) was used to express protease-producing activity.

Identification of protease-producing aerobic bacteria: Morphological observation and physiological and biochemical identification of bacterial strains were performed according to *Bergey' s Manual of Determinative Bacteriology* (8th edition). The 16S rDNA identification method was as follows: Target strain DNA was extracted using the TIANamp Bacteria DNA Kit (Tiangen Biotech) following the manufacturer' s instructions. Using the DNA as template, the 16S rDNA fragment was amplified with universal primers: forward primer 27F: 5' -AGAGTTTGATC(C/A)TGGCTCAG-3' and reverse primer 1492R: 5' -GGTTACCTTGTTACGACTT-3' . The reaction mixture contained 21 µL ddH2O, 25 µL Mix, 1 µL each of forward and reverse primers, and 2 µL DNA template. PCR amplification conditions were: 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 90 s; followed by 72 °C for 7 min and storage at 4 °C. PCR products were sequenced by BGI Genomics.

Statistical Analysis

Statistical analysis was performed using Excel 2010 and SPSS 19.0 software. Data are expressed as mean ± standard error (mean ± SE), with significance level set at $P < 0.05$. When significant differences were detected, t-tests were used for comparative analysis of intra-group and inter-group differences.

Effects of Soybean Meal Replacement on Growth Performance of Channa argus

As shown in Table 2 , the final body weight, weight gain rate, and specific growth rate of *Channa argus* in D1 group were significantly higher than those in D2 and D3 groups ($P < 0.05$), and these parameters in D2 group were significantly higher than those in D3 group ($P < 0.05$). Survival rates in D1, D2, and D3 groups were 88.00%, 86.67%, and 88.00%, respectively. Replacement of fish meal with different proportions of soybean meal had no significant effect on survival rate of *Channa argus* ($P > 0.05$).

Table 2 Effects of replacement of fish meal by soybean meal on growth performance of *Channa argus.* In the same column, values with no letter or the same letter superscripts mean no significant difference ($P > 0.05$), while different lowercase letter superscripts mean significant difference ($P < 0.05$). The same as Table 3 .

Effects of Soybean Meal Replacement on Numbers of Protease-Producing Aerobic Bacteria in Channa argus Intestine

Through cultivation of intestinal microbiota from each group, a total of 21 protease-producing aerobic bacterial strains were isolated. Specifically, 16 strains were from D1 group, 19 from D2 group, and 20 from D3 group. The strain numbers are shown in Table 3 . Strains P1003, P1006, P1015, P1016, and P1020 were not detected in D1 group; strains P1002 and P1020 were not detected in D2 group; and strain P1002 was not detected in D3 group.

Based on strain screening, the protease-producing activity of each aerobic bacterial strain was measured, with results expressed as R/r values shown in Table 3 . Statistical analysis indicated significant differences in R/r values among different strains within the same group ($P < 0.05$). Strains with stronger protease-producing activity in D1 group were P1007, P1008, and P1009, while in D2 and D3 groups they were P1009 and P1012. Notably, strain P1009 exhibited the strongest protease-producing activity across all three groups, with R/r values of 2.91, 3.22, and 3.15 in D1, D2, and D3 groups, respectively. Strains with the weakest protease-producing activity in D1 group included P1002, P1010, P1014, and P1019; in D2 group included P1003, P1010, and P1014; and in D3 group included P1003, P1014, and P1019.

Effects of Soybean Meal Replacement on Protease-Producing Activity of Aerobic Bacteria in Channa argus Intestine

As shown in Table 4 , replacement of fish meal with soybean meal significantly decreased the protease-producing activity of strains P1004 and P1018 ($P < 0.05$), but significantly increased the protease-producing activity of strains P1009 and P1012 ($P < 0.05$). Additionally, as the proportion of fish meal replaced by soybean meal increased, the protease-producing activity of strain P1018 decreased significantly ($P < 0.05$).

Identification of Strain P1009 with Strongest Protease-Producing Activity

When cultured on freshwater fish medium, strain P1009 formed small, centrally raised colonies and was Gram-stain negative. Its physiological and biochemical characteristics are shown in Table 5 . Arginine dihydrolase test, oxidase test, glucose fermentation test, and citrate test results were all positive, while lysine decarboxylase test and maltose oxidation test results were negative. 16S rDNA sequence analysis of strain P1009 revealed 99% homology with *Pseudomonas aeruginosa.* Combined with physiological and biochemical identification results, strain P1009 was identified as *Pseudomonas aeruginosa.*

Discussion

Fish meal has long been used as the primary protein source in aquafeeds due to its high protein content, richness in essential amino acids and fatty acids, presence of unknown growth factors, good palatability, and high digestibility. In this study, weight gain rate and specific growth rate of *Channa argus* in the fish meal-only control group (D1 group) were significantly higher than those in soybean meal replacement groups (D2 and D3 groups), fully demonstrating that fish meal is a high-quality protein source for aquafeeds [13]. When soybean meal replaced different proportions of fish meal, resulting in dietary soybean meal levels of 35% and 75%, the growth of *Channa argus* was significantly affected, which may be related to certain antinutritional factors present in soybean, such as soybean agglutinin, trypsin inhibitors, and saponins [14].

High-throughput sequencing technologies have revealed that vertebrate digestive tracts harbor vast microbial communities. In humans, for example, the colon accumulates bacteria numbering 10 times the human cell count and 100 times the human gene count, with up to 1,150 species [15], forming a complex microecosystem in the intestine [16]. The gut microbiota, host, and intestinal environment (such as feed/food and habitat) form an interactive and interdependent "triangular" relationship, participating in feed digestion, nutrient absorption, and energy metabolism [17]. In this study, we screened 21 aerobic bacterial strains with protease-producing capability from the intestine of *Channa argus.* Wang et al. [18] analyzed enzyme-producing bacteria in the digestive tracts of wild and cultured silver pomfret (*Pampus argenteus*), finding 16 enzyme-producing strains from wild silver pomfret (44% of culturable bacteria produced protease) and 22 strains from cultured silver pomfret (70% of culturable bacteria produced protease). Yang et al. [19] cultured microorganisms from the intestine of Pacific white shrimp (*Litopenaeus vannamei*) and obtained 27 protease-producing strains. These results demonstrate that intestinal microorganisms can secrete digestive enzymes to assist the host in nutrient digestion and absorption, thereby improving feed utilization efficiency [20].

Ray et al. [21] found that *Catla catla*, *Cirrhinus mrigala*, and *Labeo rohita* raised in the same environment but fed different diets exhibited different types and activities of enzymes produced by their digestive tract microbiota, proving that fish intestinal microbiota structure undergoes "structure-function" changes with dietary alterations. In this study, replacement of fish meal with different proportions of soybean meal altered the species and numbers of protease-producing aerobic bacteria in the intestine of *Channa argus*. For example, strains P1002 were not detected in D2 and D3 groups; strains P1003, P1006, P1015, and P1016 were not detected in D1 group; and strain P1020 was not detected in D1 and D2 groups. Meanwhile, the protease-producing activity of some strains was also significantly affected, with soybean meal replacement enhancing the protease-producing activity of strains P1009 and P1012. Studies on other fish species have also demonstrated that dietary protein source significantly affects intestinal microbiota structure. For instance, replacing 30% of fish meal with soybean meal in diets for gilthead sea bream (*Sparus aurata* L.) significantly increased the abundance of Cyanobacteria and *Lactobacillus* but significantly decreased Synergistetes [22]. Gajardo et al. [23] fed Atlantic salmon (*Salmo salar* L.) diets with different plant protein sources replacing fish meal and found that the fish meal-only control group had higher abundances of *Bacillus*, *Streptococcus*, *Clostridium*, and *Cetobacterium* in the intestine. These results may be related to the nutritional requirements of microbial growth, as plant protein sources contain more indigestible fiber and low-molecular-weight oligosaccharides that favor the growth of microorganisms such as lactic acid bacteria that utilize these compounds as metabolic substrates [24-25].

Probiotics are valuable biotechnological products with significant practical applications. Common aquaculture probiotics mainly include lactic acid bacteria and *Bacillus* species, with protease-producing *Bacillus* being the most widely used due to its important roles in promoting growth, enhancing disease resistance, and improving water quality. Additionally, other bacterial species isolated from aquaculture animal intestines can be developed as potential probiotics. In this study, *Pseudomonas aeruginosa* isolated from the intestine of *Channa argus* exhibited high protease-producing activity. Chythanya et al. [26] investigated the inhibitory effects of *Pseudomonas aeruginosa* extracellular products against shrimp pathogens including *Vibrio harveyi*, *V. parahaemolyticus*, and *V. fluvialis*, confirming the antibacterial activity of *P. aeruginosa* against these pathogens. Vijayan et al. [27] found that *P. aeruginosa* effectively inhibited *Vibrio* and *Aeromonas* species. Given the important functions of *P. aeruginosa* in protease secretion and pathogen inhibition, it could be considered as a potential candidate for probiotic development. Meanwhile, considering that soybean meal replacement of fish meal induced changes in both protease-producing strains and their activities in the intestine of *Channa argus*, the influence of dietary ingredients should be taken into account during probiotic development to maximize probiotic functionality. It should be noted that some protease-producing strains, such as *Hafnia alvei* and *Aeromonas veronii*, are opportunistic pathogens [28-29].

It is estimated that only 10% of microorganisms in vertebrate digestive tracts can be identified, and only 1% can be cultured under laboratory conditions

[30-31]. Studies on freshwater fish such as angelfish (*Pterophyllum scalare*) and oscars (*Astronotus ocellatus*), as well as marine fish such as southern flounder (*Paralichthys lethostigma*), have also found that intestinal anaerobic bacteria play important roles in nutrient digestion and absorption, providing various enzymes including carbohydrases, phosphatases, lipases, and proteases to assist fish in digesting and absorbing nutrients [32]. Therefore, the basic culture method used in this study to analyze protease-producing strains from the intestine of *Channa argus* has certain limitations. Additionally, since only freshwater fish culture medium was used for screening protease-producing strains, some culturable bacteria may not have grown normally. Thus, further development of probiotics from the intestine of *Channa argus* will depend on improved bacterial culture methods and experimental techniques.

Conclusion

1. The intestine of *Channa argus* harbors a rich diversity of protease-producing aerobic bacteria with varying protease-producing capabilities. Among these, strains P1009, P1008, P1007, P1012, and P1015 showed stronger protease-producing activity, with R/r values ranging from 2.44 to 3.22.
2. Replacement of fish meal with soybean meal significantly decreased the protease-producing activity of strains P1004 and P1018, but significantly increased the protease-producing activity of strains P1009 and P1012. As the proportion of fish meal replaced by soybean meal increased, the protease-producing activity of strain P1018 decreased significantly, demonstrating the plasticity of intestinal microbiota structure in response to dietary changes.
3. Strain P1009, which exhibited the strongest protease-producing activity, was identified as *Pseudomonas aeruginosa* through 16S rDNA sequence analysis. Based on the characteristics of *P. aeruginosa*, it can be considered as a potential candidate for probiotic development.

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