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Selection of One Probiotic Bacterium and Assessment of its Effects on Digestive Enzyme activity of Young Pufferfish, *Takifugu Rubripes*

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Research Article

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Abstract

In order to select the potential prbiotics for the culture of the pufferfish *Takifugu rubripes*, 30 bacterial strains were isolated from the intestine of juvenile pufferfish. Four strains were screened based on the isolates producing a variety of enzymes (protease, amylase and lipase) and/or having antagonistic activity against the pathogen *Vibro harveyi*. Hemolysis was tested in four strains and the results showed that F15 did not secrete hemolysin, without potential pathogenicity. F15 was proven to be safe for young pufferfish challenged by intraperitoneal injection. It was identified as *Pseudoalteromonas* sp. by 16S rRNA gene sequencing. Young pufferfish were assigned to three groups (three tanks per group) and received the control diet (basal diet) and diets supplemented with F15 at 10^7 and 10^9 cells g^{-1}. After 14 days of feeding, all pufferfish fed the F15-supplemented diets had a significant enhanced intestinal and hepatopancreas pepsin, trypsin, lipase and amylase activities as compared with the controls. Moreover, the intestinal pepsin and amylase activities and the hepatopancreas four digestive enzymes activities of pufferfish fed the 10^9 cells g^{-1} F15 diet were significantly higher than those of pufferfish fed the 10^7 cells g^{-1} F15 diet. It can be concluded that *Pseudoalteromonas sp.*15 can improve intestinal and hepatopancreas digestive enzyme activity of young pufferfish.

Keywords: *Takifugu rubripes*; Probiotics; Selection; *Pseudoalteromonas sp.*; Digestive Enzyme Activity

Introduction

Pufferfish *Takifugu rubripes* is one of the most important farmed fish species in China. The annual output was about 50,000 tons with a value of more than 10 billion Yuan RMB according to the 2016 data of Fugu branch of China Fisheries Association. Due to the expansion and intensification of pufferfish farming, various diseases occur frequently, causing serious economic losses. Probiotics are usually live microorganisms that, when administered in adequate amounts, confer a health benefits on host. The advancement of probiotics research and their applications in fish farming industries were reviewed by Banerjee and Ray [1,2]. Many probiotics such as *Bacillus* sp. [1], *Brevibacillus brevis* [3],

Enterococcus faecium [4], *Kocuria* sp. and *Rhodococcus* sp. [5], *Lactobacillus rhamnosus* [6], *Lactococcus lactis* [7], *Pseudoalteromonas* sp. [8], *Shewanella putrefaciens* [9] were used in marine fish aquaculture. Nevertheless, no information about the application of probiotics for the pufferfish. Previously, *Vibrio harveyi* was proven to be the pathogen of pufferfish [10]. In the present study, one potential probiotic strain F15 was isolated from the intestine of juvenile pufferfish, and the antibacterial activity of F15 against the pathogen *V. harveyi in vitro* and the effect of F15 digestive enzyme activity of young pufferfish were evaluated.

Materials and Methods

Bacterial Isolates

Healthy juvenile pufferfish (~1 g) were obtained from a local hatchery farm (Dalian Fugu Fishery Co. Ltd., Dalian, China). The intestines were removed from seven fish and homogenized in a sterile glass homogenizer with sterile physiological saline solution (0.9 g sodium chloride dissolved in 100 mL distilled water, pH 7.0). Appropriate dilutions were prepared and 0.01 mL volumes were spread over the surface of plates of ZoBell 2216E agar with incubation at 25°C for 5–7 days. A total of 30 colonies were picked randomly from the plates. Pure cultures were obtained by repeated subculture on new agar plates and stored at –80°C as suspensions in 30% (v/v) glycerol.

Enzyme-Producing Isolates

Thirty strains were inoculated into media containing varied substrates to screen isolates producing extracellular protease, amylase and lipase according to the methods of Zhao, et al. [11] and Zhang, et al. [12]. For proteolytic activity, the isolates were inoculated on casein supplemented agar plates and incubated at 28°C for 72 h. Appearances of clear zones around the colonies were recorded. To assess amylolytic activity, the isolates were inoculated on soluble starch supplemented agar plates and were incubated at 28°C for 72 h. Appearances of clear zones around the colonies were recorded after Lugol's iodine solution was flooded on the culture plates. For lipolytic activity, the isolates were inoculated on Tween 80 supplemented agar plates and were incubated at 28°C for 72 h. Appearances of opaque halos around the colonies were recorded.

Antagonism Study

The antagonistic activity of 30 test strains was examined against the indicator pathogenic bacterium *V. harveyi* following a method described by zhao, et al. [11]. Briefly, a culture of the indicator bacterium was spread on ZoBell 2216E agar plates, and then the test strains were inoculated on the plates. The plates were incubated at 28°C and zones of inhibition around the test strains colonies were observed and recorded after 24 h.

Hemolytic Test

The bacterial isolates were spotted onto blood-agar plates and incubated at 28°C for 24 h. The plates were observed for hemolytic reaction. Strains that produced no change on the agar plates around the colonies were considered to be non-hemolytic and strains displaying clean hemolysis zones around colonies were considered to be hemolytic (β-haemolysis).

Safety of a Probiotic Strain Evaluation

The safety of F15 was evaluated using healthy pufferfish (~22 g) by a challenge study. Pufferfish were acclimated to the rearing conditions for two weeks. Then pufferfish were distributed randomly into 2 plastic tanks (200 L), each tank containing 15 pufferfish. The bacterium was cultured in ZoBell 2216E broth at 25°C with constant shaking until the early stationary phase before centrifuging at 1000×*g* for 10 min at 4°C, washing twice and re-suspending in 0.9% (w/v) saline. The pufferfish from one tank were intraperitoneally injected with 0.2 mL of F15 fresh culture suspension containing 10^9 cells mL^{-1} as determined by means of a haemocytometer slide. Rest one tank served as a control and injected with 0.2 mL sterile saline. All pufferfish were fed with basal diet containing the mixture of surimi from frozen *Ammodytes personatus* and fish meal (4:1, w/w), and kept under observation for 30 days, and the disease symptoms and mortality were recorded.

Feeding Trial

The safety test showed that F15 did not induce disease symptoms and mortality of fish. The F15 was cultured in ZoBell 2216E broth at 25°C with constant shaking until the early stationary phase. The cellular suspension was centrifuged and the recovered pellet re-suspending in 0.9% (w/v) saline was incorporated into the diet. A basal diet containing the mixture of surimi from frozen *A. personatus* and fish meal (4:1, w/w) was used as a control. The experimental diets were prepared by supplementing graded doses of F15 at 10^7 and 10^9 cells g^{-1} of diet, respectively, which were prepared every day in order to guarantee the vitality of F15.

Young pufferfish were purchased from Dalian Tianzheng Industry Co. Ltd., Dalian, China. The pufferfish were transferred to the laboratory and acclimatized in a concrete pool filled with 25 m^3 aerated filtered seawater at ~20°C for

2 weeks. Then fish of similar sizes (22.13 ± 1.47 g, mean ± SD) were reared in 9, 200 L plastic tanks, at a density of 15 pufferfish per tank. Pufferfish were assigned to three groups (three tanks per group) and received the control diet (basal diet) and diets supplemented with F15 at 10^7 and 10^9 cells g^{-1}. During the period of 2 weeks feeding, all pufferfish were fed with diets twice (08:00 and 14:00) daily at a rate of 2%–2.5% body weight. Uneaten feed residue and feces were removed by siphoning 1 h after each feeding. Water in each tank was replaced with fresh sand-filtered seawater twice a day. Aeration was provided to maintain dissolve oxygen levels at >5 mg L^{-1}. Water temperature ranged from 20°C to 24°C, salinity from 32.4 to 32.8 and acidity from pH 7.9 to 8.1. All experimental protocols were approved by the Experimental Animal Ethics Committee of Dalian Ocean University, China.

Sample Collection and Digestive Enzyme Assay

At the end of the feeding trial, pufferfish were starved for 48 h and anaesthetized administration of an overdose of MS-222. The intestines /hepatopancreas from six fish were removed, rinsed three times with sterile 0.9% (w/v) saline, pooled and immediately stored at –80°C for further analysis. Frozen intestinal/hepatopancreas homogenates were thawed at 4°C and homogenized in nine volumes of sterile 0.9% (w/v) saline using a manual glass homogenizer. The homogenates were then centrifuged and the supernatants were used for digestive enzyme activity analysis. The soluble protein content of the supernatants of intestinal/ hepatopancreas homogenates were measured according to the method described by Bradford [13] using bovine serum albumin as standard.

Pepsin, trypsin, amylase and lipase activities were measured by colorimetric analysis using commercial test kits (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions except that the temperature for the reaction was changed to room temperature (~25°C).

Statistical Analysis

Statistical analysis was performed using the SPSS 22.0 for windows. The differences in digestive enzyme activity between different diets were compared using one-way analysis of variance. If significance was detected, Tukey's multiple range test was used to compare the means between groups. Differences were considered significant if $P < 0.05$.

Results

Selection of Probiotic Bacteria

A total of 30 bacterial strains were isolated from the intestine of juvenile pufferfish. Strains F04, F05, F09 and F15 produced protease, amylase and lipase (Table 1). The *in vitro* antibacterial assay showed that the strain F15 induced an inhibition zone against *V. harveyi* (Table 1). Hemolysis was tested in four strains and the results showed that F15 did not secrete hemolysin. The intraperitoneal injection of F15 was thought to be harmless to the pufferfish although three pufferfish (one injected with suspension of F15, two injected with sterile saline) died during the 30 days of post-challenge observation. Nevertheless, no disease symptoms were found in the skin, intestine and liver tissues of the dead pufferfish. Thus, the cause of pufferfish death should be that the pufferfish kill each other during rearing. Pufferfish were ferocious in nature and mutual biting occurred from teeth growth of juveniles to adults [14]. F15 was identified as *Pseudoalteromonas* sp. by 16S rRNA gene sequencing [15].

Strains	Protease activity	Amylase activity	Lipase activity	Antagonistic activity
F04	1.95	2.58	1.52	_
F05	2.09	2.14	1.48	_
F09	2	3.34	1.5	_
F15	3.44	2.23	2.13	5.34

Dh denotes diameter of hydrolysis zone; Dc denotes colony diameter; Di denotes diameter of inhibition zone

Table 1: Qualitative extracellular enzyme activity (Dh/ Dc) and antagonistic activity (Di/Dc) produced by the bacterial strains from the intestines of juvenile pufferfish *Takifugu rubripes*.

Enzymatic Activity

After 14 days of feeding, pufferfish fed with F15 at $10^7$and 10^9 cells g^{-1} of diet showed a significant increase in intestinal pepsin, trypsin, lipase and amylase activities compared with those fed the control diet (Table 2). Moreover, the intestinal pepsin and amylase activities of pufferfish fed the 10^9 cells g^{-1} F15 diet were significantly higher than those of pufferfish fed the 10^7 cells g^{-1} F15 diet (Table 2).

	Pepsin activity (U/mg prot)	Trypsin activity(U/mg prot)	Lipase activity(U/g prot)	Amylase activity (U/mg prot)
Control	13.31 ± 0.26[a]	876.19 ± 11.23[a]	8.23 ± 0.02[a]	0.33 ± 0.02[a]
10^7 cells/g	16.47 ± 0.25[b]	975.75 ± 9.00[b]	10.15 ± 0.04[b]	0.42 ± 0.01[b]
10^9 cells/g	18.24 ± 0.21[c]	1002.46 ± 47.34[b]	10.08 ± 0.02[b]	0.55 ± 0.01[c]

Means with different superscript lowercase letters in the same column differ significantly (P < 0.05) among different groups. Data represents means ± SDs (n = 3).

Table 2: Intestinal digestive enzyme activity of pufferfish fed with control diet and F15-supplemented diets at 10^7 and 10^9 cells g^{-1} for 14 days.

After 14 days of feeding, all pufferfish fed the F15-supplemented diets had a significant enhanced hepatopancreas pepsin, trypsin, lipase and amylase activities as compared with the controls (Table 3). Moreover, there were significant differences in four digestive enzymes activities in pufferfish fed with two doses of F15.

	Pepsin activity(U/mg prot)	Trypsin activity(U/mg prot)	Lipase activity(U/g prot)	Amylase activity(U/mg prot)
Control	11.68 ± 0.21[a]	847.55 ± 1.54[a]	9.26 ± 0.09[a]	1.11 ± 0.02[a]
10^7 cells/g	14.10 ± 0.10[b]	917.75 ± 10.61[b]	12.23 ± 0.07[b]	1.19 ± 0.02[b]
10^9 cells/g	15.19 ± 0.20[c]	983.64 ± 10.54[c]	14.13 ± 0.17[c]	1.28 ± 0.02[c]

Means with different superscript lowercase letters in the same column differ significantly (P < 0.05) among different groups. Data represents means ± SDs (n = 3).

Table 3: Hepatopancreas digestive enzyme activity of pufferfish fed with control diet and F15-supplemented diets at 10^7 and 10^9 cells g^{-1} for 14 days.

Discussion

In the present study, F15 was selected as a probiotic of young pufferfish based on the results of enzyme production, antagonistic activity, hemolytic activity and challenge test. Firstly, a variety of enzymes including protease, amylase, and lipase produced by F15 were efficient at metabolizing a large range of proteins, carbohydrates and lipids, and this was one of the reasons for its selection as a probiotic to improve digestive enzyme activity [16]. Secondly, antagonistic activity against pathogenic bacteria is also often used as an effective way to select potential probiotics *in vitro*. In this study, F15 inhibited the growth of pufferfish pathogen *V. harveyi*. Previously, it had been demonstrated that F15 showed antagonistic activity against Yesso scallop pathogen *Vibrio splendidus* [15]. The inhibitory activity of F15 may be attributed to its ability to produce antimicrobial substances. Thirdly, hemolytic assay was performed to exclude the potential pathogenic strains [17]. Our present study confirmed that F15 was non hemolytic. Finally, a small scale challenge test was employed to to ensure the safety of the candidate to the host [18]. In this study, F15 was proven to be safe to pufferfish because it had no harmful effect on pufferfish survival at the concentrations tested (10^8 cells).

Digestive enzymes play an important role in food digestion and absorption. Abalone (*Haliotis midae*) fed with kelp supplemented with *Pseudoalteromonas* sp. C4 showed higher alginate lyase activity in the crop and stomach in comparison to those fed a standard kelp diet, which may be related to the contribution of strain C4 to the pool of polysaccharolytic enzymes available in the digestive tract for the digestion of kelp ingested by the abalone [19]. Major digestive enzymes reported in pufferfish intestines and hepatopancreas were protease, lipase, and amylase. If the productions of these enzymes increase, there may be an opportunity to improve overall body metabolism. In the present study, pufferfish fed with the diet containing F15 exhibited improvement in digestive enzyme activity compared to those fed the control diet. The improved digestive enzymes activities may be due to the wide range of exoenzymes secreted by the probiont and/or due to endogenous enzymes synthesized by pufferfish as a result of F15 stimulation [16]. In addition, the effect of F15 on digestive enzymes was dose-dependent. The dietary bacterium that transited in the digestive tract might be active to modulate the response of the host, depending on the dose supplied. Similar results were observed in the effects of *Pseudoalteromonas* sp. BC228 on intestinal trypsin and lipase activities of sea cucumber (*Apostichopus japonicus*)

[16].

Conclusion

F15 displays properties of good probiotics, including the production of extracellular enzymes, growth inhibition of pathogens, non-hemolytic and safety. Dietary administration of F15 could improve the digestive enzyme activity of pufferfish and a 10^9 cells g^{-1} supplement of F15 was recommended for *T. rubripes* based on the results of the present study. Future studies should be focused on growth performance, feed utilization, immune response and resistance against pufferfish pathogens for exploring the feasibility of its commercial application in pufferfish farming industry.

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