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Selection of prospective probiotic bacteria from the intestines of Rabbitfish, *Siganus guttatus* as a fermenter for fish feed raw materials

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Abstract. Rabbitfish is classified as herbivorous fish by utilizing plants as a staple food, so it has the potential to contain microflora in the digestive tract. The study aimed to select bacteria from the intestines of Rabbitfish as probiotic candidates for degradation of feed ingredients. At the beginning of the study, microbial dissolution of the intestines of 10 Rabbitfish measured weight and body length 127 ±20.1 g and 191.4±1.17 cm, healthy and not deformed, obtained from the waters around the island of Salemo, Regency. Pangkep. At this stage, they have successfully isolated 72 isolates. Then make a selection of bacteria based on the activity of extracellular enzymes by testing the hydrolysis ability of starch using Zobel media adding 1% soluble starch, protein hydrolysis using Zobel media adding 1% skim milk, cellulose hydrolysis using Zobel media adding 1% CMC. The ability of the insulator to hydrolyze starch ranges from 0.4 cm; to protein to 2.4 cm; cellulose to 1.5 cm. Based on these results, 10 isolates were selected as probiotic candidates, namely 411; 413; 422; 427; 430; 434; 437; 452 and 455. The next stage of the pathogenicity test of 10 selected isolates. Each isolate with a density of 10^6 CFU/mL was injected into 5 juveniles of healthy Rabbitfish intramuscularly as much as 0.1 mL. Monitoring is carried out daily for 10 days after infection to evaluate clinical signs of the disease in test fish. The results obtained showed that no symptoms of pain, injury, or death were found, so it was concluded that the bacterial isolate was not pathogenic.

1. Introduction

In nature, rabbitfish are herbivorous fish that consume a lot of aquatic plants, such as *Enhalus sp, Padina sp, Gelidium, Sargassum sp, Chaetomorpha sp, Enteromorpha sp, Cladophoropsis sp, Helimeda sp, Caulerpa sp, Eucheuma sp, and Sargassum sp*. As herbivorous fish, they generally have a digestive tract measuring 1.6 to 8.0 times their body length [1]. The anatomy and physiology of the gastrointestinal tract is an essential component that determines the qualitative and quantitative aspects of the microbiota [2]. The intestine is the central part of the digestive tract because it functions as a place for the digestive process and absorption of food substances [3]. According to [4], the digestion of food depends on three main factors: (1) the food eaten and the extent to which the food is susceptible to the effects of digestive enzymes, (2) the activity of the digestive enzymes, and (3) the length of time the food is in contact with the digestive enzymes. One of the roles of microflora in the digestive tract of herbivorous fish is to produce cellulase enzymes that will degrade cellulose more quickly [3]. Feeding habits and trophic levels of fish affect the composition of microbes that live in the digestive tract of fish [5]. Cellulose-degrading bacteria such as Clostridium, Citrobacter, and Leptotrichia are dominant in herbivorous fish [5]. Several types of bacteria found in the digestive tract of animals have an essential role in increasing feed utilization, fish health, and improving the quality of the environment and microorganisms [6]. In addition, several bacterial florae in the digestive tract play an essential role and produce several types of enzymes in the digestive tract that may act as probiotic candidates.

When selected, probiotic microbes' requirements include not being pathogenic. Probiotics are live microbial supplements that compete with pathogenic bacteria, are sensitive to antibiotics, disturb the host or consumers (humans and other animals), and do not disturb the balance of the local ecosystem, which has a beneficial effect on the microbial community in the environment. Easy to maintain and reproduce, and can survive and breed in the intestines of fish [6–8]. In increasing the nutritional value of feed, probiotics can produce several exogenous enzymes that are useful to help digest feed, such as amylase, protease, lipase, and cellulase enzymes [9–12].

Several studies have shown that probiotics can be isolated from the digestive tract of fish such as milkfish [13], gourami - *Osphronemus goramy* [14], duck grouper [15], catfish [16], tilapia - *Oreochromis niloticus* [17,18], snakehead fish - *Channa striatus* [19], other freshwater fish (*Catla catla*, *Labeo rohita*, *Cirrhinus mrigala*, and *Cyprinus carpio*) [20], shrimp digestive tract - *Litopenaeus vannamei* [21] and mollusk - *Anadara tuberculosa* [22]. Many uses of probiotics from the digestive tract of fish have been carried out, including milkfish [13], vaname shrimp [23], jelawat [24], and tilapia [25]. Its application to feed showed significant results for growth and feed efficiency. As reported [26], the basic principle of probiotics is to utilize the ability of microbes to facilitate absorption by the digestive tract of fish. Bacterial strains derived from the intestines of these fish are potential probiotic candidates because these bacteria can stick to the intestinal wall and have adapted to environmental conditions in the intestines. These bacteria are also used to compete with pathogenic bacteria for nutrients.

Based on the results of these studies, we tried to isolate microbes from the intestines of rabbitfish because, until now, there has been no research that has identified these microbes. Therefore, this study aims to research to select bacteria from the intestines of rabbitfish as probiotic candidates for the degradation of feed ingredients.

2. Materials and methods

2.1. Research implementation
This research activity was carried out at the Laboratory of Microbiology of Environmental and Fish Health, Laboratory of Nutrition, and Feed Technology of Maros. The rabbitfish were obtained from Samalona Island, Pangkep Regency. The fish was used as a sample for isolation of probiotic candidate microbes obtained from nature capture (waters around Selemo island, Pangkep Regency). Ten healthy rabbitfish with an average weight of (127±20.1 g) and average length (19.14±1.17 cm) were used in this study.

2.2. Microbial isolation procedure
The isolation procedure for lactic acid bacteria was carried out using MRS (de Man, Rogosa and Sharpe) broth media, which refers to the method performed on land animals as described in [27] and in combination with procedures for isolating bacteria from the digestive tract of fish such as conducted by [28–32]. Taking the contents of the digestive tract of rabbitfish as a source of inoculum was carried out by removing the digestive tract (intestines), then the intestines were crushed to remove the contents, 1 g was taken and immediately put into a test tube which already contained 9 mL of MRS broth. The samples were then incubated on a shaking incubator for 2 days. After incubation for 48 hours, 1 mL of the sample was taken and put into 9 mL of sterile physiological (0.85% NaCl) solution [13] and homogenized (1-time dilution). Subsequent dilutions were carried out in stages up to 7 times the dilution. After being homogenized, from

each diluent tube (from 104-10^6), 0.1 mL of a solution was taken; and distributed in petri dishes containing MRS Agar which was made in duplicate. This culture is then incubated at room temperature for 24-48 hours. The growing bacterial colonies were identified based on color, shape, and size differences. Each colony type was isolated on slanted MRSA media for further testing and harvesting and then stored in 1% skim milk as pure isolate stock in the freezer.

2.3. Selection of bacteria based on substrate hydrolysis ability

The selection of bacteria isolated from the intestines of rabbitfish refers to the presence or absence of amylase, protease, and cellulase enzyme activity. These enzymes play an essential role in hydrolyzing substrates containing starch, protein, and crude fibre (cellulose). As a first step to determine the enzyme's activity, a substrate hydrolysis test containing starch, protein, and crude fibre was carried out to isolate bacteria from the intestines of rabbitfish. The hydrolysis test of materials containing starch (amylase enzyme activity) was carried out using media containing soluble starch, the hydrolysis test of materials containing protein (protease enzyme activity) was carried out using Zobel media added with 1% skim milk, while the hydrolysis test of materials containing fibre Crude or cellulose (cellulase enzyme activity) was carried out using Zobel media plus 1% Carboxy Methyl Cellulose (CMC) then dripped with Congo red. Each bacterial isolate that produced a clear zone around the colony was positive for enzymes, and isolates with the highest clear zone were selected as probiotic candidates for fermenting seaweed-based feed ingredients.

2.4. Pathogenicity test of candidate probiotic bacteria

The pathogenicity test used juvenile rabbitfish with an average size of 45g/head. The container used is a round tank with a volume of 100 L, which has been filled with 30ppt salinity seawater with a density of 5 fish per tank. The fish were reared for five days to adapt during adaptation, and the fish were fed a self-formulated feed in the hope that the feed did not contain antibiotics. After that, each isolate was injected into a group of healthy rabbitfish (consisting of five fish in each tank) intramuscularly as much as 0.1 mL with a bacterial content of 10^6 CFU/mL. Monitoring was carried out every day for 10 days after infection to determine clinical signs of disease in the test fish.

2.5. Data analysis

The data obtained from this study were in the form of microbial isolation from fish intestines, isolate data based on the ability to hydrolyze each substrate, and the data from the pathogenicity test were analyzed descriptively in tabular form.

3. Results

The natural rabbitfish caught around the waters of P. Salemo, Pangkep, as many as 10 fish with the size as presented in Table 1. The size of the fish is classified as juvenile size, which is still in a fast growth phase, where the average total length of fish is 19.41 ±1.7 cm, with an average total length of the intestine 56.5±5.97 cm. Based on these results, it is shown that, on average, of the ten samples observed, the length of the intestine is relatively three times the length of the body of the rabbitfish. As reported by [33] that rabbitfish with a total length of 16.1 cm have an intestine length of about 42.5 cm. The same thing was reported by [3]; [34] that the length of the intestine is often related to the food, where the intestine is part of the digestive tract which has a crucial role in the process of simplifying food through physical and chemical mechanisms, besides that the intestine also acts as a place for growth. And the proliferation of microflora that a role in secreting various types of enzymes that function to hydrolyze food in the intestine.

Table 1. Bodyweight, total length, intestine length, and gut weight of rabbitfish were taken from their intestines.

Code	Weight (g)	Total length (cm)	Intestine length (cm)	Intestine weight (g)
1	140	20.5	59	12.45
2	116	18.7	60	12.86
3	110	18.8	69	10.12
4	145	20.1	58	9.13
5	150	20.5	55	12.07
6	130	20	53	12.03
7	110	18.9	51	8.9
8	150	20.5	60	11.02
9	130	19.3	51	10.99
10	90	16.8	49	5.98
Average	127.1±20.10	19.41±1.17	56.5±5.97	10.6±2.10

The test animals were obtained from nature, so that during their life, they obtained food entirely from nature in the form of seaweed and seagrass. These two types of plants generally had high crude fibre and low protein content, so they could not rely solely on their organs to hydrolyse these two types of food. Digestive tract only. As reported by [35]; [36] that the leading food of rabbitfish is macrophytes such as seagrass, macroalgae, and microalgae such as Bacillariophyceae, Chlorophyceae, Cyanophyceae, and Dinophyceae) while complementary foods are ciliates, bivalves, gastropods, crustaceans (Copepods), seagrass, algae, and mosses.

The results of the characterization of bacteria from the intestines of rabbitfish obtained several isolates that have the potential as 13 biotic candidates based on the extracellular enzyme activity test of the isolates as presented in Table 2. Based on the table it can be seen that the ability of isolates to hydrolyze starch substrate (starch) optimally grows only up to 24 hours, so not found after 48 hours. Meanwhile, for the activity of isolates in degrading protein sources (skim milk) starting from 24 - 48 hours, activity was still found in hydrolyzing the substrate. Even the activity of isolates in degrading cellulose substrate for 6-7 days was still found to be positive. This indicated that some of these isolates had the activity/ability to utilize cellulose substrate (crude fibre).

Table 2. Hydrolysis test results of starch, skim milk, and CMC as indicators of amylase, protease, and cellulase enzyme activity from isolates of rabbitfish gut bacteria.

Isolate code	Amylase (cm) 24 hours	Proteinase (cm)					Media CMC (cm)			Cellulose enzyme activity (cm)
		24 hours		48 hours			6 days	7 days		
	Amylase enzyme activity (cm)	Colony diameter (cm)	Proteinase enzyme activity (cm)	Colony diameter (cm)	Clear zone diameter (cm)	Proteinase enzyme activity (cm)	Growing	Clear zone diameter (cm)	Colony diameter (cm)	
411	-	1	0.4	1.4		1.4	+	3.2	1.1	2.1
413	1.6	-	-	-	-	-	-	-	-	-
422	-	1.3	0.6	1.2	0.6	1.35	+	1.2	1	1
427	-	1.1	0.35	1.45	0.5	0.95	+	0.8	0.2	0.6
430	0.2	0	1.05	1.05	0.85	0.2	+	0.9	3.9	0.1
434	-	0	0.95	0.95	0.65	0.3	+	2.3	0.2	1.1
437	0.3	1.6	1.01	2.25	0.56	1.75	+	1.35	0.2	1.15
452	0.3	1.65	1.09	2.1	0.64	1.6	+	1	1.3	1
455	0.4	1.35	2.2	2.4	0.78	1.4	+	1.5	3	1.5
464	-	0.8	0.7	1.5	0	1.5	+	3.1	0	0.1

The analysis of the characteristics of the fish's blood components shows a reasonably healthy condition, so it can be ascertained that the candidate probiotic bacteria are safe and not pathogenic. As reported by several researchers [31]; [37] that the microflora of the digestive tract is mainly commensal. It utilizes the relationship with the host and interacts with various microbial species in the digestive tract both antagonistically and synergistically. In addition, the microflora in the digestive tract of fish also has an essential role in the physiological function of the digestive tract of fish and the mechanism of protection against pathogenic bacteria. [38] also reported that the native microflora of the digestive tract has a mutualistic relationship with its host, namely, using the host as a place of life. The advantages for the host are (1) generally, microbes eat waste or use waste materials; (2) many gut bacteria can synthesize vitamins, secrete enzymes, and aid in the digestion of nutrients; (3) the presence of native microbes tends to suppress the growth of pathogenic bacteria to protect the host against disease and stimulate immune function.

The bacterial characteristics were tested for the pathogenicity of each type of bacterial isolate against juvenile rabbitfish, which was carried out for 7 days. There were no fish experiencing illness/injury/death symptoms, so it was concluded that the bacterial isolates were not pathogenic. In addition, to ensure the health condition of the fish that had been injected with the bacterial isolate, on day 7, 1 ml of fish blood samples were taken from each injected isolate. The parameters observed included the condition of red, white, hematocrit, and leukocrit blood cells, as presented in Table 3.

Table 3. Types of isolates, conditions, hematocrit and leukokrit in rabbitfish after pathogenicity test.

Isolate	Hematocrit (%)	Leukocrit (%)
413	54.6±1.9	45.4±1.9
437	45.1±14.1	54.9±14.1
452	58±0	42.2±0
427	49.9±6.5	50.1±6.5
411	68.6±27.6	31.5±27.6
422	42.9±24.8	57.1±24.8
430	41.9±8.6	58.1±8.6
464	61.5±20	38.5±20
455	57.1±28.6	42.9±28.6
471	61.6±4.8	38.4±4.8

4. Conclusion

The results of the selection of microbial isolation in the digestive tract obtained as many as 72 bacterial isolates. Based on the hydrolysis ability test on each substrate, 10 isolates were likely to be probiotic candidates, namely 411; 413; 422; 427; 430; 434; 437; 452; 455, and 464. Based on the pathogenicity test, no pathogenic isolates were found.

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