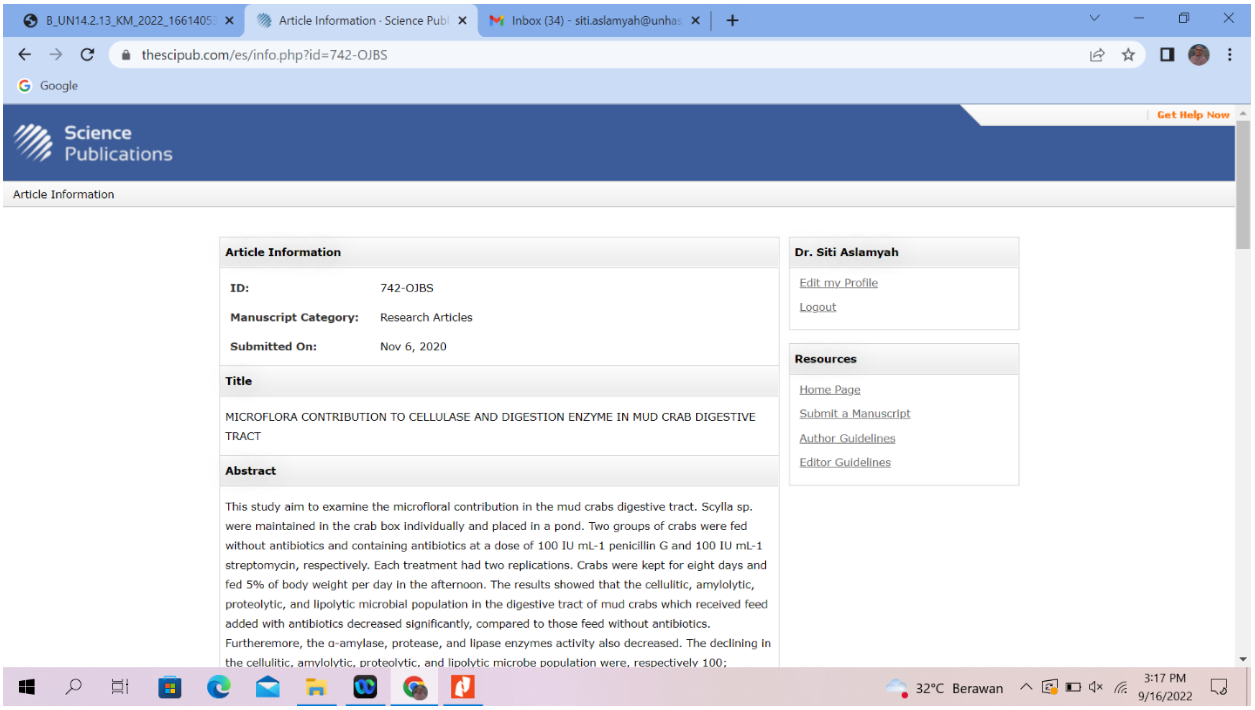

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Title

MICROFLORA CONTRIBUTION TO CELLULASE AND DIGESTION ENZYME IN MUD CRAB DIGESTIVE TRACT

Abstract

This study aim to examine the microfloral contribution in the mud crabs digestive tract. Scylla sp. were maintained in the crab box individually and placed in a pond. Two groups of crabs were fed without antibiotics and containing antibiotics at a dose of 100 IU mL-1 penicillin G and 100 IU mL-1 streptomycin, respectively. Each treatment had two replications. Crabs were kept for eight days and fed 5% of body weight per day in the afternoon. The results showed that the cellulitic, amylolytic, proteolytic, and lipolytic microbial population in the digestive tract of mud crabs which received feed added with antibiotics decreased significantly, compared to those feed without antibiotics. Furtheremore, the α-amylase, protease, and lipase enzymes activity also decreased. The declining in the cellulitic, amylolytic, proteolytic, and lipolytic microbe population were, respectively 100;

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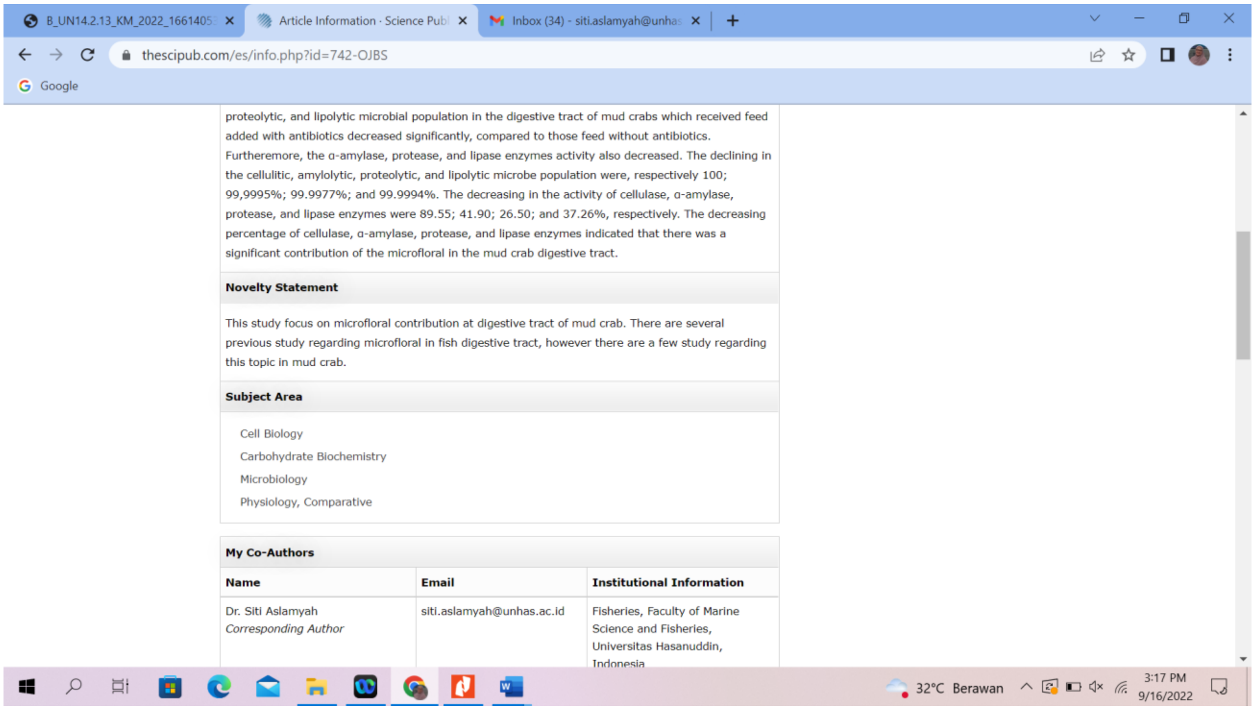

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99,9995%; 99.9977%; and 99.9994%. The decreasing in the activity of cellulase, α-amylase, protease, and lipase enzymes were 89.55; 41.90; 26.50; and 37.26%, respectively. The decreasing percentage of cellulase, α-amylase, protease, and lipase enzymes indicated that there was a significant contribution of the microfloral in the mud crab digestive tract.

Novelty Statement

This study focus on microfloral contribution at digestive tract of mud crab. There are several previous study regarding microfloral in fish digestive tract, however there are a few study regarding this topic in mud crab.

Subject Area

Cell Biology
Carbohydrate Biochemistry
Microbiology
Physiology, Comparative

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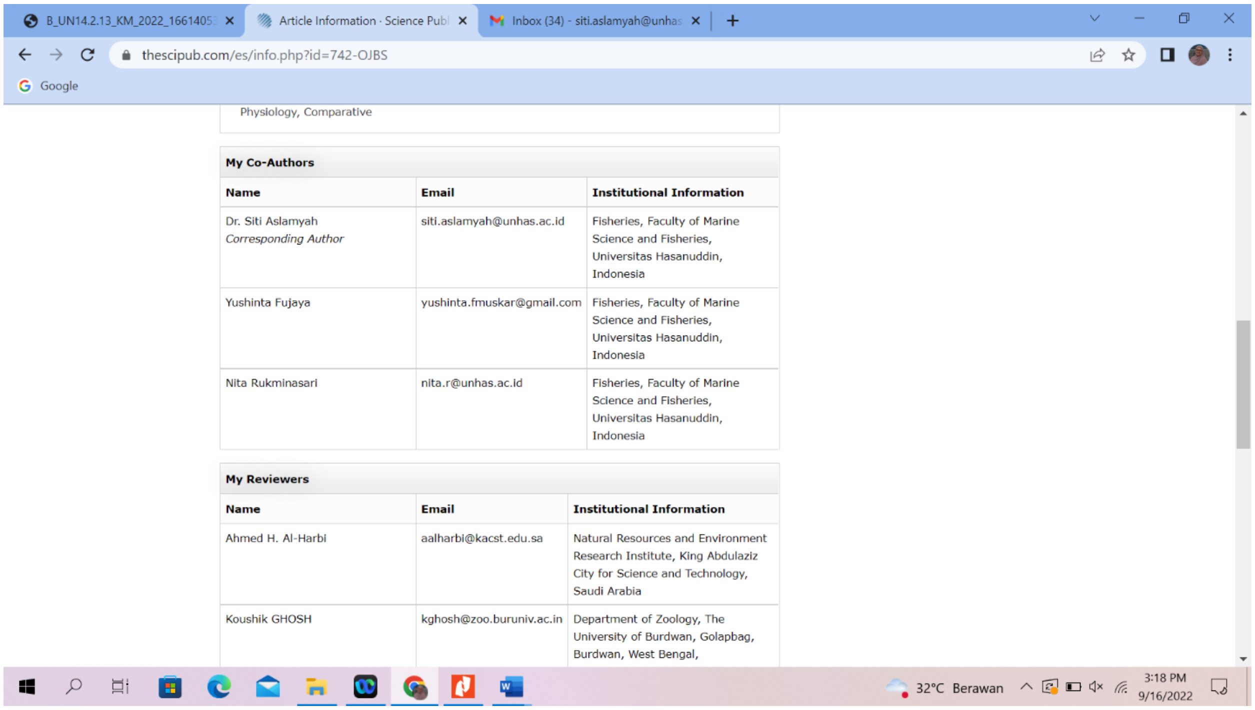

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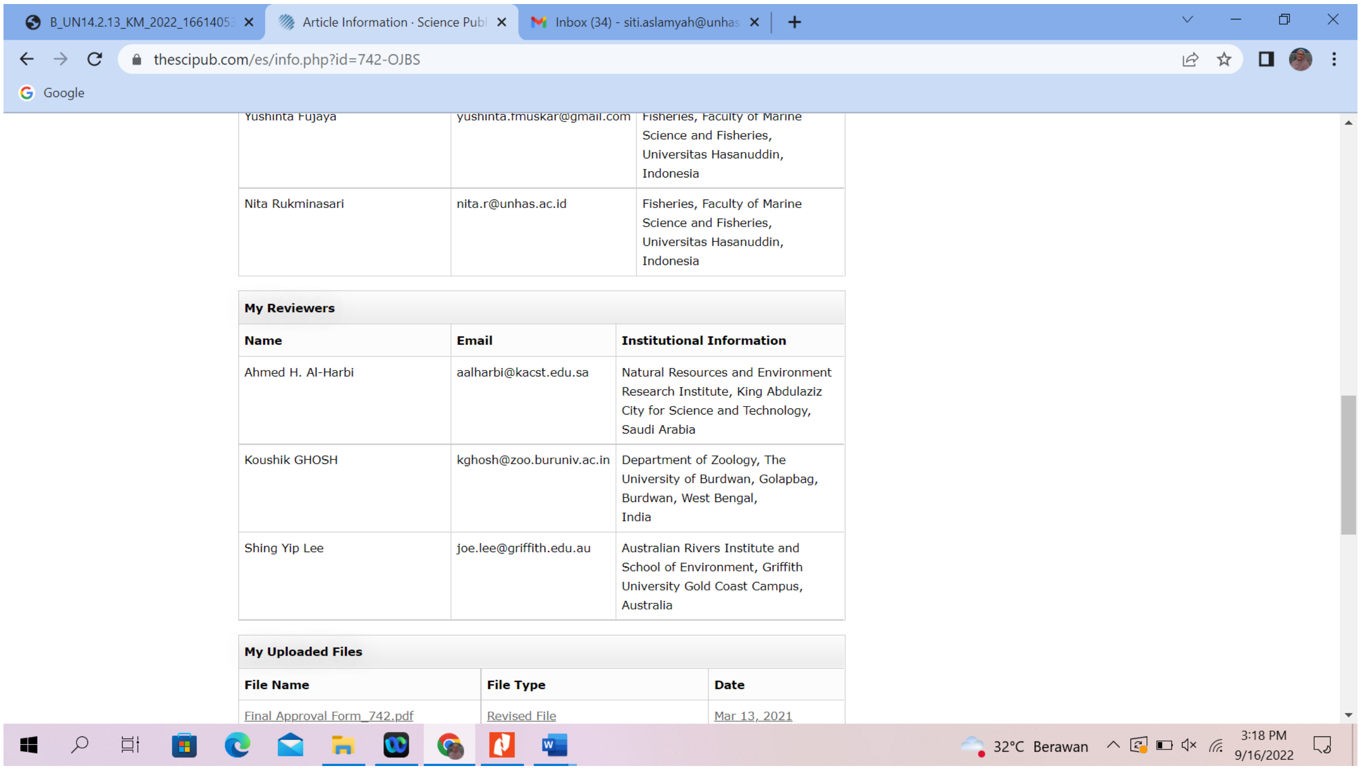

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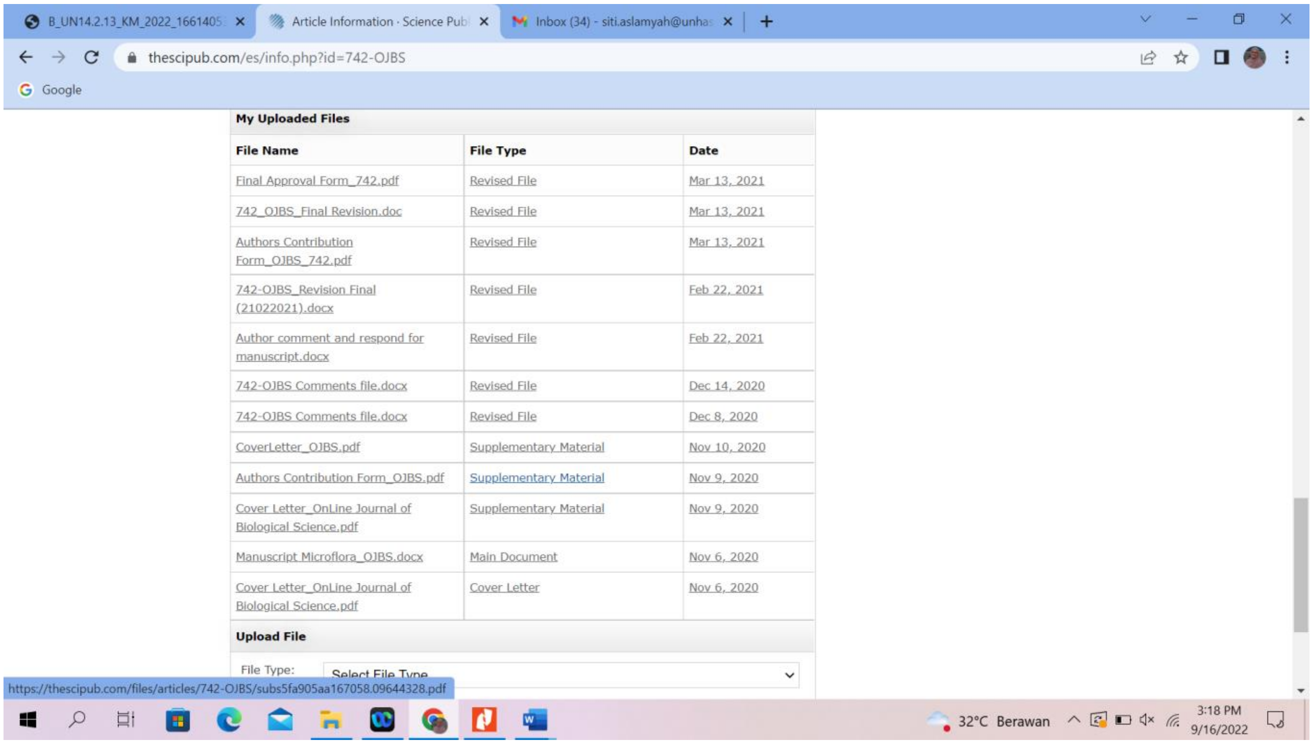

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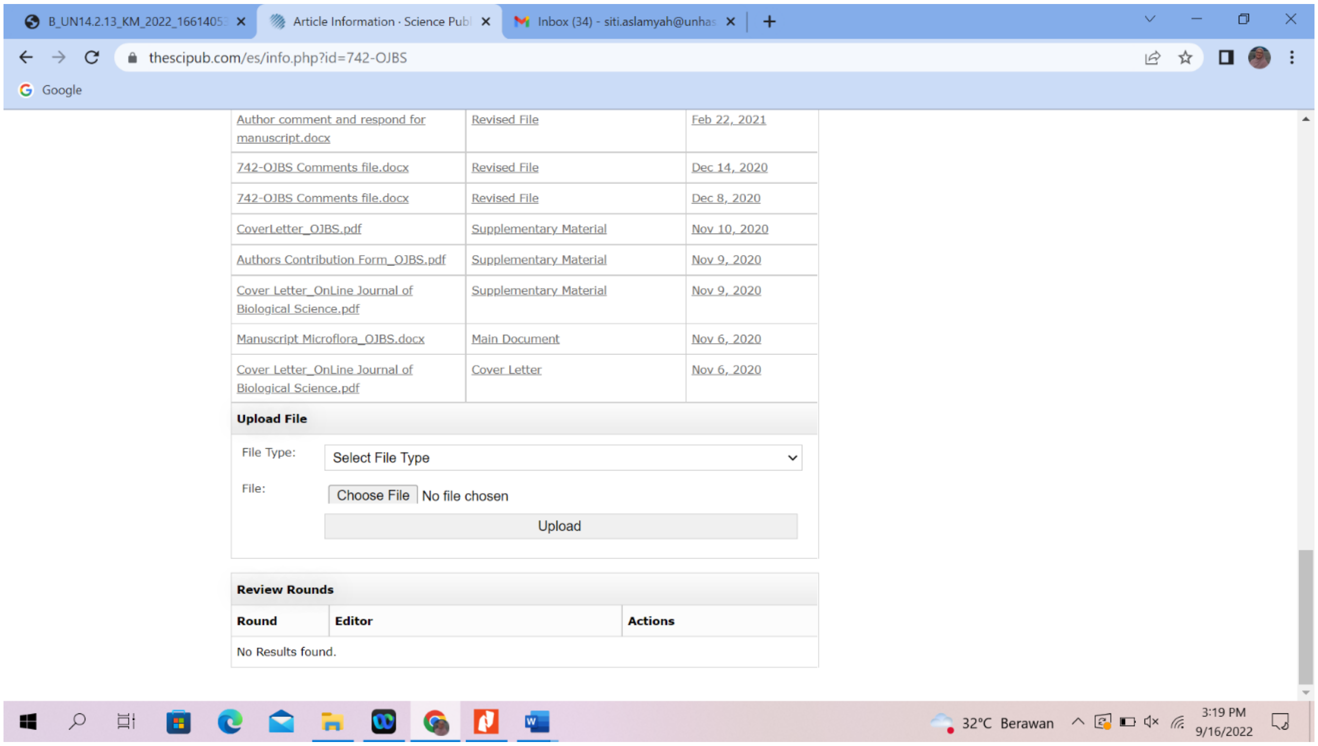

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Original Research Paper

MICROFLORA CONTRIBUTION TO CELLULASE AND DIGESTION ENZYME IN MUD CRAB DIGESTIVE TRACT

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Abstract: This study aim to examine the microfloral contribution in the mud crabs digestive tract. Scylla sp. were maintained in the crab box individually and placed in a pond. Two groups of crabs were fed without antibiotics and containing antibiotics at a dose of 100 IU mL-1 penicillin G and 100 IU mL-1 streptomycin, respectively. Each treatment had two replications. Crabs were kept for eight days and fed 5% of body weight per day in the afternoon. The results showed that the cellulitic, amylolytic, proteolytic, and lipolytic microbial population in the digestive tract of mud crabs which received feed added with antibiotics decreased significantly, compared to those feed without antibiotics. Furtheremore, the α-amylase, protease, and lipase enzymes activity also decreased. The declining in the cellulitic, amylolytic, proteolytic, and lipolytic microbe population were, respectively 100; 99,9995%; 99.9977%; and 99.9994%. The decreasing in the activity of cellulase, α-amylase, protease, and lipase enzymes were 89.55; 41.90; 26.50; and 37.26%, respectively. The decreasing percentage of cellulase, α-amylase, protease, and lipase enzymes indicated that there was a significant contribution of the microfloral in the mud crab digestive tract.

Keywords: mud crab, microflora, cellulitic, proteolytic, amylolytic, lipolytic

Introduction

The indigenous microflora of fish in aquaculture has previously been studied for many purposes including descriptions of microbial spoilage, correlation between environment and fish microflora (Horsley, 1973), the monitoring of changes in fish farms (Allen et al., 1983), the nutritional role of the intestinal flora (Hansen et al., 1992), and the antibiotic resistance profile of the indigenous flora (Spanggaard et al., 2000).

It is generally recognized that the intestinal flora of endothermic animal serves both as a digestive function and as a protection barrier against disease (Sissons, 1989). The intestinal flora of fish has as a consequence received much attention by several authors (Ringø et al., 1995; Sugita et al., 1997). The composition of the intestinal microflora in fish is influenced by, or directly derived from, the microflora of the food (Grisez et al., 1997). Fish with abundant and diverse microflora have considerable opportunities to adapt to changing nutritional substrates and to assimilate food better, and thus enhance their adaptive possibilities (Al-Harbi and Uddin, 2005).

The main component in animal feed is protein, carbohydrate and fat. To increase an absorption and circulation throughout the body through circulatory system, required a simplification process into smaller molecules. According to Bakke et al., (2010) a chemical feed simplification process is also called hydrolysis, involving digestive enzymes as biological catalysts. The main feed components in the form of protein, fat and carbohydrates are broken down into simple compounds which are the constituent components. Steinberg (2018) explained that the hydrolysis of macro nutrients into micro nutrients in the digestive system was occurred due to the presence of digestive enzymes, namely proteases, amylase, carbohydrase, lipase, and stomach acid. Those compounds are produced by the stomach, intestines, liver, and pancreas. In addition, several researchers reported that there was a cellulase enzyme activities in several species of aquatic animals. The presence of the

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cellulase enzyme is closely related to the presence of microflora in the digestive tract (Bui and Lee, 2015; Xue et al., 1999). The discovery of the enzyme cellulase in the digestive tract of aquatic animals allows these animals to digest feed containing fiber.

Previous studies showed that in addition to endogenous digestive enzymes, exogenous digestive enzymes are also found from microflora that live in mutualism symbiosis with aquatic animals in their digestive tract (Das et al., 2014; Ganguly and Prasad, 2012; Pond et al., 2006; Xue et al., 1999). Microflora in the digestive tract of aquatic animals is thought to be not only the microbes that contribute to the production of cellulase enzymes, but also contribute to other digestive enzymes, such as protease, amylase, and lipase. In fish, the intestinal microflora has been regarded as fulfilling several roles. A nutritional function has been suggested, in which bacteria break down ingested foods to individual components such as vitamins or amino acids (Pond et al., 2006). However, there is lack of study that has been conducted related to microflora activities in mud crab digestive tract. This experiment was carried out with reference to the experiments that had been conducted by previous researchers. The aim of study was to examine the microflora contribution in the mud crab's digestive tract., namely in contributing of cellulase enzymes and digestive enzymes including α-amylase, protease, and exogenous lipase.

Material and methods

The experiment was conducted at Education Pond, Universitas Hasanuddin, Barru Regency on May – June 2020. Crab feed making, analysis for microbial population, dan enzyme activity were conducted at Biotechnology Fisheries and Marine Laboratory, Center for Research Activities, Universitas Hasanuddin.

The number of mud crab (Scylla spp.) samples were 60 crabs. There was two treatments and three replicates for each treatment. There was 30 crabs For each treatment and replicate. The initial crab weight was 95,56±2,87 g and carapace width was 8,69±2,95 mm. Crab samples were obtained from crab fishermen and local crab supplier. Before experiment started, crab samples were acclimated in the pond for one week. After acclimatization process, crabs were fasted for 24 hours. Fasting process for crab samples was removing remaining feed in the crab body. Before treatment, crab was weighted for recorded an initial body weight using electric balance. Carapace width was measured using caliper. Crab samples were then tagged in carapace dorsal using a marker for easily to do the observation, after that crab was put into crab box. Crab was growing out individually into the crab box (the dimension of box: 21 cm x 15 cm x 8 cm). Crabs box was put into polyethylene pipa and set on surface pond with the depth of pond was ± 100 cm. The water replacement was done everyday following daily high and low tide (minimal 10% of water replacement). Water quality parameters (temperature, salinity, DO and pH) was measured daily.

Method of experiment was referred to (Xue et al., 1999) and (Aslamyah, 2006). Crab samples divided into two groups, namely crab fed with feed with antibiotic penicillin G with the dose of 100 IU mL-1 and 100 IU mL-1 streptomisin per kg feed and crab samples that fed with feed without antibiotic. Crab was fed twice a day. Crab was maintained into crab box for eight days and fed as amount of 5% from total body weight. We used pellet form for crab feed, with the nutritional contents of feed, namely protein 41,93%; BETN (29,33%), fiber 7,82%; fat 7,43%; dan DE 2767,63 kkal/kg.

Parameters were measured in this study was microbial population, cellulose enzyme and digestive enzyme activity that were conducted at the end of experiment. The method for measuring microbial population of amylolithic dan lypolithic referred to Aslamyah (2006). Analysis for cellulose enzyme and digestive enzyme (α-amilase, protease, dan lipase) activity was referred to Aslamyah (2006). Analysis method for cellulose enzyme activity following Miller (1959), α-amilase enzyme and protease enzyme activity was analyzed following (Aebi, 1974), lipase enzyme activity (Borlongan, 1990). Data was analyzed descriptively using mean and standard deviation.

Results

The measurement results for microbial population (cellutithic, amylolithic, proteolithic and lipolithic (cfu mL-1) and enzyme activity (cellulase, α-amilase, protease, dan lipase (U/g minute-1) was shown at a Tabel 1 and 2. The results of the Paired-Sampels T showed that there were significant differences in the microbial population and enzyme activity between the two groups of tested crabs. Table 1 showed that there was decreasing significantly of microbial population (cellutithic, amylolithic, proteolithic and lipolithic) in mud crab digestive tract who give a feed with antibiotic than those of feed without antibiotic. The decreasing of microbial population was almost 100% of all parameters. This trend also occurred for enzymatic activity (cellulose, □-amilase, protease, and lipase) (Table 2). The decreasing of Penurunan celulase, □-amilase, protease, and lipase accounting for 89,55; 41,90; 26,50; and37,26%, respectively.

Tabel 1. Microbial population of cellutithic, amylolithic, proteolithic and lipolithic (cfu mL^{-1}) in mud crab in the end of experiment

Treatment	Replicate	Microbial population (cfu mL^{-1})			
		Celllulithic	Amylolithic	Proteolithic	Lipolithic
Feed without antibiotic	1	$1,3 \times 10^{11}$	$8,3 \times 10^{10}$	$9,2 \times 10^{10}$	$7,8 \times 10^{10}$
	2	$9,6 \times 10^{10}$	$8,7 \times 10^{10}$	$9,8 \times 10^{10}$	$5,5 \times 10^{10}$
	Mean	$1,13 \times 10^{11}$	$8,5 \times 10^{10}$	$9,5 \times 10^{10}$	$6,65 \times 10^{10}$
Feed with antibiotic	1	$4,3 \times 10^{3}$	$9,8 \times 10^{4}$	$1,2 \times 10^{5}$	$3,4 \times 10^{4}$
	2	$3,9 \times 10^{3}$	$6,8 \times 10^{5}$	$4,2 \times 10^{6}$	8.2×10^{5}
	Mean	$4,1 \times 10^{3}$	$3,9 \times 10^{5}$	$2,2 \times 10^{6}$	$4,3 \times 10^{5}$

Tabel 2. Digestive enzyme activity (cellulase, α-amilase, protease, dan lipase (U/g/menit) kepiting bakau pada akhir percobaan

Treatment	Replicate	Enzym activity (U/g $minute^{-1}$)			
		Selulase	α-Amilase	Selulase	α-Amilase
Feed without antibiotic	1	24.36	18.55	32.58	15.36
	2	26.19	21.47	28.41	17.68
	Mean	25.275	20.01	30.495	16.52
Feed with antibiotic	1	4.7	13.65	25.61	9.6
	2	0.58	9.6	19.22	11.13
	Mean	2.64	11.625	22.415	10.365

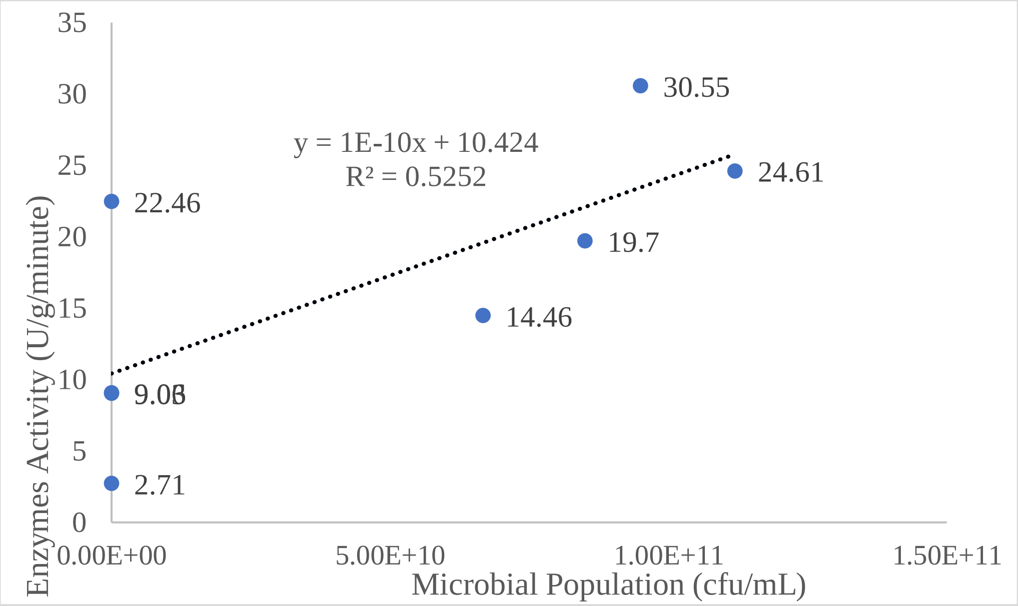

Figure 1. The correlation graph of microbial populations and enzyme activity in the digestive tract of mud crabs

The regression analysis showed that the correlation between the microbial population and enzyme activity was linear with regression equation: y = 1E-10x + 10,424; R = 0.5252. Figure 1 showed that the increasing microbial population followed by the increasing the enzyme activity.

Discussion

The investigation of the contribution of microflora in the digestive tract of mud crabs showed that the presence of cellulase enzymes in the digestive tract of mud crabs was thought to be a contribution to the microflora of the digestive tract. Our result showed that a high results of the contribution of cellulase enzymes derived from cellulitic microbes accounting for 22.64 IU g-1 minute-1 or 89.55%. The use of antibiotics to prove the role of microflora in the digestive tract of animals as reported by (Das and Tripathi, 1991; Xue et al., 1999). Tetracycline and penicillin are types of antibiotics that can kill gram-positive bacteria, while streptomycin is a type of antibiotic that can kill gram-negative bacteria. Das and Tripathi (1991) reported decreased cellulase activity when grass carp were fed with tetracyclines. Cherac quadricarinatus which was fed with 100 IU mL-1 penicillin G. and 100 IU mL-1 streptomycin per kg of feed for 8 days, showed a decreasing in cellulase enzyme activity in the digestive tract by 40%, as well as a decreasing in the bacterial population by 94% compared to controls (Xue et al., 1999). The presence of this cellulase enzyme enables the mud crab to digest feed fiber. According to (Allan and Fielder, 2004) digestibility of crabs on fiber and all vegetable feed raw materials is very high, ranging from 94.4–96.1%. Our result found that there was a contribution of digestive enzymes α-amylase, protease, and microbaial lipase derived from microflora in the digestive tract of mud crabs. The contribution of amylolytic, proteolytic, and lipolytic microbes to the enzymes of cellulase, α-amylase, protease, and lipase in the digestive tract of mud crabs were 8.34; 8.08 and 6.16 IU g-1 minute-1 . This finding indicated that in addition to the digestive enzymes α -amylase, protease, and endogenous lipase secreted by the digestive tract and glands, there were also digestive enzymes α -amylase, protease, and exogenous lipase originating from the microflora in the digestive tract. The contribution of fish digestive tract microflora was also reported by (Aslamyah, 2006). Microbial in the digestive tract of carp in the omnivore phase was higher than in the carnivore phase. The contribution of the digestive enzyme's protease, α-amylase, and microbial lipase in carnivorous gourami were 25.21; 25.22; and 18.27%, repectively. However, it was lower than the contribution of the digestive enzyme's protease, α-amylase, and microbial lipase derived from microflora in the digestive tract of milkfish. In milkfish, the contribution of the digestive enzymes' protease, α-amylase, and microbial lipase were 36.12; 41.33; and 22.51% (Aslamyah, 2006). Lazado et al (2012) reported GP21 (Pseudomonas sp.) And GP12 (Psychrobacter sp.), which was two bacteria isolated from the gastrointestinal tract of Atlantic cod, GP21 capable in producing amylase, chitinase, cellulase and protease, whereas GP12 can only produce chitinase and protease. Furthermore, GP21 and GP12, potential probiotic organisms that could support digestion of Atlantic cod.

The microflora in the digestive tract was originated from detritus that consumed by mud crabs. In general, the types of microbes that found in the digestive tract of mud crabs was from cultivation media and or fom pond sediments. Al-Harbi and Uddin (2005) stated that there was a positive correlation between the composition of bacteria in the gills and digestive tract of fish with the composition of bacteria in water and sediment pond.

Detritus contains a lot of microorganisms that play a role in contributing exogenous digestive enzymes to degrade feed nutrients consumed by mud crabs. These microorganisms are also a source of additional nutrients for mud crabs. This finding was in line with previous study by Xue et al. (1999) who found that the growth of several species freshwater Cherac quadricarinatus reared in soil ponds was better than those reared in tank ponds. This difference was interpreted as Cherac quadricarinatus had an ability to obtain additional nutrients from the detritus material at the bottom of the pond which is not found in the tank pond and / or utilized the microorganisms that was presented in detritus to support digestif activities.

The microflora was in the digestive tract lives in mutualism symbiosis with the host and is in balance, namely between beneficial microbes and pathogenic microbes. These microflora also interact with each other between various microbial species in the digestive tract, both antagonistically and synergistically. The interactions that occurred are very important in maintaining the balance of the digestive tract microflora. It will have a positive effect for the host. Douillet and Langdon (1994) reported that on oyster cultivation, there was a high growth rate which was associated with the contribution of bacteria supplying 1) essential nutrients which were not found in some individuals in the algae population; 2) enzymes that could improve the digestive process of larvae. The microflora in the intestines of bivalve larvae was obtained in optimal proportions to produce extracellular enzymes such as proteases and lipases

The estimation of a relationship between the habit of water animals eating detritus and the presence of microflora in the digestive tract was also reported by Xue et al. (1999). Liu et al., (2016) reported that the trophic level of the host affected the structure and composition of the intestinal

microbiota, metabolic capacity and enzyme activity of the intestinal content. Clostridium, Citrobacter and Leptotrichia cellulose degrading bacteria were dominant in herbivores, while Cetobacterium and Halomonas protease-producing bacteria were dominant in carnivores. In addition, cellulase and amylase activity in herbivorous fish was significantly higher than in carnivorous fish, whereas trypsin activity in carnivorous fish was much higher than in herbivorous fish.

Conclusions

Cellulase, amylolytic, proteolytic, and lipolytic microbes in the digestive tract of mud crabs play an important role in the physiological function of the digestive tract, namely contributing to the enzymes cellulase, □-amylase, protease, and endogenous lipase which were 89.55; 41.90; 26.50; and 37.26%, respectively. Furthermore, the role of the gastrointestinal microflora in contributing to cellulase enzymes and exogenous digestive enzymes amylase, protease, and lipase can be demonstrated. The microflora of the digestive tract is thought to have originated from the detritus eaten by mud crabs.

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Conflict of Interest: Author Siti Aslamyah, Yushinta Fujaya dan Nita Rukminasari have received research grant from Universitas Hasanuddin. The authors declare that they have no conflict of interest.

Ethical approval:

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Author’s Contributions

Siti Aslmayah (SA), Yushinta Fujaya (YF) and Nita Rukminasari (NR) conceived and designed an experiment method. SA and YF conducted experiment. NR conducted samples analysis. SA, YF and NR analyzed data. SA and YF wrote the manuscript and NR have done final editing and proofreading of manuscript. All authors read and approved the manuscript.

Ethics

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

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Original Research Paper

MICROFLORA CONTRIBUTION TO CELLULASE AND DIGESTION ENZYME IN MUD CRAB DIGESTIVE TRACT

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Abstract: This study aim to examine the microfloral contribution in the mud crabs digestive tract. Scylla sp. were maintained in the crab box individually and placed in a pond. Two groups of crabs were fed without antibiotics and containing antibiotics at a dose of 100 IU mL-1 penicillin G and 100 IU mL-1 streptomycin, respectively. Each treatment had two replications. Crabs were kept for eight days and fed 5% of body weight per day in the afternoon. The results showed that the cellulitic, amylolytic, proteolytic, and lipolytic microbial population in the digestive tract of mud crabs which received feed added with antibiotics decreased significantly, compared to those feed without antibiotics. Furthermore, the α-amylase, protease, and lipase enzymes activity also decreased. The declining in the cellulitic, amylolytic, proteolytic, and lipolytic microbe population were, respectively 100; 99,9995%; 99.9977%; and 99.9994%. The decreasing in the activity of cellulase, α-amylase, protease, and lipase enzymes were 89.55; 41.90; 26.50; and 37.26%, respectively. The decreasing percentage of cellulase, α-amylase, protease, and lipase enzymes indicated that there was a significant contribution of the microfloral in the mud crab digestive tract.

Keywords: mud crab, microflora, cellulitic, proteolytic, amylolytic, lipolytic

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Introduction

The indigenous microflora of fish in aquaculture has previously been studied for many purposes including descriptions of microbial spoilage, correlation between environment and fish microflora (Horsley, 1973), the monitoring of changes in fish farms (Allen et al., 1983), the nutritional role of the intestinal flora (Hansen et al., 1992), and the antibiotic resistance profile of the indigenous flora (Spanggaard et al., 2000).

It is generally recognized that the intestinal flora of endothermic animal serves both as a digestive function and as a protection barrier against disease (Sissons, 1989). The intestinal flora of fish has as a consequence received much attention by several authors (Ringø et al., 1995; Sugita et al., 1997). The composition of the intestinal microflora in fish is influenced by, or directly derived from, the microflora of the food (Grisez et al., 1997). Fish with abundant and diverse microflora have considerable opportunities to adapt to changing nutritional substrates and to assimilate food better, and thus enhance their adaptive possibilities (Al-Harbi and Uddin, 2005).

The main component in animal feed is protein, carbohydrate and fat. To increase an absorption and circulation throughout the body through circulatory system, required a simplification process into smaller molecules. According to Bakke et al., (2010) a chemical feed simplification process is also called hydrolysis, involving digestive enzymes as biological catalysts. The main feed components in the form of protein, fat and carbohydrates are broken down into simple compounds which are the constituent components. Steinberg (2018) explained that the hydrolysis of macro nutrients into micro nutrients in the digestive system was occurred due to the presence of digestive enzymes, namely proteases, amylase, carbohydrase, lipase, and stomach acid. Those compounds are produced by the stomach, intestines, liver, and pancreas. In addition, several researchers reported that there was a cellulase enzyme activities in several species of aquatic animals. The presence of the

cellulase enzyme is closely related to the presence of microflora in the digestive tract (Bui and Lee, 2015; Xue et al., 1999). The discovery of the enzyme cellulase in the digestive tract of aquatic animals allows these animals to digest feed containing fiber.

Previous studies showed that in addition to endogenous digestive enzymes, exogenous digestive enzymes are also found from microflora that live in mutualism symbiosis with aquatic animals in their digestive tract (Das et al., 2014; Ganguly and Prasad, 2012; Pond et al., 2006; Xue et al., 1999). Microflora in the digestive tract of aquatic animals is thought to be not only the microbes that contribute to the production of cellulase enzymes, but also contribute to other digestive enzymes, such as protease, amylase, and lipase. In fish, the intestinal microflora has been regarded as fulfilling several roles. A nutritional function has been suggested, in which bacteria break down ingested foods to individual components such as vitamins or amino acids (Pond et al., 2006). However, there is lack of study that has been conducted related to microflora activities in mud crab digestive tract. This experiment was carried out with reference to the experiments that had been conducted by previous researchers. The aim of study was to examine the microflora contribution in the mud crab's digestive tract., namely in contributing of cellulase enzymes and digestive enzymes including α-amylase, protease, and exogenous lipase.

Material and methods

The experiment was conducted at Education Pond, Universitas Hasanuddin, Barru Regency on May – June 2020. Crab feed making, analysis for microbial population, dan enzyme activity were conducted at Biotechnology Fisheries and Marine Laboratory, Center for Research Activities, Universitas Hasanuddin.

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The number of mud crab (Scylla spp.) samples were 60 crabs. There was two treatments and three replicates for each treatment. There was 30 crabs For each treatment and replicate. The initial crab weight was 95,56±2,87 g and carapace width was 8,69±2,95 mm. Crab samples were obtained from crab fishermen and local crab supplier. Before experiment started, crab samples were acclimated in the pond for one week. After acclimatization process, crabs were fasted for 24 hours. Fasting process for crab samples was removing remaining feed in the crab body. Before treatment, crab was weighted for recorded an initial body weight using electric balance. Carapace width was measured using caliper. Crab samples were then tagged in carapace dorsal using a marker for easily to do the observation, after that crab was put into crab box. Crab was growing out individually into the crab box (the dimension of box: 21 cm x 15 cm x 8 cm). Crabs box was put into polyethylene pipa and set on surface pond with the depth of pond was ± 100 cm. The water replacement was done everyday following daily high and low tide (minimal 10% of water replacement). Water quality parameters (temperature, salinity, DO and pH) was measured daily.

Method of experiment was referred to (Xue et al., 1999) and (Aslamyah, 2006). Crab samples divided into two groups, namely crab fed with feed with antibiotic penicillin G with the dose of 100 IU mL-1 and 100 IU mL-1 streptomisin per kg feed and crab samples that fed with feed without antibiotic. Crab was fed twice a day. Crab was maintained into crab box for eight days and fed as amount of 5% from total body weight. We used pellet form for crab feed, with the nutritional contents of feed, namely protein 41,93%; BETN (29,33%), fiber 7,82%; fat 7,43%; dan DE 2767,63 kkal/kg.

Parameters were measured in this study was microbial population, cellulose enzyme and digestive enzyme activity that were conducted at the end of experiment. The method for measuring microbial population of amylolithic dan lypolithic referred to Aslamyah (2006). Analysis for cellulose enzyme and digestive enzyme (α-amilase, protease, dan lipase) activity was referred to Aslamyah (2006). Analysis method for cellulose enzyme activity following Miller (1959), α-amilase enzyme and protease enzyme activity was analyzed following (Aebi, 1974), lipase enzyme activity (Borlongan, 1990). Data was analyzed descriptively using mean and standard deviation.

Results

The measurement results for microbial population (cellutithic, amylolithic, proteolithic and lipolithic (cfu mL-1) and enzyme activity (cellulase, α-amilase, protease, dan lipase (U/g minute-1) was shown at a Tabel 1 and 2. The results of the Paired-Sampels T showed that there were significant differences in the microbial population and enzyme activity between the two groups of tested crabs. Table 1 showed that there was decreasing significantly of microbial population (cellutithic, amylolithic, proteolithic and lipolithic) in mud crab digestive tract who give a feed with antibiotic than those of feed without antibiotic. The decreasing of microbial population was almost 100% of all parameters. This trend also occurred for enzymatic activity (cellulose, □-amilase, protease, and lipase) (Table 2). The decreasing of Penurunan celulase, □-amilase, protease, and lipase accounting for 89,55; 41,90; 26,50; and37,26%, respectively.

Tabel 1. Microbial population of cellutithic, amylolithic, proteolithic and lipolithic (cfu mL^{-1}) in mud crab in the end of experiment

Treatment	Replicate	Microbial population (cfu mL^{-1})			
		Celllulithic	Amylolithic	Proteolithic	Lipolithic
Feed without antibiotic	1	1,3 x 10^{11}	8,3 x 10^{10}	9,2 x 10^{10}	7,8 x 10^{10}
	2	9,6 x 10^{10}	8,7 x 10^{10}	9,8 x 10^{10}	5,5 x 10^{10}
	Mean	1,13 x 10^{11}	8,5 x 10^{10}	9,5 x 10^{10}	6,65 x 10^{10}
Feed with antibiotic	1	4,3 x 10^{3}	9,8 x 10^{4}	1,2 x 10^{5}	3,4 x 10^{4}
	2	3,9 x 10^{3}	6,8 x 10^{5}	4,2 x 10^{6}	8.2 x 10^{5}
	Mean	4,1 x 10^{3}	3,9 x 10^{5}	2,2 x 10^{6}	4,3 x 10^{5}

Tabel 2. Digestive enzyme activity (cellulase, α-amilase, protease, dan lipase (U/g/menit) kepiting bakau pada akhir percobaan

Treatment	Replicate	Enzym activity (U/g minute^{-1})			
		Selulase	α-Amilase	Selulase	α-Amilase
Feed without antibiotic	1	24.36	18.55	32.58	15.36
	2	26.19	21.47	28.41	17.68
	Mean	25.275	20.01	30.495	16.52
Feed with antibiotic	1	4.7	13.65	25.61	9.6
	2	0.58	9.6	19.22	11.13
	Mean	2.64	11.625	22.415	10.365

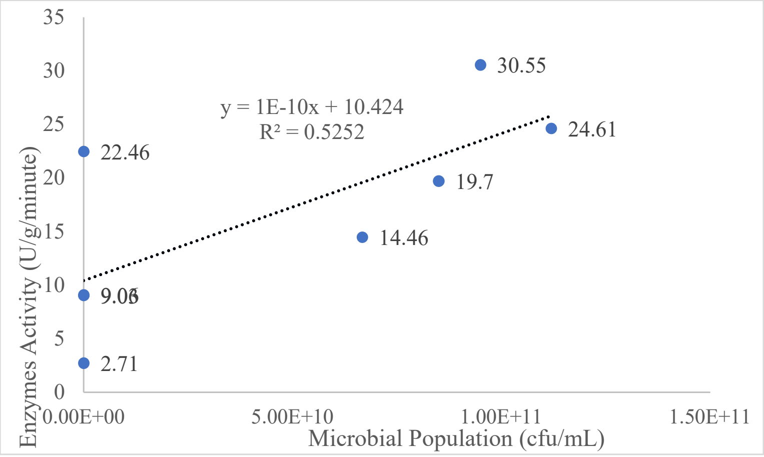

Figure 1. The correlation graph of microbial populations and enzyme activity in the digestive tract of mud crabs

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The regression analysis showed that the correlation between the microbial population and enzyme activity was linear with regression equation: y = 1E-10x + 10,424; R = 0.5252. Figure 1 showed that the increasing microbial population followed by the increasing the enzyme activity.

Discussion

The investigation of the contribution of microflora in the digestive tract of mud crabs showed that the presence of cellulase enzymes in the digestive tract of mud crabs was thought to be a contribution to the microflora of the digestive tract. Our result showed that a high results of the contribution of cellulase enzymes derived from cellulitic microbes accounting for 22.64 IU g-1 minute-1 or 89.55%. The use of antibiotics to prove the role of microflora in the digestive tract of animals as reported by (Das and Tripathi, 1991; Xue et al., 1999). Tetracycline and penicillin are types of antibiotics that can kill gram-positive bacteria, while streptomycin is a type of antibiotic that can kill gram-negative bacteria. Das and Tripathi (1991) reported decreased cellulase activity when grass carp were fed with tetracyclines. Cherac quadricarinatus which was fed with 100 IU mL-1 penicillin G. and 100 IU mL-1 streptomycin per kg of feed for 8 days, showed a decreasing in cellulase enzyme activity in the digestive tract by 40%, as well as a decreasing in the bacterial population by 94% compared to controls (Xue et al., 1999). The presence of this cellulase enzyme enables the mud crab to digest feed fiber. According to (Allan and Fielder, 2004) digestibility of crabs on fiber and all vegetable feed raw materials is very high, ranging from 94.4–96.1%. Our result found that there was a contribution of digestive enzymes α-amylase, protease, and microbaial lipase derived from microflora in the digestive tract of mud crabs. The contribution of amylolytic, proteolytic, and lipolytic microbes to the enzymes of cellulase, α-amylase, protease, and lipase in the digestive tract of mud crabs were 8.34; 8.08 and 6.16 IU g-1 minute-1 . This finding indicated that in addition to the digestive enzymes α -amylase, protease, and endogenous lipase secreted by the digestive tract and glands, there were also digestive enzymes α -amylase, protease, and exogenous lipase originating from the microflora in the digestive tract. The contribution of fish digestive tract microflora was also reported by (Aslamyah, 2006). Microbial in the digestive tract of carp in the omnivore phase was higher than in the carnivore phase. The contribution of the digestive enzyme's protease, α-amylase, and microbial lipase in carnivorous gourami were 25.21; 25.22; and 18.27%, repectively. However, it was lower than the contribution of the digestive enzyme's protease, α-amylase, and microbial lipase derived from microflora in the digestive tract of milkfish. In milkfish, the contribution of the digestive enzymes' protease, α-amylase, and microbial lipase were 36.12; 41.33; and 22.51% (Aslamyah, 2006). Lazado et al (2012) reported GP21 (Pseudomonas sp.) And GP12 (Psychrobacter sp.), which was two bacteria isolated from the gastrointestinal tract of Atlantic cod, GP21 capable in producing amylase, chitinase, cellulase and protease, whereas GP12 can only produce chitinase and protease. Furthermore, GP21 and GP12, potential probiotic organisms that could support digestion of Atlantic cod.

The microflora in the digestive tract was originated from detritus that consumed by mud crabs. In general, the types of microbes that found in the digestive tract of mud crabs was from cultivation media and or fom pond sediments. Al-Harbi and Uddin (2005) stated that there was a positive correlation between the composition of bacteria in the gills and digestive tract of fish with the composition of bacteria in water and sediment pond.

Detritus contains a lot of microorganisms that play a role in contributing exogenous digestive enzymes to degrade feed nutrients consumed by mud crabs. These microorganisms are also a source of additional nutrients for mud crabs. This finding was in line with previous study by Xue et al. (1999) who found that the growth of several species freshwater Cherac quadricarinatus reared in soil ponds was better than those reared in tank ponds. This difference was interpreted as Cherac quadricarinatus had an ability to obtain additional nutrients from the detritus material at the bottom of the pond which is not found in the tank pond and / or utilized the microorganisms that was presented in detritus to support digestif activities.

The microflora was in the digestive tract lives in mutualism symbiosis with the host and is in balance, namely between beneficial microbes and pathogenic microbes. These microflora also interact with each other between various microbial species in the digestive tract, both antagonistically and synergistically. The interactions that occurred are very important in maintaining the balance of the digestive tract microflora. It will have a positive effect for the host. Douillet and Langdon (1994) reported that on oyster cultivation, there was a high growth rate which was associated with the contribution of bacteria supplying 1) essential nutrients which were not found in some individuals in the algae population; 2) enzymes that could improve the digestive process of larvae. The microflora in the intestines of bivalve larvae was obtained in optimal proportions to produce extracellular enzymes such as proteases and lipases

The estimation of a relationship between the habit of water animals eating detritus and the presence of microflora in the digestive tract was also reported by Xue et al. (1999). Liu et al., (2016) reported that the trophic level of the host affected the structure and composition of the intestinal

microbiota, metabolic capacity and enzyme activity of the intestinal content. Clostridium, Citrobacter and Leptotrichia cellulose degrading bacteria were dominant in herbivores, while Cetobacterium and Halomonas protease-producing bacteria were dominant in carnivores. In addition, cellulase and amylase activity in herbivorous fish was significantly higher than in carnivorous fish, whereas trypsin activity in carnivorous fish was much higher than in herbivorous fish.

Conclusions

Cellulase, amylolytic, proteolytic, and lipolytic microbes in the digestive tract of mud crabs play an important role in the physiological function of the digestive tract, namely contributing to the enzymes cellulase, -amylase, protease, and endogenous lipase which were 89.55; 41.90; 26.50; and 37.26%, respectively. Furthermore, the role of the gastrointestinal microflora in contributing to cellulase enzymes and exogenous digestive enzymes amylase, protease, and lipase can be demonstrated. The microflora of the digestive tract is thought to have originated from the detritus eaten by mud crabs.

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Conflict of Interest: Author Siti Aslamyah, Yushinta Fujaya dan Nita Rukminasari have received research grant from Universitas Hasanuddin. The authors declare that they have no conflict of interest.

Ethical approval:

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Author's Contributions

Siti Aslmayah (SA), Yushinta Fujaya (YF) and Nita Rukminasari (NR) conceived and designed an experiment method. SA and YF conducted experiment. NR conducted samples analysis. SA, YF and NR analyzed data. SA and YF wrote the manuscript and NR have done final editing and proofreading of manuscript. All authors read and approved the manuscript.

Ethics

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

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Original Research Paper

MICROFLORA CONTRIBUTION TO CELLULASE AND DIGESTION ENZYME IN MUD CRAB DIGESTIVE TRACT

Siti Aslamyah*), Yushinta Fujaya and Nita Rukminasari

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Abstract: This study aim to examine the microfloral contribution in the mud crabs digestive tract. Scylla sp. were maintained in the crab box individually and placed in a pond. Two groups of crabs were fed without antibiotics and containing antibiotics at a dose of 100 IU mL-1 penicillin G and 100 IU mL-1 streptomycin, respectively. Each treatment had two replications. Crabs were kept for eight days and fed 5% of body weight per day in the afternoon. The results showed that the cellulitic, amylolytic, proteolytic, and lipolytic microbial population in the digestive tract of mud crabs which received feed added with antibiotics decreased significantly, compared to those feed without antibiotics. Furtheremore, the α-amylase, protease, and lipase enzymes activity also decreased. The declining in the cellulitic, amylolytic, proteolytic, and lipolytic microbe population were, respectively 100; 99,9995%; 99.9977%; and 99.9994%. The decreasing in the activity of cellulase, α-amylase, protease, and lipase enzymes were 89.55; 41.90; 26.50; and 37.26%, respectively. The decreasing percentage of cellulase, α-amylase, protease, and lipase enzymes indicated that there was a significant contribution of the microfloral in the mud crab digestive tract.

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Keywords: mud crab, microflora, cellulitic, proteolytic, amylolytic, lipolytic

Introduction

The indigenous microflora of fish in aquaculture has previously been studied for many purposes including descriptions of microbial spoilage, correlation between environment and fish microflora (Horsley, 1973), the monitoring of changes in fish farms (Allen et al., 1983), the nutritional role of the intestinal flora (Hansen et al., 1992), and the antibiotic resistance profile of the indigenous flora (Spanggaard et al., 2000).

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It is generally recognized that the intestinal flora of endothermic animal serves both as a digestive function and as a protection barrier against disease (Sissons, 1989). The intestinal flora of fish has as a consequence received much attention by several authors (Ringø et al., 1995; Sugita et al., 1997). The composition of the intestinal microflora in fish is influenced by, or directly derived from, the microflora of the food (Grisez et al., 1997). Fish with abundant and diverse microflora have considerable opportunities to adapt to changing nutritional substrates and to assimilate food better, and thus enhance their adaptive possibilities (Al-Harbi and Uddin, 2005).

The main component in animal feed is protein, carbohydrate and fat. To increase an absorption and circulation throughout the body through circulatory system, required a simplification process into smaller molecules. According to Bakke et al., (2010) a chemical feed simplification process is also called hydrolysis, involving digestive enzymes as biological catalysts. The main feed components in the form of protein, fat and carbohydrates are broken down into simple compounds which are the constituent components. Steinberg (2018) explained that the hydrolysis of macro nutrients into micro nutrients in the digestive system was occurred due to the presence of digestive enzymes, namely proteases, amylase, carbohydrase, lipase, and stomach acid. Those compounds are produced by the stomach, intestines, liver, and pancreas. In addition, several researchers reported that there was a cellulase enzyme activities in several species of aquatic animals. The presence of the

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cellulase enzyme is closely related to the presence of microflora in the digestive tract (Bui and Lee, 2015; Xue et al., 1999). The discovery of the enzyme cellulase in the digestive tract of aquatic animals allows these animals to digest feed containing fiber.

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Previous studies showed that in addition to endogenous digestive enzymes, exogenous digestive enzymes are also found from microflora that live in mutualism symbiosis with aquatic animals in their digestive tract (Das et al., 2014; Ganguly and Prasad, 2012; Pond et al., 2006; Xue et al., 1999). Microflora in the digestive tract of aquatic animals is thought to be not only the microbes that contribute to the production of cellulase enzymes, but also contribute to other digestive enzymes, such as protease, amylase, and lipase. In fish, the intestinal microflora has been regarded as fulfilling several roles. A nutritional function has been suggested, in which bacteria break down ingested foods to individual components such as vitamins or amino acids (Pond et al., 2006). However, there is lack of study that has been conducted related to microflora activities in mud crab digestive tract. This experiment was carried out with reference to the experiments that had been conducted by previous researchers. The aim of study was to examine the microflora contribution in the mud crab's digestive tract., namely in contributing of cellulase enzymes and digestive enzymes including α-amylase, protease, and exogenous lipase.

Material and methods

The experiment was conducted at Education Pond, Universitas Hasanuddin, Barru Regency on May – June 2020. Crab feed making, analysis for microbial population, dan enzyme activity were conducted at Biotechnology Fisheries and Marine Laboratory, Center for Research Activities, Universitas Hasanuddin.

The number of mud crab (Scylla spp.) samples were 60 crabs. There was two treatments and three replicates for each treatment. There was 30 crabs For each treatment and replicate. The initial crab weight was 95,56±2,87 g and carapace width was 8,69±2,95 mm. Crab samples were obtained from crab fishermen and local crab supplier. Before experiment started, crab samples were acclimated in the pond for one week. After acclimatization process, crabs were fasted for 24 hours. Fasting process for crab samples was removing remaining feed in the crab body. Before treatment, crab was weighted for recorded an initial body weight using electric balance. Carapace width was measured using caliper. Crab samples were then tagged in carapace dorsal using a marker for easily to do the observation, after that crab was put into crab box. Crab was growing out individually into the crab box (the dimension of box: 21 cm x 15 cm x 8 cm). Crabs box was put into polyethylene pipa and set on surface pond with the depth of pond was ± 100 cm. The water replacement was done everyday following daily high and low tide (minimal 10% of water replacement). Water quality parameters (temperature, salinity, DO and pH) was measured daily.

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Method of experiment was referred to (Xue et al., 1999) and (Aslamyah, 2006). Crab samples divided into two groups, namely crab fed with feed with antibiotic penicillin G with the dose of 100 IU mL-1 and 100 IU mL-1 streptomisin per kg feed and crab samples that fed with feed without antibiotic. Crab was fed twice a day. Crab was maintained into crab box for eight days and fed as amount of 5% from total body weight. We used pellet form for crab feed, with the nutritional contents of feed, namely protein 41,93%; BETN (29,33%), fiber 7,82%; fat 7,43%; dan DE 2767,63 kkal/kg.

Parameters were measured in this study was microbial population, cellulose enzyme and digestive enzyme activity that were conducted at the end of experiment. The method for measuring microbial population of amylolithic dan lypolithic referred to Aslamyah (2006). Analysis for cellulose enzyme and digestive enzyme (α-amilase, protease, dan lipase) activity was referred to Aslamyah (2006). Analysis method for cellulose enzyme activity following Miller (1959), α-amilase enzyme and protease enzyme activity was analyzed following (Aebi, 1974), lipase enzyme activity (Borlongan, 1990). Data was analyzed descriptively using mean and standard deviation.

Results

The measurement results for microbial population (cellutithic, amylolithic, proteolithic and lipolithic (cfu mL-1) and enzyme activity (cellulase, α-amilase, protease, dan lipase (U/g minute-1) was shown at a Tabel 1 and 2. The results of the Paired-Sampels T showed that there were significant differences in the microbial population and enzyme activity between the two groups of tested crabs. Table 1 showed that there was decreasing significantly of microbial population (cellutithic, amylolithic, proteolithic and lipolithic) in mud crab digestive tract who give a feed with antibiotic than those of feed without antibiotic. The decreasing of microbial population was almost 100% of all parameters. This trend also occurred for enzymatic activity (cellulose, □-amilase, protease, and lipase) (Table 2). The decreasing of Penurunan celulase, □-amilase, protease, and lipase accounting for 89,55; 41,90; 26,50; and37,26%, respectively.

Tabel 1. Microbial population of cellutithic, amylolithic, proteolithic and lipolithic (cfu mL^{-1}) in mud crab in the end of experiment

Treatment	Replicate	Microbial population (cfu mL^{-1})			
		Celllulithic	Amylolithic	Proteolithic	Lipolithic
Feed without antibiotic	1	1,3 x 10^{11}	8,3 x 10^{10}	9,2 x 10^{10}	7,8 x 10^{10}
	2	9,6 x 10^{10}	8,7 x 10^{10}	9,8 x 10^{10}	5,5 x 10^{10}
	Mean	1,13 x 10^{11}	8,5 x 10^{10}	9,5 x 10^{10}	6,65 x 10^{10}
Feed with antibiotic	1	4,3 x 10^{3}	9,8 x 10^{4}	1,2 x 10^{5}	3,4 x 10^{4}
	2	3,9 x 10^{3}	6,8 x 10^{5}	4,2 x 10^{6}	8.2 x 10^{5}
	Mean	4,1 x 10^{3}	3,9 x 10^{5}	2,2 x 10^{6}	4,3 x 10^{5}

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Tabel 2. Digestive enzyme activity (cellulase, α-amilase, protease, dan lipase (U/g/menit) kepiting bakau pada akhir percobaan

Treatment	Replicate	Enzym activity (U/g minute^{-1})			
		Selulase	α-Amilase	Selulase	α-Amilase
Feed without antibiotic	1	24.36	18.55	32.58	15.36
	2	26.19	21.47	28.41	17.68
	Mean	25.275	20.01	30.495	16.52
Feed with antibiotic	1	4.7	13.65	25.61	9.6
	2	0.58	9.6	19.22	11.13
	Mean	2.64	11.625	22.415	10.365

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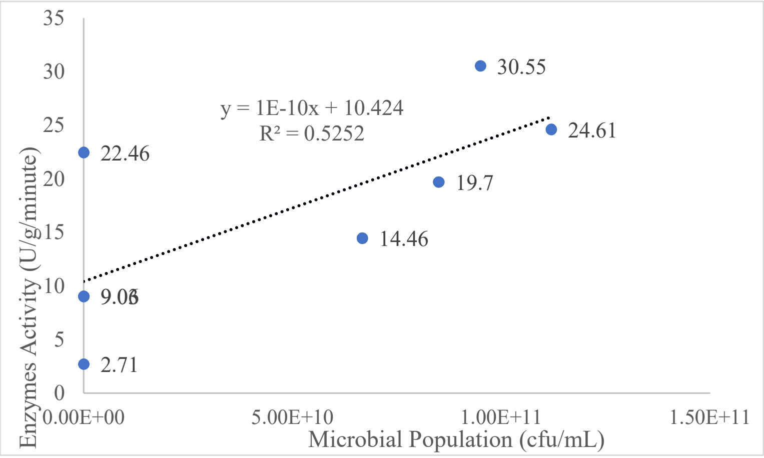

Figure 1. The correlation graph of microbial populations and enzyme activity in the digestive tract of mud crabs

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The regression analysis showed that the correlation between the microbial population and enzyme activity was linear with regression equation: $y = 1E-10x + 10,424$; $R = 0.5252$. Figure 1 showed that the increasing microbial population followed by the increasing the enzyme activity.

Discussion

The investigation of the contribution of microflora in the digestive tract of mud crabs showed that the presence of cellulase enzymes in the digestive tract of mud crabs was thought to be a contribution to the microflora of the digestive tract. Our result showed that a high results of the contribution of cellulase enzymes derived from cellulitic microbes accounting for 22.64 IU g-1 minute-1 or 89.55%. The use of antibiotics to prove the role of microflora in the digestive tract of animals as reported by (Das and Tripathi, 1991; Xue et al., 1999). Tetracycline and penicillin are types of antibiotics that can kill gram-positive bacteria, while streptomycin is a type of antibiotic that can kill gram-negative bacteria. Das and Tripathi (1991) reported decreased cellulase activity when grass carp were fed with tetracyclines. Cherac quadricarinatus which was fed with 100 IU mL-1 penicillin G. and 100 IU mL-1 streptomycin per kg of feed for 8 days, showed a decreasing in cellulase enzyme activity in the digestive tract by 40%, as well as a decreasing in the bacterial population by 94% compared to controls (Xue et al., 1999). The presence of this cellulase enzyme enables the mud crab to digest feed fiber. According to (Allan and Fielder, 2004) digestibility of crabs on fiber and all vegetable feed raw materials is very high, ranging from 94.4–96.1%. Our result found that there was a contribution of digestive enzymes α-amylase, protease, and microbaial lipase derived from microflora in the digestive tract of mud crabs. The contribution of amylolytic, proteolytic, and lipolytic microbes to the enzymes of cellulase, α-amylase, protease, and lipase in the digestive tract of mud crabs were 8.34; 8.08 and 6.16 IU g-1 minute-1 . This finding indicated that in addition to the digestive enzymes α -amylase, protease, and endogenous lipase secreted by the digestive tract and glands, there were also digestive enzymes α -amylase, protease, and exogenous lipase originating from the microflora in the digestive tract. The contribution of fish digestive tract microflora was also reported by (Aslamyah, 2006). Microbial in the digestive tract of carp in the omnivore phase was higher than in the carnivore phase. The contribution of the digestive enzyme's protease, α-amylase, and microbial lipase in carnivorous gourami were 25.21; 25.22; and 18.27%, repectively. However, it was lower than the contribution of the digestive enzyme's protease, α-amylase, and microbial lipase derived from microflora in the digestive tract of milkfish. In milkfish, the contribution of the digestive enzymes' protease, α-amylase, and microbial lipase were 36.12; 41.33; and 22.51% (Aslamyah, 2006). Lazado et al (2012) reported GP21 (Pseudomonas sp.) And GP12 (Psychrobacter sp.), which was two bacteria isolated from the gastrointestinal tract of Atlantic cod, GP21 capable in producing amylase, chitinase, cellulase and protease, whereas GP12 can only produce chitinase and protease. Furthermore, GP21 and GP12, potential probiotic organisms that could support digestion of Atlantic cod.

The microflora in the digestive tract was originated from detritus that consumed by mud crabs. In general, the types of microbes that found in the digestive tract of mud crabs was from cultivation media and or fom pond sediments. Al-Harbi and Uddin (2005) stated that there was a positive correlation between the composition of bacteria in the gills and digestive tract of fish with the composition of bacteria in water and sediment pond.

Detritus contains a lot of microorganisms that play a role in contributing exogenous digestive enzymes to degrade feed nutrients consumed by mud crabs. These microorganisms are also a source of additional nutrients for mud crabs. This finding was in line with previous study by Xue et al. (1999) who found that the growth of several species freshwater Cherac quadricarinatus reared in soil ponds was better than those reared in tank ponds. This difference was interpreted as Cherac quadricarinatus had an ability to obtain additional nutrients from the detritus material at the bottom of the pond which is not found in the tank pond and / or utilized the microorganisms that was presented in detritus to support digestif activities.

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The microflora was in the digestive tract lives in mutualism symbiosis with the host and is in balance, namely between beneficial microbes and pathogenic microbes. These microflora also interact with each other between various microbial species in the digestive tract, both antagonistically and synergistically. The interactions that occurred are very important in maintaining the balance of the digestive tract microflora. It will have a positive effect for the host. Douillet and Langdon (1994) reported that on oyster cultivation, there was a high growth rate which was associated with the contribution of bacteria supplying 1) essential nutrients which were not found in some individuals in the algae population; 2) enzymes that could improve the digestive process of larvae. The microflora in the intestines of bivalve larvae was obtained in optimal proportions to produce extracellular enzymes such as proteases and lipases

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The estimation of a relationship between the habit of water animals eating detritus and the presence of microflora in the digestive tract was also reported by Xue et al. (1999). Liu et al., (2016) reported that the trophic level of the host affected the structure and composition of the intestinal

microbiota, metabolic capacity and enzyme activity of the intestinal content. Clostridium, Citrobacter and Leptotrichia cellulose degrading bacteria were dominant in herbivores, while Cetobacterium and Halomonas protease-producing bacteria were dominant in carnivores. In addition, cellulase and amylase activity in herbivorous fish was significantly higher than in carnivorous fish, whereas trypsin activity in carnivorous fish was much higher than in herbivorous fish.

Conclusions

Cellulase, amylolytic, proteolytic, and lipolytic microbes in the digestive tract of mud crabs play an important role in the physiological function of the digestive tract, namely contributing to the enzymes cellulase, □-amylase, protease, and endogenous lipase which were 89.55; 41.90; 26.50; and 37.26%, respectively. Furthermore, the role of the gastrointestinal microflora in contributing to cellulase enzymes and exogenous digestive enzymes amylase, protease, and lipase can be demonstrated. The microflora of the digestive tract is thought to have originated from the detritus eaten by mud crabs.

Acknowledgment

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Conflict of Interest: Author Siti Aslamyah, Yushinta Fujaya dan Nita Rukminasari have received research grant from Universitas Hasanuddin. The authors declare that they have no conflict of interest.

Ethical approval:

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Author's Contributions

Siti Aslmayah (SA), Yushinta Fujaya (YF) and Nita Rukminasari (NR) conceived and designed an experiment method. SA and YF conducted experiment. NR conducted samples analysis. SA, YF and NR analyzed data. SA and YF wrote the manuscript and NR have done final editing and proofreading of manuscript. All authors read and approved the manuscript.

Ethics

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

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Author comment and respond for manuscript Tanggal : 22 Februari 2021

Author comment and respond for manuscript # 742-OJBS

Reviewer	Comment	Author comment & Respond
1	1) English writing and spelling mistakes all over the MS. Few examples: cellutithic; Selulase; Amilase; for easily; decreasing in activity; mud crab., ; crabs digestive to Crabs digestive tract; there was 30 crabs; everyday; fom etc. Without antibiotic and containing antibiotic to with and without antibiotic. etc. Rewrite -The microflora was in the digestive tract lives in	All spelling mistakes have been corrected
	2) In article history (on first page), I could not get why 2014 is mentioned.	We have corrected this part
	3) Why each treatment had only two replicas? If we see the data variation, it need more number of experiments (n= 3-6).	In this experiment, we used two replicates as following a previous study by Xue et al (1999).
	4) Decrease in cellulase, amylase activity is too low so we could write it as change in activity instead of decrease. This could be an experimental variation only as the n is low.	Yes, we agreed that cellulase and amylase activities in this experiment was changing and not decreasing due too low value those parameters. We have revised this part
	5) It is not clear in introduction that why authors want to test crab here and for what aim?	We explained the reason why we conducted micro flora study in mud crab at the last paragraph of introduction.
	6) Food breakdown does not necessarily form only vitamins and amino acids. Author may correct it to more food breakdown products to mention here.	Regarding food breakdown, we explained more detail in the third paragraph of introduction.
	7) Methods : why only may-june (just 2 months) data is reported. Authors may need to explain this as limited time and number of experiments may need to artifacts.	We have already added the information regarding this issue (We put the text in highlight).
	8) What is 95,56 g weight? It is not clear what they want to write actually. Is it 9.556	We have revised this part.

	kg then ? Keep writing style same, see table 1 and 2. Sometime authors write 1,3 or 9,6 style and sometime its 24.36 or 26.19 style.	
	9) What is meaning of 30 crabs for each experiment and replicate? It does not make it clear that how many crabs were in a single set.	30 crabs were number of crab for each treatment (including replicate).
	10) Dimensions like weight, age, size for Crabs is not written clearly.	The dimension of crab was explained in the second paragraph of material and method
	11) How was fasting done in crabs? Like removing feed?	Fasting process for crab samples was removing remaining feed in the crab body.
	12) What are the details of the feed given ?	We used pellet form for crab feed, with the nutritional contents of feed, namely protein 41.93%; Nitrogen Free Extract/NFE (29,33%), fiber 7.82%; fat 7.43%; and Digestible Energy/DE 2767.63 kkal/kg.
	13) Describing the assay done for each activity is better than referring only.	We have explained more detail regarding measurement enzyme activity and microbial population (text in highlighted)
	14) How the enzymes were prepared for these activities? Or write clearly what was tested ? I also did not see any data for the assay done without which its hard to get the MS.	We have revised this part (added information in highlighted)
	15) What do authors mean by " The decreasing of microbial population was almost 100 % of all parameters? Also where is the data shown for this?	We have revised this part.
	16) What is ˜Table 1 title meaning - at the end of experiment. Why only 2 sets are done? Is the number = 1.3 x 10 or different like 13 x 10	We measured parameters in the end of experiment. We only have 2 data sets for our experiments as mentioned in Table 1 and 2. The number written 13x10 supposed to be written 1.3 x 10.

	17) Table 2 â€“ what are the two same rows for the activity- Cellulase - cellulase or Amylase -Amylase ? 18) How was Fig 1 plotted? What are the all points plotted, kindly include in fig legend.	We have revised this table We plotted microbial population and enzyme activity data on Fig.1. We want to determine the correlation between number of microbial population and enzyme activity through regression analysis.
2	The quality of this manuscript has not accepted for publication in the journal e.g. language is not in English (some part) but showing poor consideration, figure and tables are not acceptable for publication as well. They need to be careful in writing all parts of the manuscript to reach the good quality before send out the manuscript, at least proof-reading should be done.	We have sent our manuscript to professional English language editor for proofreading and editing.

Revised Author 1 Tanggal : 22 Februari 2021

OnLine Journal of Biological Science

Original Research Paper

MICROFLORA CONTRIBUTION TO CELLULASE AND DIGESTION ENZYME IN MUD CRAB DIGESTIVE TRACT

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Abstract: Microflora in the digestive tract of aquatic animals is thought to be not only the microbes that contribute to the production of cellulase enzymes but also contribute to other digestive enzymes, such as protease, amylase, and lipase. This study aims to examine the microflora contribution to the mud crab's digestive tract. Scylla sp. were maintained in the crab box individually and placed in a pond. Two groups of crabs were fed without antibiotics and containing antibiotics at a dose of 100 IU mL^{-1} penicillin G and 100 IU mL^{-1} streptomycin, respectively. Each treatment had two replications. Crabs were kept for eight days and fed 5% of body weight per day in the afternoon. The results showed that the cellulitis, amylolytic, proteolytic, and lipolytic microbial population in the digestive tract of mud crabs that received feed added with antibiotics decreased significantly compared to those feed without antibiotics. Furthermore, the α-amylase, protease, and lipase enzyme activity also decreased. The decrease in cellulase activity, α-amylase, protease, and lipase enzymes were 89.55, 41.90, 26.50, and 37.26%, respectively. The decreasing percentage of cellulase, α-amylase, protease, and lipase enzymes indicated a significant microflora contribution in the mud crab digestive tract.

Keywords: mud crab, microflora, cellulitis, proteolytic, amylolytic, lipolytic

Introduction

The indigenous microflora of fish in aquaculture has previously been studied for many purposes, including descriptions of microbial spoilage, the correlation between environment and fish microflora (Horsley, 1973), the monitoring of changes in fish farms (Allen et al., 1983), the nutritional role of the intestinal flora (Hansen et al., 1992), and the antibiotic resistance profile of the indigenous flora (Spanggaard et al., 2000).

It is generally recognized that the intestinal flora of endothermic animals serves both as a digestive function and as a protective barrier against disease (Sissons, 1989). The intestinal flora of fish has, as a consequence, received much attention from several authors (Ringø et al., 1995; Sugita et al., 1997). The composition of the intestinal microflora in fish is influenced by, or directly derived from, the microflora of the food (Grisez et al., 1997). Fish with abundant and diverse microflora have considerable opportunities to adapt to changing nutritional substrates, assimilate food better, and enhance their adaptive possibilities (Al-Harbi and Uddin, 2005).

The main component in animal feed is protein, carbohydrate, and fat. To increase absorption and circulation throughout the body through the circulatory system required a simplification process into smaller molecules. According to Bakke et al. (2010), a chemical feed simplification process is also called hydrolysis, involving digestive enzymes as biological catalysts. The main feed components in protein, fat, and carbohydrates are broken down into simple compounds, which are the constituent components.

Science Publications

Steinberg (2018) explained that the hydrolysis of macronutrients into micronutrients in the digestive system was occurred due to the presence of digestive enzymes, namely protease, amylase, carbohydrase, lipase, and stomach acid. Those compounds are produced by the stomach, intestines, liver, and pancreas. In addition, several researchers reported that there were cellulase enzyme activities in several species of aquatic animals. The presence of the cellulase enzyme is closely related to the microflora in the digestive tract (Bui and Lee, 2015; Xue et al., 1999). The discovery of the enzyme cellulase in the digestive tract of aquatic animals allows these animals to digest feed containing fiber.

Previous studies showed that in addition to endogenous digestive enzymes, exogenous digestive enzymes are also found from microflora that lives in mutualism symbiosis with aquatic animals in their digestive tract (Das et al., 2014; Ganguly and Prasad, 2012; Pond et al., 2006; Xue et al., 1999). Microflora in the digestive tract of aquatic animals is thought to be the microbes that contribute to the production of cellulase enzymes and contribute to other digestive enzymes, such as protease, amylase, and lipase. In fish, the intestinal microflora has been regarded as fulfilling several roles. A nutritional function has been suggested, in which bacteria break down ingested foods into individual components such as vitamins or amino acids (Pond et al., 2006). However, there is a lack of study conducted related to microflora activities in the mud crab digestive tract. This experiment was carried out with reference to the experiments that had been conducted by previous researchers. The aim of study was to examine the microflora contribution in the mud crab's digestive tract., namely in contributing of cellulase enzymes and digestive enzymes including α-amylase, protease, and exogenous lipase.

Material and methods

The experiment was conducted at Education Pond, Universitas Hasanuddin, Barru Regency, on May – June 2020. We only conducted the experiment for two months due to limited time and other sources of research, including funds and labor. Crab feed making, analysis for microbial population, and enzyme activity were conducted at Biotechnology Fisheries and Marine Laboratory, Center for Research Activities, Universitas Hasanuddin.

The number of mud crab (Scylla spp.) samples were 60 crabs. There were two treatments and three replicates for each treatment. There were 30 crabs for each treatment and replicate. The initial crab weight was 95.56±2.87 g and carapace width was 8.69±2.95 mm. Crab samples were obtained from crab fishers and local crab suppliers. Before the experiment started, crab samples were acclimated to the pond for one week. After the acclimatization process, crabs were fasted for 24 hours. The fasting process for crab samples was removing the remaining feed in the crab body. Before treatment, the crab was weighted for recorded an initial body weight using electric balance. Carapace width was measured using a caliper. Crab samples were then tagged in carapace dorsal using a marker for easy-to-do observation. After that, the crab was put into the crab box.

The crab was growing out individually into the crab box (the dimension of the box: 21 cm x 15 cm x 8 cm). Crabs box was put into polyethylene pipa and set on the surface pond with the depth of pond was ± 100 cm. The water replacement was done daily following daily high and low tide (minimal 10% of water replacement). Water quality parameters (temperature, salinity, dissolved oxygen, and pH) were measured daily.

The experiment method was referred to (Xue et al., 1999) and (Aslamyah, 2006). Crab samples were divided into two groups, namely crab fed with feed with antibiotic penicillin G with the dose of 100 IU mL^{-1} and 100 IU mL^{-1} streptomycin kg^{-1} feed and crab samples fed with feed without antibiotic. The crab was fed twice a day. The crab was maintained in the crab box for eight days and fed as an amount of 5% of total body weight. We used pellet form for crab feed, with the nutritional contents of the feed, namely protein 41.93%, Nitrogen Free Extract/NFE 29,33%, fiber 7.82%, fat 7.43%, and Digestible Energy /DE 2767.63 kcal/kg.

Parameters that were measured in this study were microbial population, cellulose enzyme, and digestive enzyme activity that were conducted at the end of the experiment.

Measurement microbial population

The method for measuring the microbial population of cellulitis, amylolytic, proteolytic, and lipolytic referred to Aslamyah (2006) as the detailed method: measurement of the microbial population begins with preparing the source of the inoculum from the digestive tract of the crabs. Digestive tract samples were crushed, and every 10 g of the sample was diluted with 90 mL of sterile physiological solution (0.85% NaCl). The inoculum source of 0.5 mL was inoculated into 10 mL of standard liquid media, namely Trypticase Soy Broth (TSB, Merck), which added 1% NaCl and cellulose for cellulitis, casein as an energy source for proteolytic, TSB plus 1% NaCl, and starch for amylolytic, and TSB plus 1% NaCl and fish oil for lipolytic. The culture was then incubated at 29ºC for 24 hours. Serial dilution is carried out from 10^{-2} to 10^{-10} by taking 0.05 mL from the microbial culture in liquid media and putting it in 4.95 mL of the first diluent medium, then 0.05 mL of the first diluent medium is taken and put into in 4.95 mL of second diluent medium and so on until the last diluting medium. Each dilution series is transferred as much as 0.1 mL into solid media, which consists of a mixture of TSB, 1% NaCl, agar, and its energy source. This preparation was incubated again at 29ºC for 24 to 48 hours. The microbial colonies that grew were counted in colony count (CFU / mL).

Measurement enzyme activity

Analysis for cellulase enzyme and digestive enzyme (α-amylase, protease, and lipase) activity begins with preparing a crude enzyme extract which refers to Aslamyah (2006). Activities carried out at a temperature of 0 to 4ºC with the aim of the enzyme in an inactive condition. Digestive tract samples with distilled water and dried with suction paper. Samples were taken as much as 1 g and crushed with mortar until smooth and homogenized with 10 mL of cold distilled water, then centrifuged at 15,000 rpm for 20 minutes at 4ºC. The supernatant was taken as crude enzyme extract and used as samples for enzyme activity testing.

Cellulase enzyme activity was determined using the DNS method (3,5-dinitrosalicylic acid). A total of 80 µL of crude enzyme extract plus 720 µL of 1% CMC solution substrate (in citrate phosphate buffer pH 7) was put into the Eppendorf tube and then incubated a water bath at 37°C for 60 minutes. The incubation results were added with 1200 µL of DNS reagent then put in a boiling water bath for 15 minutes. After that, put in an ice bath for 20 minutes. In this test, control was used in the form of 80 µL crude extract of the enzyme, which had been heated for 15 minutes, then added 720 µL of the substrate and 1200 µL of DNS reagent. The solution mixture is treated the same as the sample conditions above. After that, the reducing sugar formed was measured by a UV-Vis spectrophotometer at λ = 540 nm (Miller, 1959).

Measurement of protease activity was carried out based on the method of Bergmeyer and Grassi (1983). A total of 1 mL of 2% casein solution mixed with 1 mL of borate buffer (0.01 M) pH 8.0, 0.20 mL 0.05 M hydrochloric acid, and 0.20 mL crude enzyme extract, which activity will be determined. Then incubated in a water bath at 37ºC for 10 minutes, then added 2 mL of 0.1 M trichloroacetic acid (TCA). Incubated for 10 minutes, then centrifuged. The 1.5 mL portion of the filtrate was mixed with 5 mL of 0.4 M disodium carbonate and 1mL of Folin Ciocalteu's reagent and let stand for 20 minutes. Then read the absorbance at a wavelength of 578 nm.

Determination of the amylase enzyme activity value was carried out by mixing 1 mL of the enzyme with 1% starch in 1 mL of 0.05 M citrate buffer pH 5.7. Then the incubation was carried out at 37°C for 30 minutes. The reaction was stopped by adding 2 mL of DNS (3.5 dinitro salicylic acid), then heated in boiling water for 5 minutes and, after chilling, centrifuged at 3,000 rpm for 5 minutes. Furthermore, reducing sugar can be measured using a spectrophotometer at a wavelength of 540 nm. One unit of enzyme activity is defined as the number of enzymes that produce 1 µmol of glucose / per minute

Analysis method for lipase enzyme activity (Tietz and Friedreck 1966 in Borlongan, 1990), a stable lipase substrate (olive oil) 1.5 mL plus 1 mL Tris-HCl 0.1 M as a buffer with a pH of 8.0. Then add 1.0 mL of crude enzyme extract. The mixture was homogenized and incubated for 6 hours at 37ºC. The reaction is stopped by adding 3 mL of 95% ethyl alcohol. Titrate the sample with 0.01 N NaOH, using 0.9% (w/v) thymolphthalein in ethanol as an indicator. The same procedure is carried out on the blank. One lipase activity unit was defined as the volume of 0.05 N NaOH needed to neutralize the fatty acids released during 6 hours of incubation with the substrate, after correction with blanks.

Data were analyzed descriptively using mean and standard deviation.

Results

The measurement results for microbial population (cellulitis, amylolytic, proteolytic, and lipolytic CFU mL^{-1}) and enzyme activity (cellulase, α-amylase, protease, dan lipase IU g^{-1} $minute^{-1}$) was shown in Table 1 and 2. The Paired-Samples T results showed significant differences in the microbial population and enzyme activity between the two groups of tested crabs. Table 1 showed that there was decreasing significantly of the microbial population (cellulitis, amylolytic, proteolytic, and lipolytic) in mud crab digestive tract who give a feed with antibiotic than those of feed without antibiotic. This trend also occurs for the enzymatic activity of cellulose, α-amylase, protease, and lipase, as shown in Table 2. The decreasing of cellulose, α-amylase, protease, and lipase accounted for 89.55, 41.90, 26.50, and 37.26%, respectively.

The regression analysis showed that the correlation between the microbial population and enzyme activity was linear with the regression equation: y = 1E-10x + 10.424; R = 0.5252. Figure 1 showed that the increasing microbial population followed by increasing the enzyme activity.

Table 1. The microbial population of cellulitis, amylolytic, proteolytic, and lipolytic (CFU mL^{-1}) in mud crab at the end of the experiment

Treatment	Replicate	Microbial population (CFU mL^{-1})			
		Cellulitis	Amylolytic	Proteolytic	Lipolytic
Feed without antibiotic	1	1.3×10^{11}	8.3×10^{10}	9.2×10^{10}	7.8×10^{10}
	2	9.6×10^{10}	8.7×10^{10}	9.8×10^{10}	5.5×10^{10}
	Mean	1.13×10^{11}	8.5×10^{10}	9.5×10^{10}	6.65×10^{10}
Feed with antibiotic	1	4.3×10^{3}	9.8×10^{4}	1.2×10^{5}	3.4×10^{4}
	2	3.9×10^{3}	6.8×10^{5}	4.2×10^{6}	8.2×10^{5}
	Mean	4.1×10^{3}	3.9×10^{5}	2.2×10^{6}	4.3×10^{5}

Table 2. Digestive enzyme activity of cellulase, α-amylase, protease, and lipase (IU g^{-1} minute^{-1}) of mud crab at the end of the experiment

Treatment	Replicate	Enzyme activity (IU g^{-1} minute^{-1})			
		Cellulose	α-Amylase	Protease	Lipase
Feed without antibiotic	1	24.36	18.55	32.58	15.36
	2	26.19	21.47	28.41	17.68
	Mean	25.275	20.01	30.495	16.52
Feed with antibiotic	1	4.7	13.65	25.61	9.6
	2	0.58	9.6	19.22	11.13
	Mean	2.64	11.625	22.415	10.365

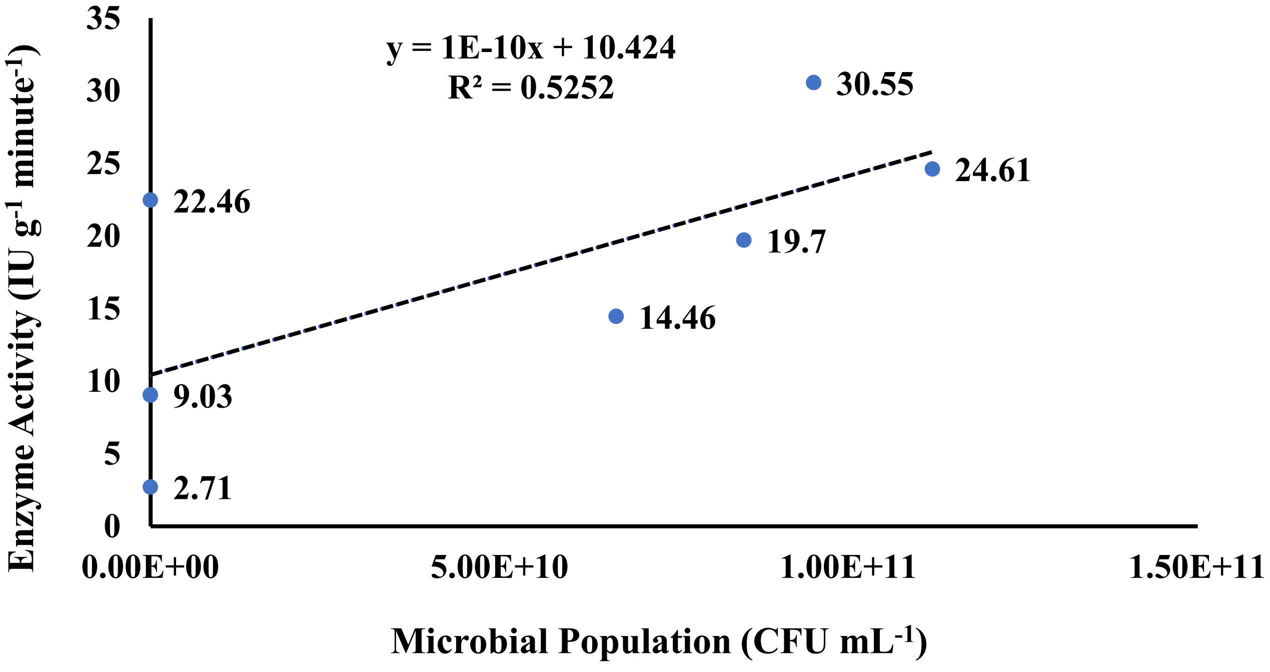

Figure 1. The regression graph of microbial populations and enzyme activity in the digestive tract of mud crabs

Discussion

The investigation of the contribution of microflora in the digestive tract of mud crabs showed that cellulase enzymes in the digestive tract of mud crabs were thought to contribute to the microflora of the digestive tract. Our result showed that a high impact of the contribution of cellulase enzymes derived from cellulitis microbes accounting for 22.64 IU g^{-1} minute^{-1} or 89.55%. The use of antibiotics to prove the role of microflora in the digestive tract of animals as reported by (Das and Tripathi, 1991; Xue et al., 1999). Tetracycline and penicillin are types of antibiotics that can kill gram-positive bacteria, while streptomycin is a type of antibiotic that can kill gram-negative bacteria. Das and Tripathi (1991) reported decreased cellulase activity when grass carp were fed with tetracyclines. *Cherac quadricarinatus,* which was fed with 100 IU mL^{-1} penicillin G. and 100 IU mL^{-1} streptomycin per kg of feed for eight days, showed a decrease in cellulase enzyme activity in the digestive tract by 40%, as well as a reduction of the bacterial population by 94% compared to controls (Xue et al., 1999). The presence of this cellulase enzyme enables the mud crab to digest feed fiber. According to (Allan and Fielder, 2004) digestibility of crabs on fiber and all vegetable feed raw materials is very high, ranging from 94.4–96.1%. Our result found a contribution of digestive enzymes α-amylase, protease, and microbial lipase derived from microflora in the digestive tract of mud crabs. The contribution of amylolytic, proteolytic, and lipolytic microbes to the enzymes of cellulase, α-amylase, protease, and lipase in the digestive tract of mud crabs were 8.34, 8.08, and 6.16 IU g^{-1} minute^{-1}. This finding indicated that in addition to the digestive enzymes α-amylase, protease, and endogenous lipase secreted by the digestive tract and glands, there were also digestive enzymes α-amylase, protease, and exogenous lipase originating from the microflora in the digestive tract. The contribution of fish digestive tract microflora was also reported by (Aslamyah, 2006). Microbial in the digestive tract of carp in the omnivore phase was higher than in the carnivore phase. The contribution of the digestive enzymes protease, α-amylase, and microbial lipase in carnivorous gourami were 25.21, 25.22, and 18.27%, respectively. However, it was lower than the contribution of the digestive enzymes protease, α-amylase, and microbial lipase derived from microflora in the digestive tract of milkfish. In milkfish, the contribution of the digestive enzymes protease, α-amylase, and microbial lipase were 36.12, 41.33, and 22.51% (Aslamyah, 2006). Lazado et al. (2012) reported GP21 (*Pseudomonas* sp.) And GP12 (*Psychrobacter* sp.), which was two bacteria isolated from the gastrointestinal tract of Atlantic cod, GP21 capable of producing amylase, chitinase, cellulase, and protease, whereas GP12 can only produce chitinase and protease. Furthermore, GP21 and GP12, potential probiotic organisms that could support the digestion of Atlantic cod.

The microflora in the digestive tract was originated from detritus consumed by mud crabs. In general, the types of microbes found in the digestive tract of mud crabs were from cultivation media and or form pond sediments. Al-Harbi and Uddin (2005) stated a positive correlation between the composition of bacteria in the gills and digestive tract of fish with the composition of bacteria in water and sediment pond.

Detritus contains many microorganisms that play a role in contributing exogenous digestive enzymes to degrade feed nutrients consumed by mud crabs. These microorganisms are also a source of additional nutrients for mud crabs. This finding was in line with a previous study by Xue et al. (1999), who found that the growth of several species of freshwater *Cherac quadricarinatus* reared in soil ponds was better than those reared in tank ponds. This difference was interpreted as *Cherac quadricarinatus* had an ability to obtain additional nutrients from the detritus material at the bottom of the pond, which is not found in the tank pond and/or utilized the presented microorganisms in detritus to support digestif activities.

The microflora was in the digestive tract lives in mutualism symbiosis with the host and is in balance, namely between beneficial microbes and pathogenic microbes. These microfloras also interact with each other between various microbial species in the digestive tract, both antagonistically and synergistically. The interactions that occurred are very important in maintaining the balance of the digestive tract microflora. It will have a positive effect on the host. Douillet and Langdon (1994) reported that on oyster cultivation, there was a high growth rate which was associated with the contribution of bacteria supplying 1) essential nutrients which were not found in some individuals in the algae population, and 2) enzymes that could improve the digestive process of larvae. The microflora in the intestines of bivalve larvae was obtained in optimal proportions to produce extracellular enzymes such as proteases and lipases

The estimation of a relationship between the habit of water animals eating detritus and the presence of microflora in the digestive tract was also reported by Xue et al. (1999). Liu et al. (2016) reported that the trophic level of the host affected the structure and composition of the intestinal microbiota, metabolic capacity, and enzyme activity of the intestinal content. *Clostridium, Citrobacter,* and *Leptotrichia* cellulose-degrading bacteria were dominant in herbivores, while *Cetobacterium* and *Halomonas* protease-producing bacteria were dominant in carnivores. In addition, cellulase and amylase activity in herbivorous fish was significantly higher than in carnivorous fish, whereas trypsin activity in carnivorous fish was much higher than in herbivorous fish.

Conclusions

Cellulase, amylolytic, proteolytic, and lipolytic microbes in the digestive tract of mud crabs play an important role in the physiological function of the digestive tract, namely contributing to the enzymes cellulase, α-amylase, protease, dan lipase (IU g^{-1} $minute^{-1}$) was shown 55, 41.90, 26.50, and 37.26%, respectively. Furthermore, the role of the gastrointestinal microflora in contributing to cellulase enzymes and exogenous digestive enzymes amylase, protease, and lipase can be demonstrated. The microflora of the digestive tract is thought to have originated from the detritus eaten by mud crabs.

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Conflict of Interest: Author Siti Aslamyah, Yushinta Fujaya dan Nita Rukminasari have received a research grant from Universitas Hasanuddin. The authors declare that they have no conflict of interest.

Ethical approval:

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Author's Contributions

Siti Aslamyah (SA), Yushinta Fujaya (YF), and Nita Rukminasari (NR) conceived and designed an experiment method. SA and YF conducted the experiment. NR conducted sample analysis. SA, YF, and NR analyzed data. SA and YF wrote the manuscript, and NR have done final editing and proofreading of the manuscript. All authors read and approved the manuscript.

Ethics

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

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Original Research Paper

Microflora Contribution to Cellulase and Digestion Enzyme in Mud Crab Digestive Tract

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Abstract: Microflora in the digestive tract of aquatic animals is thought to be not only the microbes that contribute to the production of cellulase enzymes but also contribute to other digestive enzymes, such as protease, amylase and lipase. This study aims to examine the microflora contribution to the mud crab's digestive tract. Scylla sp. were maintained in the crab box individually and placed in a pond. Two groups of crabs were fed without antibiotics and containing antibiotics at a dose of 100 IU mL^{-1} penicillin G and 100 IU mL^{-1} streptomycin, respectively. Each treatment had two replications. Crabs were kept for eight days and fed 5% of body weight per day in the afternoon. The results showed that the cellulitis, amylolytic, proteolytic and lipolytic microbial population in the digestive tract of mud crabs that received feed added with antibiotics decreased significantly compared to those feed without antibiotics. Furthermore, the α-amylase, protease and lipase enzyme activity also decreased. The decrease in cellulase activity, α-amylase, protease and lipase enzymes were 89.55, 41.90, 26.50 and 37.26%, respectively. The decreasing percentage of cellulase, α-amylase, protease and lipase enzymes indicated a significant microflora contribution in the mud crab digestive tract.

Keywords: Mud Crab, Microflora, Cellulitis, Proteolytic, Amylolytic, Lipolytic

Introduction

The indigenous microflora of fish in aquaculture has previously been studied for many purposes, including descriptions of microbial spoilage, the correlation between environment and fish microflora (Horsley, 1973), the monitoring of changes in fish farms (Allen *et al*., 1983), the nutritional role of the intestinal flora (Hansen *et al*., 1992) and the antibiotic resistance profile of the indigenous flora (Spanggaard *et al*., 2000).

It is generally recognized that the intestinal flora of endothermic animals serves both as a digestive function and as a protective barrier against disease (Sissons, 1989). The intestinal flora of fish has, as a consequence, received much attention from several authors (Ringø *et al*., 1995; Sugita *et al*., 1997). The composition of the intestinal microflora in fish is influenced by, or directly derived from, the microflora of the food (Grisez *et al*., 1997). Fish with abundant and diverse microflora have considerable opportunities to adapt to changing nutritional substrates, assimilate food better and enhance their adaptive possibilities (Al-Harbi and Uddin, 2005).

The main component in animal feed is protein, carbohydrate and fat. To increase absorption and circulation throughout the body through the circulatory system required a simplification process into smaller molecules. According to (Bakke *et al*., 2010), a chemical feed simplification process is also called hydrolysis, involving digestive enzymes as biological catalysts. The main feed components in protein, fat and carbohydrates are broken down into simple compounds, which are the constituent components. Steinberg (2017) explained that the hydrolysis of macronutrients into micronutrients in the digestive system was occurred due to the presence of digestive enzymes, namely protease, amylase, carbohydrase, lipase and stomach acid. Those compounds are produced by the stomach, intestines, liver and pancreas. In addition, several researchers reported that there were cellulase enzyme activities in several species of aquatic animals. The presence of the cellulase enzyme is closely related to the microflora in the digestive tract (Bui and Lee, 2015; Xue *et al*., 1999). The discovery of the enzyme cellulase in the digestive tract of aquatic animals allows these animals to digest feed containing fiber.

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Previous studies showed that in addition to endogenous digestive enzymes, exogenous digestive enzymes are also found from microflora that lives in mutualism symbiosis with aquatic animals in their digestive tract (Das *et al*., 2014; Ganguly and Prasad, 2012; Pond *et al*., 2006; Xue *et al*., 1999). Microflora in the digestive tract of aquatic animals is thought to be the microbes that contribute to the production of cellulase enzymes and contribute to other digestive enzymes, such as protease, amylase and lipase. In fish, the intestinal microflora has been regarded as fulfilling several roles. A nutritional function has been suggested, in which bacteria break down ingested foods into individual components such as vitamins or amino acids (Pond *et al*., 2006). However, there is a lack of study conducted related to microflora activities in the mud crab digestive tract. This experiment was carried out with reference to the experiments that had been conducted by previous researchers. The aim of study was to examine the microflora contribution in the mud crab's digestive tract., namely in contributing of cellulase enzymes and digestive enzymes including α-amylase, protease and exogenous lipase.

Materials and Methods

The experiment was conducted at Education Pond, Universitas Hasanuddin, Barru Regency, on May-June 2020. We only conducted the experiment for two months due to limited time and other sources of research, including funds and labor. Crab feed making, analysis for microbial population and enzyme activity were conducted at Biotechnology Fisheries and Marine Laboratory, Center for Research Activities, Universitas Hasanuddin.

The number of mud crab (Scylla spp.) samples were 60 crabs. There were two treatments and three replicates for each treatment. There were 30 crabs for each treatment and replicate. The initial crab weight was 95.56±2.87 g and carapace width was 8.69±2.95 mm. Crab samples were obtained from crab fishers and local crab suppliers. Before the experiment started, crab samples were acclimated to the pond for one week. After the acclimatization process, crabs were fasted for 24 h. The fasting process for crab samples was removing the remaining feed in the crab body. Before treatment, the crab was weighted for recorded an initial body weight using electric balance. Carapace width was measured using a caliper. Crab samples were then tagged in carapace dorsal using a marker for easy-to-do observation. After that, the crab was put into the crab box.

The crab was growing out individually into the crab box (the dimension of the box: 21×15×8 cm). Crabs box was put into polyethylene pipa and set on the surface pond with the depth of pond was ±100 cm. The water replacement was done daily following daily high and low tide (minimal 10% of water replacement). Water quality parameters (temperature, salinity, dissolved oxygen and pH) were measured daily.

The experiment method was referred to (Xue *et al*., 1999; Aslamyah, 2006). Crab samples were divided into two groups, namely crab fed with feed with antibiotic penicillin G with the dose of 100 and 100 IU mL^{-1} streptomycin kg^{-1} feed and crab samples fed with feed without antibiotic. The crab was fed twice a day. The crab was maintained in the crab box for eight days and fed as an amount of 5% of total body weight. We used pellet form for crab feed, with the nutritional contents of the feed, namely protein 41.93%, Nitrogen Free Extract/NFE 29,33%, fiber 7.82%, fat 7.43% and Digestible Energy/DE 2767.63 kcal/kg.

Parameters that were measured in this study were microbial population, cellulose enzyme and digestive enzyme activity that were conducted at the end of the experiment.

Measurement Microbial Population

The method for measuring the microbial population of cellulitis, amylolytic, proteolytic and lipolytic referred to (Aslamyah, 2006) as the detailed method: Measurement of the microbial population begins with preparing the source of the inoculum from the digestive tract of the crabs. Digestive tract samples were crushed and every 10 g of the sample was diluted with 90 mL of sterile physiological solution (0.85% NaCl). The inoculum source of 0.5 mL was inoculated into 10 mL of standard liquid media, namely Trypticase Soy Broth (TSB, Merck), which added 1% NaCl and cellulose for cellulitis, casein as an energy source for proteolytic, TSB plus 1% NaCl and starch for amylolytic and TSB plus 1% NaCl and fish oil for lipolytic. The culture was then incubated at 29°C for 24 h. Serial dilution is carried out from 10^{-2} to 10^{-10} by taking 0.05 mL from the microbial culture in liquid media and putting it in 4.95 mL of the first diluent medium, then 0.05 mL of the first diluent medium is taken and put into in 4.95 mL of second diluent medium and so on until the last diluting medium. Each dilution series is transferred as much as 0.1 mL into solid media, which consists of a mixture of TSB, 1% NaCl, agar and its energy source. This preparation was incubated again at 29°C for 24 to 48 h. The microbial colonies that grew were counted in colony count (CFU/mL).

Measurement Enzyme Activity

Analysis for cellulase enzyme and digestive enzyme (α-amylase, protease and lipase) activity begins with preparing a crude enzyme extract which refers to (Aslamyah, 2006). Activities carried out at a temperature of 0 to 4°C with the aim of the enzyme in an inactive condition. Digestive tract samples with distilled water and dried with suction paper. Samples were taken as much as 1 g and crushed with mortar until smooth and homogenized with 10 mL of cold distilled water, then centrifuged at 15,000 rpm for 20 minutes at 4ºC. The supernatant was taken as crude enzyme extract and used as samples for enzyme activity testing.

Cellulase enzyme activity was determined using the DNS method (3,5-dinitrosalicylic acid). A total of 80 µL of crude enzyme extract plus 720 µL of 1% CMC solution substrate (in citrate phosphate buffer pH 7) was put into the Eppendorf tube and then incubated a water bath at 37°C for 60 min. The incubation results were added with 1200 µL of DNS reagent then put in a boiling water bath for 15 min. After that, put in an ice bath for 20 min. In this test, control was used in the form of 80 µL crude extract of the enzyme, which had been heated for 15 min, then added 720 µL of the substrate and 1200 µL of DNS reagent. The solution mixture is treated the same as the sample conditions above. After that, the reducing sugar formed was measured by a UV-Vis spectrophotometer at λ = 540 nm (Miller, 1959).

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Measurement of protease activity was carried out based on the method of Bergmeyer and Grassi (1983). A total of 1 mL of 2% casein solution mixed with 1 mL of borate buffer (0.01 M) pH 8.0, 0.20 mL 0.05 M hydrochloric acid and 0.20 mL crude enzyme extract, which activity will be determined. Then incubated in a water bath at 37°C for 10 min, then added 2 mL of 0.1 M Trichloroacetic Acid (TCA). Incubated for 10 min, then centrifuged. The 1.5 mL portion of the filtrate was mixed with 5 mL of 0.4 M disodium carbonate and 1 mL of Folin Ciocalteu's reagent and let stand for 20 min. Then read the absorbance at a wavelength of 578 nm.

Determination of the amylase enzyme activity value was carried out by mixing 1 mL of the enzyme with 1% starch in 1 mL of 0.05 M citrate buffer pH 5.7. Then the incubation was carried out at 37°C for 30 min. The reaction was stopped by adding 2 mL of DNS (3.5 dinitro salicylic acid), then heated in boiling water for 5 min and, after chilling, centrifuged at 3,000 rpm for 5 min. Furthermore, reducing sugar can be measured using a spectrophotometer at a wavelength of 540 nm. One unit of enzyme activity is defined as the number of enzymes that produce 1 µmol of glucose/per minute

Analysis method for lipase enzyme activity (Tietz and Friedreck 1966; Borlongan, 1990), a stable lipase substrate (olive oil) 1.5 mL plus 1 mL Tris-HCl 0.1 M as a buffer with a pH of 8.0. Then add 1.0 mL of crude enzyme extract. The mixture was homogenized and incubated for 6 h at 37°C. The reaction is stopped by adding 3 mL of 95% ethyl alcohol. Titrate the sample with 0.01 N NaOH, using 0.9% (w/v) thymolphthalein in ethanol as an indicator. The same procedure is carried out on the blank. One lipase activity unit was defined as the volume of 0.05 N NaOH needed to neutralize the fatty acids released during 6 h of incubation with the substrate, after correction with blanks.

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Data were analyzed descriptively using mean and standard deviation.

Results

The measurement results for microbial population (cellulitis, amylolytic, proteolytic and lipolytic CFU mL^{-1}) and enzyme activity (cellulase, α-amylase, protease, dan lipase IU g^{-1} minute^{-1}) was shown in Table 1 and 2. The Paired-Samples T results showed significant differences in the microbial population and enzyme activity between the two groups of tested crabs. Table 1 showed that there was decreasing significantly of the microbial population (cellulitis, amylolytic, proteolytic and lipolytic) in mud crab digestive tract who give a feed with antibiotic than those of feed without antibiotic. This trend also occurs for the enzymatic activity of cellulose, α-amylase, protease and lipase, as shown in Table 2. The decreasing of cellulose, α-amylase, protease and lipase accounted for 89.55, 41.90, 26.50 and 37.26%, respectively.

The regression analysis showed that the correlation between the microbial population and enzyme activity was linear with the regression equation: y = 1E-10× + 10.424; R = 0.5252. Figure 1 showed that the increasing microbial population followed by increasing the enzyme activity.

Table 1: The microbial population of cellulitis, amylolytic, proteolytic and lipolytic (CFU mL^{-1}) in mud crab at the end of the experiment

		Microbial population (CFU mL^{-1})			
Treatment	Replicate	Cellulitis	Amylolytic	Proteolytic	Lipolytic
Feed without antibiotic	1	1.3×10^{11}	8.3×10^{10}	9.2×10^{10}	7.8×10^{10}
	2	9.6×10^{10}	8.7×10^{10}	9.8×10^{10}	5.5×10^{10}
	Mean	1.13×10^{11}	8.5×10^{10}	9.5×10^{10}	6.65×10^{10}
Feed with antibiotic	1	4.3×10^{3}	9.8×10^{4}	1.2×10^{5}	3.4×10^{4}
	2	3.9×10^{3}	6.8×10^{5}	4.2×10^{6}	8.2×10^{5}
	Mean	4.1×10^{3}	3.9×10^{5}	2.2×10^{6}	4.3×10^{5}

Table 2: Digestive enzyme activity of cellulase, α-amylase, protease and lipase (IU g^{-1} $minute^{-1}$) of mud crab at the end of the experiment

		Enzyme activity (IU g^{-1} $minute^{-1}$)			
Treatment	Replicate	Cellulose	α-Amylase	Protease	Lipase
Feed without antibiotic	1	24.360	18.550	32.580	15.360
	2	26.190	21.470	28.410	17.680
	Mean	25.275	20.010	30.495	16.520
Feed with antibiotic	1	4.700	13.650	25.610	9.600
	2	0.580	9.600	19.220	11.130
	Mean	2.640	11.625	22.415	10.365

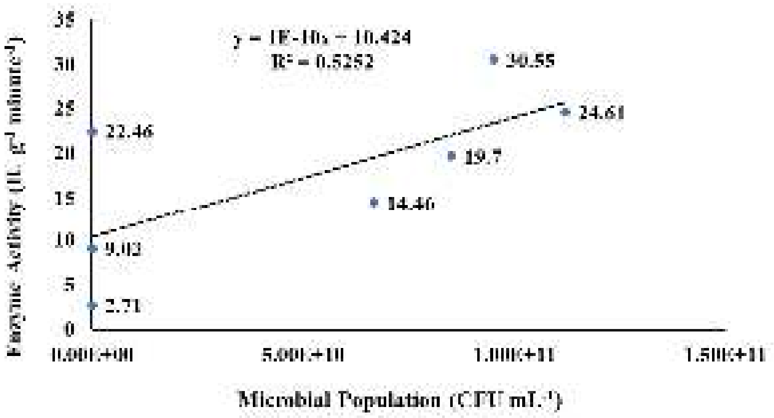

Fig. 1: The regression graph of microbial populations and enzyme activity in the digestive tract of mud crabs

Discussion

The investigation of the contribution of microflora in the digestive tract of mud crabs showed that cellulase enzymes in the digestive tract of mud crabs were thought to contribute to the microflora of the digestive tract. Our result showed that a high impact of the contribution of cellulase enzymes derived from cellulitis microbes accounting for 22.64 IU g^{-1} $minute^{-1}$ or 89.55%. The use of antibiotics to prove the role of microflora in the digestive tract of animals as reported by (Das and Tripathi, 1991; Xue *et al*., 1999). Tetracycline and penicillin are types of antibiotics that can kill gram-positive bacteria, while streptomycin is a type of antibiotic that can kill gram-negative bacteria. Das and Tripathi (1991) reported decreased cellulase activity when grass carp were fed with tetracyclines. *Cherac quadricarinatus,* which was fed with 100 IU mL^{-1} penicillin G. and 100 IU mL^{-1} streptomycin per kg of feed for eight days, showed a decrease in cellulase enzyme activity in the digestive tract by 40%, as well as a reduction of the bacterial population by 94% compared to controls (Xue *et al*., 1999). The presence of this cellulase enzyme enables the mud crab to digest feed fiber. According to (Allan and Fielder, 2003) digestibility of crabs on fiber and all vegetable feed raw materials is very high, ranging from 94.4-96.1%. Our result found a contribution of digestive enzymes α-amylase, protease and microbial lipase derived from microflora in the digestive tract of mud crabs. The contribution of amylolytic, proteolytic and lipolytic microbes to the enzymes of cellulase, α-amylase, protease and lipase in the digestive tract of mud crabs were 8.34, 8.08 and 6.16 IU g^{-1} $minute^{-1}$. This finding indicated that in addition to the digestive enzymes α-amylase, protease and endogenous lipase secreted by the digestive tract and glands, there were also digestive enzymes α-amylase, protease and exogenous lipase originating from the microflora in the digestive tract. The contribution of fish digestive tract microflora was also reported by (Aslamyah, 2006). Microbial in the digestive tract of carp in the omnivore phase was higher than in the carnivore phase. The contribution of the digestive enzymes protease, α-amylase and microbial lipase in carnivorous gourami were 25.21, 25.22 and 18.27%, respectively. However, it was lower than the contribution of the digestive enzymes protease, α-amylase and microbial lipase derived from microflora in the digestive tract of milkfish. In milkfish, the contribution of the digestive enzymes protease, α-amylase and microbial lipase were 36.12, 41.33 and 22.51% (Aslamyah, 2006). Lazado *et al*. (2012) reported GP21 (*Pseudomonas* sp.) And GP12 (*Psychrobacter* sp.), which was two bacteria isolated from the gastrointestinal tract of Atlantic cod, GP21 capable of producing amylase, chitinase, cellulase and protease, whereas GP12 can only produce chitinase and protease. Furthermore, GP21 and GP12, potential probiotic organisms that could support the digestion of Atlantic cod.

The microflora in the digestive tract was originated from detritus consumed by mud crabs. In general, the types of microbes found in the digestive tract of mud crabs were from cultivation media and or form pond sediments. Al-Harbi and Uddin (2005) stated a positive correlation between the composition of bacteria in the gills and digestive tract of fish with the composition of bacteria in water and sediment pond.

Detritus contains many microorganisms that play a role in contributing exogenous digestive enzymes to degrade feed nutrients consumed by mud crabs. These microorganisms are also a source of additional nutrients for mud crabs. This finding was in line with a previous study by (Xue *et al*., 1999), who found that the growth of several species of freshwater *Cherac quadricarinatus* reared in soil ponds was better than those reared in tank ponds. This difference was interpreted as *Cherac quadricarinatus* had an ability to obtain additional nutrients from the detritus material at the bottom of the pond, which is not found in the tank pond and/or utilized the presented microorganisms in detritus to support digestif activities.

The microflora was in the digestive tract lives in mutualism symbiosis with the host and is in balance, namely between beneficial microbes and pathogenic microbes. These microfloras also interact with each other between various microbial species in the digestive tract, both antagonistically and synergistically. The interactions that occurred are very important in maintaining the balance of the digestive tract microflora. It will have a positive effect on the host. Douillet and Langdon (1994) reported that on oyster cultivation, there was a high growth rate which was associated with the contribution of bacteria supplying (1) essential nutrients which were not found in some individuals in the algae population and (2) enzymes that could improve the digestive process of larvae. The microflora in the intestines of bivalve larvae was obtained in optimal proportions to produce extracellular enzymes such as proteases and lipases

The estimation of a relationship between the habit of water animals eating detritus and the presence of microflora in the digestive tract was also reported by (Xue *et al*., 1999). Liu *et al*. (2016) reported that the trophic level of the host affected the structure and composition of the intestinal microbiota, metabolic capacity and enzyme activity of the intestinal content. *Clostridium, Citrobacter and Leptotrichia* cellulose-degrading bacteria were dominant in herbivores, while *Cetobacterium* and *Halomonas* protease-producing bacteria were dominant in carnivores. In addition, cellulase and amylase activity in herbivorous fish was significantly higher than in carnivorous fish, whereas trypsin activity in carnivorous fish was much higher than in herbivorous fish.

Conclusion

Cellulase, amylolytic, proteolytic and lipolytic microbes in the digestive tract of mud crabs play an important role in the physiological function of the digestive tract, namely contributing to the enzymes cellulase, α-amylase, protease, dan lipase (IU g^{-1} $minute^{-1}$) was shown 55, 41.90, 26.50 and 37.26%, respectively. Furthermore, the role of the gastrointestinal microflora in contributing to cellulase enzymes and exogenous digestive enzymes amylase, protease and lipase can be demonstrated. The microflora of the digestive tract is thought to have originated from the detritus eaten by mud crabs.

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Conflict of Interest

Author Siti Aslamyah, Yushinta Fujaya dan Nita Rukminasari have received a research grant from Universitas Hasanuddin. The authors declare that they have no conflict of interest.

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Author's Contributions

Siti Aslamyah: Designed an experiment method, conducted experiment, data analysis and interpretation, writing a draft manuscript, submitting a manuscript.

Yushinta Fujaya: Designed an experiment method, data interpretation, final editing and proofreading of manuscript before submitting.

Nita Rukminasari: Conducted data analysis and interpretation, writing a draft manuscript, submitting a manuscript.

Ethics

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

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OnLine Journal of Biological Sciences

Original Research Paper

Microflora Contribution to Cellulase and Digestion Enzyme in Mud Crab Digestive Tract

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Abstract: Microflora in the digestive tract of aquatic animals is thought to be not only the microbes that contribute to the production of cellulase enzymes but also contribute to other digestive enzymes, such as protease, amylase and lipase. This study aims to examine the microflora contribution to the mud crab's digestive tract. Scylla sp. were maintained in the crab box individually and placed in a pond. Two groups of crabs were fed without antibiotics and containing antibiotics at a dose of 100 IU mL^{-1} penicillin G and 100 IU mL^{-1} streptomycin, respectively. Each treatment had two replications. Crabs were kept for eight days and fed 5% of body weight per day in the afternoon. The results showed that the cellulitis, amylolytic, proteolytic and lipolytic microbial population in the digestive tract of mud crabs that received feed added with antibiotics decreased significantly compared to those feed without antibiotics. Furthermore, the α-amylase, protease and lipase enzyme activity also decreased. The decrease in cellulase activity, α-amylase, protease and lipase enzymes were 89.55, 41.90, 26.50 and 37.26%, respectively. The decreasing percentage of cellulase, α-amylase, protease and lipase enzymes indicated a significant microflora contribution in the mud crab digestive tract.

Keywords: Mud Crab, Microflora, Cellulitis, Proteolytic, Amylolytic, Lipolytic

Introduction

The indigenous microflora of fish in aquaculture has previously been studied for many purposes, including descriptions of microbial spoilage, the correlation between environment and fish microflora (Horsley, 1973), the monitoring of changes in fish farms (Allen *et al*., 1983), the nutritional role of the intestinal flora (Hansen *et al*., 1992) and the antibiotic resistance profile of the indigenous flora (Spanggaard *et al*., 2000).

It is generally recognized that the intestinal flora of endothermic animals serves both as a digestive function and as a protective barrier against disease (Sissons, 1989). The intestinal flora of fish has, as a consequence, received much attention from several authors (Ringø *et al*., 1995; Sugita *et al*., 1997). The composition of the intestinal microflora in fish is influenced by, or directly derived from, the microflora of the food (Grisez *et al*., 1997). Fish with abundant and diverse microflora have considerable opportunities to adapt to changing nutritional substrates, assimilate food better and enhance their adaptive possibilities (Al-Harbi and Uddin, 2005).

The main component in animal feed is protein, carbohydrate and fat. To increase absorption and circulation throughout the body through the circulatory system required a simplification process into smaller molecules. According to (Bakke *et al*., 2010), a chemical feed simplification process is also called hydrolysis, involving digestive enzymes as biological catalysts. The main feed components in protein, fat and carbohydrates are broken down into simple compounds, which are the constituent components. Steinberg (2017) explained that the hydrolysis of macronutrients into micronutrients in the digestive system was occurred due to the presence of digestive enzymes, namely protease, amylase, carbohydrase, lipase and stomach acid. Those compounds are produced by the stomach, intestines, liver and pancreas. In addition, several researchers reported that there were cellulase enzyme activities in several species of aquatic animals. The presence of the cellulase enzyme is closely related to the microflora in the digestive tract (Bui and Lee, 2015; Xue *et al*., 1999). The discovery of the enzyme cellulase in the digestive tract of aquatic animals allows these animals to digest feed containing fiber.

Science Publications

Previous studies showed that in addition to endogenous digestive enzymes, exogenous digestive enzymes are also found from microflora that lives in mutualism symbiosis with aquatic animals in their digestive tract (Das *et al*., 2014; Ganguly and Prasad, 2012; Pond *et al*., 2006; Xue *et al*., 1999). Microflora in the digestive tract of aquatic animals is thought to be the microbes that contribute to the production of cellulase enzymes and contribute to other digestive enzymes, such as protease, amylase and lipase. In fish, the intestinal microflora has been regarded as fulfilling several roles. A nutritional function has been suggested, in which bacteria break down ingested foods into individual components such as vitamins or amino acids (Pond *et al*., 2006). However, there is a lack of study conducted related to microflora activities in the mud crab digestive tract. This experiment was carried out with reference to the experiments that had been conducted by previous researchers. The aim of study was to examine the microflora contribution in the mud crab's digestive tract., namely in contributing of cellulase enzymes and digestive enzymes including α-amylase, protease and exogenous lipase.

Materials and Methods

The experiment was conducted at Education Pond, Universitas Hasanuddin, Barru Regency, on May-June 2020. We only conducted the experiment for two months due to limited time and other sources of research, including funds and labor. Crab feed making, analysis for microbial population and enzyme activity were conducted at Biotechnology Fisheries and Marine Laboratory, Center for Research Activities, Universitas Hasanuddin.

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The experiment method was referred to (Xue *et al*., 1999; Aslamyah, 2006). Crab samples were divided into two groups, namely crab fed with feed with antibiotic penicillin G with the dose of 100 and 100 IU mL^{-1} streptomycin kg^{-1} feed and crab samples fed with feed without antibiotic. The crab was fed twice a day. The crab was maintained in the crab box for eight days and fed as an amount of 5% of total body weight. We used pellet form for crab feed, with the nutritional contents of the feed, namely protein 41.93%, Nitrogen Free Extract/NFE 29,33%, fiber 7.82%, fat 7.43% and Digestible Energy/DE 2767.63 kcal/kg.

Parameters that were measured in this study were microbial population, cellulose enzyme and digestive enzyme activity that were conducted at the end of the experiment.

Measurement Microbial Population

The method for measuring the microbial population of cellulitis, amylolytic, proteolytic and lipolytic referred to (Aslamyah, 2006) as the detailed method: Measurement of the microbial population begins with preparing the source of the inoculum from the digestive tract of the crabs. Digestive tract samples were crushed and every 10 g of the sample was diluted with 90 mL of sterile physiological solution (0.85% NaCl). The inoculum source of 0.5 mL was inoculated into 10 mL of standard liquid media, namely Trypticase Soy Broth (TSB, Merck), which added 1% NaCl and cellulose for cellulitis, casein as an energy source for proteolytic, TSB plus 1% NaCl and starch for amylolytic and TSB plus 1% NaCl and fish oil for lipolytic. The culture was then incubated at 29°C for 24 h. Serial dilution is carried out from 10^{-2} to 10^{-10} by taking 0.05 mL from the microbial culture in liquid media and putting it in 4.95 mL of the first diluent medium, then 0.05 mL of the first diluent medium is taken and put into in 4.95 mL of second diluent medium and so on until the last diluting medium. Each dilution series is transferred as much as 0.1 mL into solid media, which consists of a mixture of TSB, 1% NaCl, agar and its energy source. This preparation was incubated again at 29°C for 24 to 48 h. The microbial colonies that grew were counted in colony count (CFU/mL).

Measurement Enzyme Activity

Analysis for cellulase enzyme and digestive enzyme (α-amylase, protease and lipase) activity begins with preparing a crude enzyme extract which refers to (Aslamyah, 2006). Activities carried out at a temperature of 0 to 4°C with the aim of the enzyme in an inactive condition. Digestive tract samples with distilled water and dried with suction paper. Samples were taken as much as 1 g and crushed with mortar until smooth and homogenized with 10 mL of cold distilled water, then centrifuged at 15,000 rpm for 20 min at 4°C. The supernatant was taken as crude enzyme extract and used as samples for enzyme activity testing.

Cellulase enzyme activity was determined using the DNS method (3,5-dinitrosalicylic acid). A total of 80 µL of crude enzyme extract plus 720 µL of 1% CMC solution substrate (in citrate phosphate buffer pH 7) was put into the Eppendorf tube and then incubated a water bath at 37°C for 60 min. The incubation results were added with 1200 µL of DNS reagent then put in a boiling water bath for 15 min. After that, put in an ice bath for 20 min. In this test, control was used in the form of 80 µL crude extract of the enzyme, which had been heated for 15 min, then added 720 µL of the substrate and 1200 µL of DNS reagent. The solution mixture is treated the same as the sample conditions above. After that, the reducing sugar formed was measured by a UV-Vis spectrophotometer at $\lambda = 540$ nm (Miller, 1959).

Measurement of protease activity was carried out based on the method of Bergmeyer and Grassi (1983). A total of 1 mL of 2% casein solution mixed with 1 mL of borate buffer (0.01 M) pH 8.0, 0.20 mL 0.05 M hydrochloric acid and 0.20 mL crude enzyme extract, which activity will be determined. Then incubated in a water bath at 37°C for 10 min, then added 2 mL of 0.1 M Trichloroacetic Acid (TCA). Incubated for 10 min, then centrifuged. The 1.5 mL portion of the filtrate was mixed with 5 mL of 0.4 M disodium carbonate and 1 mL of Folin Ciocalteu's reagent and let stand for 20 min. Then read the absorbance at a wavelength of 578 nm.

Determination of the amylase enzyme activity value was carried out by mixing 1 mL of the enzyme with 1% starch in 1 mL of 0.05 M citrate buffer pH 5.7. Then the incubation was carried out at 37°C for 30 min. The reaction was stopped by adding 2 mL of DNS (3.5 dinitro salicylic acid), then heated in boiling water for 5 min and, after chilling, centrifuged at 3,000 rpm for 5 min. Furthermore, reducing sugar can be measured using a spectrophotometer at a wavelength of 540 nm. One unit of enzyme activity is defined as the number of enzymes that produce 1 µmol of glucose/per minute

Analysis method for lipase enzyme activity (Tietz and Friedreck 1966; Borlongan, 1990), a stable lipase substrate (olive oil) 1.5 mL plus 1 mL Tris-HCl 0.1 M as a buffer with a pH of 8.0. Then add 1.0 mL of crude enzyme extract. The mixture was homogenized and incubated for 6 h at 37°C. The reaction is stopped by adding 3 mL of 95% ethyl alcohol. Titrate the sample with 0.01 N NaOH, using 0.9% (w/v) thymolphthalein in ethanol as an indicator. The same procedure is carried out on the blank. One lipase activity unit was defined as the volume of 0.05 N NaOH needed to neutralize the fatty acids released during 6 h of incubation with the substrate, after correction with blanks.

Data were analyzed descriptively using mean and standard deviation.

Results

The measurement results for microbial population (cellulitis, amylolytic, proteolytic and lipolytic CFU mL^{-1}) and enzyme activity (cellulase, α-amylase, protease, dan lipase IU g^{-1} $minute^{-1}$) was shown in Table 1 and 2. The Paired-Samples T results showed significant differences in the microbial population and enzyme activity between the two groups of tested crabs. Table 1 showed that there was decreasing significantly of the microbial population (cellulitis, amylolytic, proteolytic and lipolytic) in mud crab digestive tract who give a feed with antibiotic than those of feed without antibiotic. This trend also occurs for the enzymatic activity of cellulose, α-amylase, protease and lipase, as shown in Table 2. The decreasing of cellulose, α-amylase, protease and lipase accounted for 89.55, 41.90, 26.50 and 37.26%, respectively.

The regression analysis showed that the correlation between the microbial population and enzyme activity was linear with the regression equation: y = 1E-10× +10.424; R = 0.5252. Figure 1 showed that the increasing microbial population followed by increasing the enzyme activity.

Table 1: The microbial population of cellulitis, amylolytic, proteolytic and lipolytic (CFU mL^{-1}) in mud crab at the end of the experiment

		Microbial population (CFU mL^{-1})			
Treatment	Replicate	Cellulitis	Amylolytic	Proteolytic	Lipolytic
Feed without antibiotic	1	1.3×10^{11}	8.3×10^{10}	9.2×10^{10}	7.8×10^{10}
	2	9.6×10^{10}	8.7×10^{10}	9.8×10^{10}	5.5×10^{10}
	Mean	1.13×10^{11}	8.5×10^{10}	9.5×10^{10}	6.65×10^{10}
Feed with antibiotic	1	4.3×10^{3}	9.8×10^{4}	1.2×10^{5}	3.4×10^{4}
	2	3.9×10^{3}	6.8×10^{5}	4.2×10^{6}	8.2×10^{5}
	Mean	4.1×10^{3}	3.9×10^{5}	2.2×10^{6}	4.3×10^{5}

Table 2: Digestive enzyme activity of cellulase, α-amylase, protease and lipase (IU g^{-1} $minute^{-1}$) of mud crab at the end of the experiment

		Enzyme activity (IU g^{-1} $minute^{-1}$)			
Treatment	Replicate	Cellulose	α-Amylase	Protease	Lipase
Feed without antibiotic	1	24.360	18.550	32.580	15.360
	2	26.190	21.470	28.410	17.680
	Mean	25.275	20.010	30.495	16.520
Feed with antibiotic	1	4.700	13.650	25.610	9.600
	2	0.580	9.600	19.220	11.130
	Mean	2.640	11.625	22.415	10.365

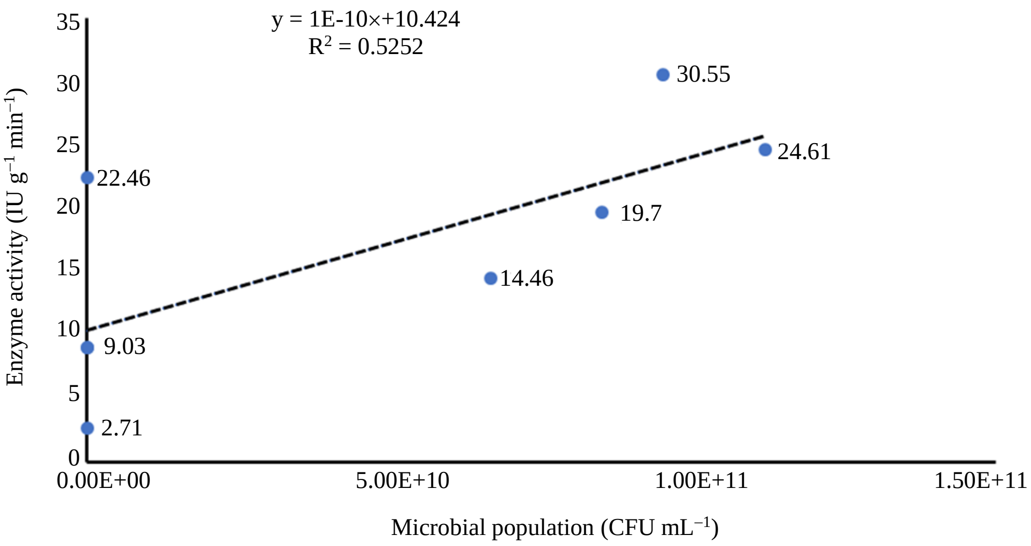

Fig. 1: The regression graph of microbial populations and enzyme activity in the digestive tract of mud crabs

Discussion

The investigation of the contribution of microflora in the digestive tract of mud crabs showed that cellulase enzymes in the digestive tract of mud crabs were thought to contribute to the microflora of the digestive tract. Our result showed that a high impact of the contribution of cellulase enzymes derived from cellulitis microbes accounting for 22.64 IU g^{-1} $minute^{-1}$ or 89.55%. The use of antibiotics to prove the role of microflora in the digestive tract of animals as reported by (Das and Tripathi, 1991; Xue *et al.*, 1999). Tetracycline and penicillin are types of antibiotics that can kill gram-positive bacteria, while streptomycin is a type of antibiotic that can kill gram-negative bacteria. Das and Tripathi (1991) reported decreased cellulase activity when grass carp were fed with tetracyclines. *Cherac quadricarinatus*, which was fed with 100 IU mL^{-1} penicillin G. and 100 IU mL^{-1} streptomycin per kg of feed for eight days, showed a decrease in cellulase enzyme activity in the digestive tract by 40%, as well as a reduction of the bacterial population by 94% compared to controls (Xue *et al.*, 1999). The presence of this cellulase enzyme enables the mud crab to digest feed fiber. According to (Allan and Fielder, 2003) digestibility of crabs on fiber and all vegetable feed raw materials is very high, ranging from 94.4-96.1%. Our result found a contribution of digestive enzymes α-amylase, protease and microbial lipase derived from microflora in the digestive tract of mud crabs. The contribution of amylolytic, proteolytic and lipolytic microbes to the enzymes of cellulase, α-amylase, protease and lipase in the digestive tract of mud crabs were 8.34, 8.08 and 6.16 IU g□1 minute□1. This finding indicated that in addition to the digestive enzymes α-amylase, protease and endogenous lipase secreted by the digestive tract and glands, there were also digestive enzymes α-amylase, protease and exogenous lipase originating from the microflora in the digestive tract. The contribution of fish digestive tract microflora was also reported by (Aslamyah, 2006). Microbial in the digestive tract of carp in the omnivore phase was higher than in the carnivore phase. The contribution of the digestive enzymes protease, α-amylase and microbial lipase in carnivorous gourami were 25.21, 25.22 and 18.27%, respectively. However, it was lower than the contribution of the digestive enzymes protease, α-amylase and microbial lipase derived from microflora in the digestive tract of milkfish. In milkfish, the contribution of the digestive enzymes protease, α-amylase and microbial lipase were 36.12, 41.33 and 22.51% (Aslamyah, 2006). Lazado *et al.* (2012) reported GP21 (*Pseudomonas* sp.) And GP12 (*Psychrobacter* sp.), which was two bacteria isolated from the gastrointestinal tract of Atlantic cod, GP21 capable of producing amylase, chitinase, cellulase and protease, whereas GP12 can only produce chitinase and protease. Furthermore, GP21 and GP12, potential probiotic organisms that could support the digestion of Atlantic cod.

The microflora in the digestive tract was originated from detritus consumed by mud crabs. In general, the types of microbes found in the digestive tract of mud crabs were from cultivation media and or form pond sediments. Al-Harbi and Uddin (2005) stated a positive correlation between the composition of bacteria in the gills and digestive tract of fish with the composition of bacteria in water and sediment pond.

Detritus contains many microorganisms that play a role in contributing exogenous digestive enzymes to degrade feed nutrients consumed by mud crabs. These microorganisms are also a source of additional nutrients for mud crabs. This finding was in line with a previous study by (Xue *et al*., 1999), who found that the growth of several species of freshwater *Cherac quadricarinatus* reared in soil ponds was better than those reared in tank ponds. This difference was interpreted as *Cherac quadricarinatus* had an ability to obtain additional nutrients from the detritus material at the bottom of the pond, which is not found in the tank pond and/or utilized the presented microorganisms in detritus to support digestif activities.

The microflora was in the digestive tract lives in mutualism symbiosis with the host and is in balance, namely between beneficial microbes and pathogenic microbes. These microfloras also interact with each other between various microbial species in the digestive tract, both antagonistically and synergistically. The interactions that occurred are very important in maintaining the balance of the digestive tract microflora. It will have a positive effect on the host. Douillet and Langdon (1994) reported that on oyster cultivation, there was a high growth rate which was associated with the contribution of bacteria supplying (1) essential nutrients which were not found in some individuals in the algae population and (2) enzymes that could improve the digestive process of larvae. The microflora in the intestines of bivalve larvae was obtained in optimal proportions to produce extracellular enzymes such as proteases and lipases

The estimation of a relationship between the habit of water animals eating detritus and the presence of microflora in the digestive tract was also reported by (Xue *et al*., 1999). Liu *et al*. (2016) reported that the trophic level of the host affected the structure and composition of the intestinal microbiota, metabolic capacity and enzyme activity of the intestinal content. *Clostridium*, *Citrobacter* and *Leptotrichia* cellulose-degrading bacteria were dominant in herbivores, while *Cetobacterium* and *Halomonas* protease-producing bacteria were dominant in carnivores. In addition, cellulase and amylase activity in herbivorous fish was significantly higher than in carnivorous fish, whereas trypsin activity in carnivorous fish was much higher than in herbivorous fish.

Conclusion

Cellulase, amylolytic, proteolytic and lipolytic microbes in the digestive tract of mud crabs play an important role in the physiological function of the digestive tract, namely contributing to the enzymes cellulase, α-amylase, protease, dan lipase (IU g^{-1} $minute^{-1}$) was shown 55, 41.90, 26.50 and 37.26%, respectively. Furthermore, the role of the gastrointestinal microflora in contributing to cellulase enzymes and exogenous digestive enzymes amylase, protease and lipase can be demonstrated. The microflora of the digestive tract is thought to have originated from the detritus eaten by mud crabs.

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Author’s Contributions

Siti Aslamyah: Designed an experiment method, conducted experiment, data analysis and interpretation, writing a draft manuscript, submitting a manuscript.

Yushinta Fujaya: Designed an experiment method, data interpretation, final editing and proofreading of manuscript before submitting.

Nita Rukminasari: Conducted data analysis and interpretation, writing a draft manuscript, submitting a manuscript.

Conflict of Interest

Author Siti Aslamyah, Yushinta Fujaya dan Nita Rukminasari have received a research grant from Universitas Hasanuddin. The authors declare that they have no conflict of interest.

Ethics

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

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