

The Role of Agarolytic Bacteria in Enhancing Physiological Function for Digestive System of Abalone (*Haliotis asinina*)

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ABSTRACT

Physiological function of digestive system for herbivorous animal depended on polysaccharolytic microbial activities in the gastrointestinal tract. The aims of this research was to elucidate the role of agarolytic bacteria, *Vibrio* sp. strain Alg3.1-Abn1.2, as exogenous agarase producer to improve nutritional status of abalone. Agar content in feed and feses of abalone was determined by gravimetric. Bomb calorimetric analysis was used to evaluate gross energy status of abalone feed and feses. Dinitrosalicylic acid used to measure *in situ* agarase activity and viable cell count was used to determine whether the probiotics Alg3.1 and Abn1.2 can colonize the gastrointestinal tract of *H. asinina*. Growth performance of gnotobiotic and normal *H. asinina* were evaluated and compared with abalone fed cake-*Gracilaria* diet supplemented with mix culture Alg3.1-Abn1.2 strains. The result of this research showed agar and gross energy content in the feses of fed probiotic abalone was lower. The number of culturable cell reisolated from *H. asinina* fed probiotic-supplemented cake for 14 days ranged from 10^6 - 10^7 cfu/g material. Agarase activity in the *H. asinina* digestive tract was significantly higher ($P < 0.05$) in *H. asinina* fed probiotic-supplemented cake compared to *H. asinina* fed unsupplemented cake. *H. asinina* fed a diet supplemented with mix culture Alg3.1-Abn1.2 strains exhibited a significantly increased ($P < 0.05$) growth rate compared to *H. asinina* fed standard diet under laboratory conditions. The growth rate of antibiotic-treated *H. asinina* was extremely poor in comparison to *H. asinina* that had not treated with antibiotics when fed an unsupplemented diet, reflecting the importance of gastrointestinal microflora in *H. asinina* growth.

KEYWORD: abalone growth, agarase activity, agarolytic bacteria, probiotic

INTRODUCTION

Abalone (*Haliotis asinina*) was a seaweed-eating marine herbivores from the red algae (rhodophyta), green (Chlorophyta) or chocolate (phaeophyta). To digest seaweed, abalone had enzymes to degrade the cell wall in digestive tract [1] to be able to use agar-agar, alginate, carrageenan as a source of energy [2].

The ability of abalone to digest seaweed was also aided by exogenous polysaccharase enzymes contributed by enteric bacteria in the digestive tract of abalone. Group of enteric bacteria played an important role in the provision of nutrition abalone with hydrolysing complex polysaccharides into simple molecules that absorbed by the abalone. 70-90% of the bacteria activity produced enzymes polysaccharolytic extracellularly secreted into the digestive tract of abalone [2]. Catabolism of monosaccharides by enteric bacteria produce large amounts of acetic acid and a format that can be used as a source of energy or the synthesis of amino acid precursors by abalone [3][4].

In his review [5] mentioned that some groups of marine bacteria produced extracellular agarase enzymes that degraded agar into agarooligosaccharide and galactose. The introduction of these bacteria in the abalone *H. asinina* expected to increase the 'pool' digestive enzymes and improved the digestibility of agar which was a major component of abalone feed.

Studies on the application of probiotic bacterial enzyme provider polysaccharase on cultured abalone had reported. [6] Alginolytic used in bacterium *Pseudoalteromonas* sp. C4 lines on preservation *H. midae* gave natural feed appears to significantly increase the speed of growth and alginate lyase enzyme activity *in situ* in the GI tract compared to control abalone. While the application of bacteria and yeasts that selected stomach on cultured abalone *H. midae* increased to 34% growth when compared with controls [7].

In this paper, we examined the role of bacterial agarase agarolytic as a provider of exogenous enzymes that helped the digestive system abalone degraded agar into simpler compounds ready absorption and its potential as probiotics for abalone *H. asinina* growth.

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MATERIALS AND METHODS

Preparation of Bacterial Culture

Bacterial isolates used rifampicin resistant mutant strains Alg3.1Rf^R and Abn1.2Rf^R in the form of mixed cultures. Bacterial isolates had grown on basal salt medium B (medium B plus called, contains: NaNO₃, 2; K₂HPO₄, 0.5; MgSO₄·7H₂O, 0.2; CaCl₂·2H₂O, 0.02; MnSO₄·H₂O, 0.02; FeSO₄·7H₂O, 0.02; yeast extract, 0.5; peptone 0.2; agarose, 2) and incubated at 29 ° C while shaken 120 rounds per minute. Furthermore, the final concentration adjusted to 10⁸ cfu/ml.

Feed Preparing

The feed made in the form of pudding and added fish flour, fish oil and soy flour. Seaweed derived from the production of Marine Aquaculture Center of Lombok. As for feed preparation procedure followed the procedure [6] with modifications in the form of *Gracilaria* by 50% (w / v) and mixed with agar-agar (2.5% w / v: 2% agar and 0.5% bactoagar swallow Merck). The mixture sterilized by autoclave 121 °C for 15 minutes. After a rather cold, some of the feed added: (A) probiotics Alg3.1Rf^R -Abn1.2Rf^R with concentration 10⁸ cfu/ml, (B) gave antibiotics rifampicin (100 µg/ml), (C) probiotics Alg3.1Rf^R -Abn1.2Rf^R with concentration 10⁸ cfu/ml and antibiotics rifampicin (100 µg/ml), and (D) did not give probiotics or antibiotics (called standar feed).

Animal test and gnotobiotic abalone preparation

Abalone used in this study came from the production of abalone hatchery and Mariculture Center Lombok. The animal preparation procedure as follows: abalone maintained at 26-28 ° C in polyethylene tanks contained 98 L seawater filtered and aerated continuously. Abalone acclimatized for 2 weeks before being used for research, during this process only abalone fed *Gracilaria* pudding.

Gnotobiotik abalone was not contained good bacteria in the digestive tract and the outer body. Making abalone gnotobiotik performed as follows: prior to the experiment done all equipment disinfected with a solution of 50-100 ppm chlorine. as much as 6 aquarium (10 liters) used as a container maintenance, and flowed sea water and air (filter stone) at a constant rate (400 ml / min). The equipment used for the collection of samples sterilized with 70% ethanol. Into each aquarium filled with 12 heads of abalone. Then abalone were give antibiotic treatment to reduce enteric bacteria in the digestive tract by means of abalone fasted for 2 days and then were give feed containing antibiotics for 4 days. After the first 2 days of 24-hour water flow stopped and put into the aquarium antibiotics (ampicillin, 500 mg / L, rifampicin 125 mg / L, and chloramphenicol, 250 mg / l). The next day added back on antibiotics for 24 hours and still given feed containing antibiotics (rifampicin 100 mg / mL). After 24 hours of treatment, the aquarium water replaced with new water.

Agar concentration determination in feed and feces

The calculation of the levels of the agar in abalone feed and feces was performed according to Indonesian National Standard (SNI 01-4497-1998), as follows: A total of 10 grams tallus pieces of dried *Gracilaria* washed with distilled water and then drained. Sample then inserted into the round bottom flask, added 100 ml of NaOH solution (2-6%). Flask heated for 2 hours at a temperature of 90oC. Examples filtered and washed again with distilled water and then added a few drops of 0.1 M HCl to neutralize the excess base (to pH 7). Example transferred to the "pressure cooker" which contains 500 ml of H₂O and extracted for 2 hours at a temperature of 100 oC. Done extraction, filtering had done immediately in hot conditions and the filtrate collected in a stainless container and immediately frozen in a refrigerator. The gel heated at 60 ° C for 2 hours, then weighed.

Gross energy

Gross energy (EB) was the amount of heat that released when a foodstuff was totally oxidized in 'Bomb Calorimeter 25-30 atmosphere containing oxygen [8].

Abalone Growth Test

This experiment followed the procedure used by [6] modified. Prior to the experiment performed all equipment disinfected with a solution of 50-100 ppm chlorine. Furthermore, as many as 12 aquarium 10-liter used as a container maintenance and seawater flowed at a constant rate of 400 ml / min. Twelfth aquaria were divided into 2 groups: group A (using abalone gnotobiotik) consisted of 2 treatments, namely K0 = given feed containing the antibiotic rifampicin 100 mg / L (called Gnotobiotik), and treatment K1 = given feed containing 5% probiotic Alg.1Rf^R - Abn1.2Rf^R with concentration of 10⁸ cfu/ml + antibiotic rifampicin 100 mg/L (called GnotobiotikPlus); group B (using normal abalone), consisted of 2 treatment, ie K2 = given feed containing 5% probiotic Alg3.1Rf^R - Abn1.2Rf^R with concentration of 10⁸ cfu/ml (called Normalplus) and K3 = treatment were fed a standard that did not contain antibiotics and probiotics (called Normal). Each treatment consisted of 3 aquarium containing 10 abalone, approximately 25-36 mm.

To maintain water quality, aquarium cleaned once every 2 days with menyiphon unconsumed feed and faecal abalone with tool surface sterilized with 70% ethanol. Abalone fed as much as 30% of body weight every 2 days. Probiotic treatment had done once every 2 days during the first 14 days of maintenance and further provided

each week. Abalone feces and residual feed weighed, the length being measured and the weight of abalone shells were weighed after 2 months of maintenance.

In situ agarase activity

Maintenance and gastrointestinal surgery

Experimental design used in this activity similar to that applied to the growth of the test, which consisted of 4 treatments and 3 replications (K0 = gnotobiotic, K1 = gnotobioticplus, K2 = normalplus, dan K3 = normal) and abalone maintained for 14 days. Furthermore, as many as 4 tails abalone dissected for each tub, the digestive tract is taken, weighed and homogenized in buffer 0.2 M citric acid / 0.2 M phosphate, pH 5.4 at a volume of 2 ml / g tissue. Then the sample centrifuged at 6000 rpm for 15 minutes to remove food debris and cellular debris, and the supernatant sample dialyzed for 48 hours using a solvent buffer 0.2 M citric acid / 0.2 M phosphate, pH 5.4 at 4° C with 2 times the replacement buffer.

Determination of agarase activity

The supernatant contained the crude extract enzyme agarase activity tested using the DNS method (Miller 1959) as follows: as much as 5 ml (0.2%, w/v) agarose in 20 mM sodium phosphate (pH 7.5) heated at 100 °C for 2 min and then cooled at a temperature of 30 °C. The reaction carried out by mixing 0.5 ml of the specimen with 0.5 ml enzyme substrate for 30 minutes at a temperature of 29 °C. Then added 1.0 ml of DNS and heated in boiling water in a water-bath for 10-15 minutes, then cooled in cold water for 10 minutes. Strength reduction agarase monitored with a spectrophotometer at a wavelength of 540 nm. One unit of agarase activity defined as the amount of enzyme that produces 1 mol galactose per second at 29 °C under the conditions described above (Dybkaer et al. 2001).

Enumeration of total bacteria and probiotics

The total number of bacteria, probiotic bacteria in the aquarium and the digestive tract of abalone calculated using the spread plate method (TPC) that had grown on media Marine Agar, and for probiotics bacterial count used MA added by antibiotics rifampicin 100 µg/ml.

Data analysis

Variable growth of juvenile abalone observed includes a weighted average initial and final (g), the initial and final shell length (mm), and feed intake (g). Based on these data calculated biomass growth (g), the relative growth (%), feed efficiency (%) and feed conversion.

RESULTS AND DISCUSSION

Data in Table 1 showed that the percentage of digestible agar and digestible energy in the agarolytic bacteria Alg3.1Rf^R-Abn1.2Rf^R-supplemented abalone higher when compared with the abalone consumed only standard *Gracilaria* cake.

Table 1 indicated that there had an increase in the availability of nutrients in the gastrointestinal tract. Agarase bacteria on the cake *Gracilaria*-containing Alg3.1Rf^R Abn1.2Rf^R hydrolyzed agar on the cake, consequently more energy was available, in particular agar degradation products, so it was more easily absorbed by the digestive tract of abalone.

Agarase Activity In Situ

To determine whether the Alg3.1Rf^R-Abn1.2Rf^R strain contributed to the 'pool' of digestive enzymes in the digestive system of abalone, then tested agarase activity in situ in the abalone fed the standard *Gracilaria* cake compared to abalone consuming cake containing Alg3.1Rf^R-Abn1.2Rf^R strain (Figure 1).

Adding Alg3.1Rf^R-Abn1.2Rf^R in feed affected the activity of agarase enzymes in the digestive tract of normal abalone or gnotobiotik abalone (Figure 1). Agarase activity gnotobiotik abalone supplemented probiotics (gnotobiotikplus) 47.4% higher than gnotobiotik, whereas agarase enzyme activity in the normal abalone supplemented probiotics (normalplus) 42.3% higher than normal abalone. These data demonstrated that the production of the enzyme agarase apart from abalone also came from bacteria in the GI tract contribution abalone.

Abalone were consume probiotic-supplemented cake *Gracilaria* that increases agarase enzyme activity in the gastrointestinal tract when compared to controls, and this suggested that the strain-Abn1.2RfR Alg3.1RfR contributed to the availability of 'pool' polisakarolitik enzymes in the digestive tract to digest complex polysaccharides had eaten abalone .

Number of Total Bacteria and Probiotics

Therefore agarase enzyme activity in the digestive tract of abalone affected by the levels of exogenous agarase enzyme produced by microbes then measured the total number of bacteria and the number of good

probiotic bacteria in the gastrointestinal tract as well as the maintenance of water abalone. The results of the calculation of microbial populations in water and digestive tract had presented in Table 2.

The total number of bacteria and probiotic bacteria (agarolytic) in the gastrointestinal tract was higher than bacteria in water conservancy. This proven that mixed cultures Alg3.1Rf^R-Abn1.2Rf^R capable well colonizes in the gastrointestinal tract of abalone and helped host by breaking complex agar into simpler molecules such as agarooligosaccharides and galactose. Data in table 2 also showed that there was a natural agarolytic bacteria in the digestive tract abalone, and suspected help host in breaking agar in the seaweed cell wall.

The data in Figure 2 informed the ratio of the number of bacteria agarolytic against the total number of bacteria in the water and abalone digestive tract. Agarolytic very high ratio of bacteria in the treatment gnotobiotik added Alg3.1Rf^R-Abn1.2Rf^R with the percentage of 93.24%, whereas the normal abalone fed Alg3.1Rf^R-Abn1.2Rf^R reached 54.10% and the abalone agarolytic bacteria normally found with a ratio of 15:19% to total bacteria. This means that there were indeed naturally agarolytic bacteria in the digestive tract of abalone, and allegedly helped break down its host in agar seaweed cell wall.

Abalone growth

Abalone growth parameters treated with mix culture probiotic Abn1.2Rf^R Alg3.1Rf^R for 60 days maintenance included biomass growth (g), relative growth (%), feed efficiency (%) and feed conversion had presented in Tables 3 and 4.

An increase in biomass growth in gnotobiotik abalone and normal abalone given agarolytic bacteria is 32 and 45% repectively when compared with gnotobiotik abalone (K0). Increasing the length of the shell was a contribution agarolytic bacteria (gnotoplus), the normal microbiota (normal) and the combination agarolytic-normal microbiota (normalplus) had respectively 24, 16 and 41% when compared with no bacteria (gnotobiotic).

The effect of probiotics on growth of abalone was a direct result of the improved value of feed and feed conversion efficiency (Table 4). Gnotobiotic abalone and normal abalone got probiotics showed feed efficiency 35% and 5% better than the abalone without probiotics.

Similarly, feed conversion of probiotic-supplemented abalone had better than gnotobiotic abalone. The data showed that the probiotics in the gastrointestinal tract contributed to improving the ability of abalone convert nutrients into meat.

The percentage of digestible agar and digestible energy levels in agarolytic bacteria supplemented-abalone and higher when compared with the abalone consumes only cake *Gracilaria* standard (Table 1). These data indicate that there had an increase in the availability of nutrients in the digestive tract of abalone. Cake *Gracilaria* contained agarase bacteria hydrolyze agar on the cake, consequently provided more nutrients and energy, in particular agar degradation products. Pre-degradation of agar provided a substrate that was more easily digested by digestive enzymes abalone, thus lowering the energy required for digestion, and consequently the addition of energy available for growth.

A number of studies on enzymes in the hepatopancreas of abalone showed that the abalone itself capable of producing the enzyme cellulase, alginate lyase, karagenase, agarase, and laminarinase [1] [2] [10]. Research conducted at *H. midae* by [6] and [7] showed that probiotic supplementation increased the activity of alginate lyase enzyme, amylase and protease in the digestive tract of abalone. While in this study giving Alg3.1-Rf^R - Abn1.2-Rf^R on abalone had increased agarase enzyme activity in the gastrointestinal tract of abalone.

Increased activity of digestive enzymes in the digestive tract had improved the nutritional status of abalone through improved efficiency of digestion and thereby increased the rate of growth of abalone. Abalone consumed cake *Gracilaria* supplemented Alg3.1-Rf^R - Abn1.2-Rf^R for 14 days showed increase maintenance agarase enzyme activity in the gastrointestinal tract when compared to controls, and this suggests that the Alg3.1-Rf^R - Abn1.2-Rf^R strain contributed to the availability of 'pool' polysaccharolytic enzymes in the digestive tract to digest complex polysaccharides had eaten abalone.

Increased agarase enzyme activity in the gastrointestinal tract was directly proportional to the increase in the number of bacteria in the digestive tract agarolytic. The results of this study were consistent with reports [10] which stated that the total number of bacteria and the activity of digestive enzymes in the gastrointestinal tract *H. gigantea* had given *Pediococcus* sp. AB1 was higher compared with the control.

Bacteria presence within gastrointestinal tract of aquatic animals gave several benefits to its host. It included increasing immunity host [11], controlling endurance and infection [12] [13], increasing survival, growth, digestive enzyme activity rate [14] [15] [16] [17].

In the abalone consume red algae, the presence of *Vibrio haliotocoli* isolated from various species of abalone digestive tract, potentially contributing to a host of nutrients through degradation of alginate [18]. Alginate degradation by *V. haliotocoli* produced acetic acid which absorbed by the intestine and metabolized as a source of oxidative energy, and used as a precursor of the synthesis of proteins, sugars and fatty acids [4]. It stated by [19] that the dominant microorganisms in the digestive tract of molluscs were *Vibrio*, *Pseudomonas*, *Flavo- bacterium*, *Micrococcus*, and *Aeromonas*. This bacterial group played an important role in the physiological functions of the digestive tract, which contributes digestive enzymes in the digestive tract of its host with a wide range of activities, including the activity of agarase, cellulase, alginase, amylase, laminarinase, mannanase and protease.

Elimination of most or all bacteria in the digestive tract of water and aquatic animals (gnotobiotic organisms) adversely affected larval survival and growth of animals because of the existence of the microbiota to benefit the host by improving the metabolism of nutrients, providing vitamins and other growth factors, the production of extracellular enzymes that aid digestion or eliminating toxic residues [3] [20].

Giving probiotics improved the growth of biomass, relative growth, and increase the length of abalone shells tested. This laboratory-based growth experiments indicated that the addition of a probiotic mixture Alg3.1RfR - Abn1.2RfR improved growth rate compared with the control abalone, abalone gnotobiotik consumed only standard feed.

Unlike most of fish its energy needed meet from protein metabolism, abalone primary energy demand derived from carbohydrates [21] and abalone met by consuming natural feed containing carbohydrates of 30-60% [22]. The presence of agarolytic bacteria added availability of energy for abalone growth through degradation agar more efficiently. The high rate of feed carbohydrates can increase the growth of abalone [24] in the presence of enzymes degrader complex carbohydrates [23] and a good capacity to synthesize non-essential fatty acids from carbohydrates [4] [24].

The increasing in the growth rate of abalone was the impact of improved physiological function of digestion. This condition occurred through several mechanisms: (1) increase the availability of nutrients in the digestive tract so that it easily absorbed by the abalone, (2) an increase in 'pool' digestive enzymes in the digestive tract, and (3) the use of nutritional supplements as a source of additional bacteria [19] [25].

An increase in the abalone growth due to agarolytic bacteria inoculation in the feed was a response from carbohydrate utilization as an energy source, it was possible to show what was called the protein sparing action for growth. Proteins used for the growth and repair of damaged cells, not as a source of energy. Although carbohydrate was not a superior energy source for fish exceeds the protein and fats, carbohydrates digested from the feed exhibited what was called the protein sparing action for growth. Even [26] stated that if carbohydrates were not available in sufficient quantities in the diet, the abalone would have diverted some of the building blocks such as amino acids and unsaturated fatty acids to be burned into energy. Saving the protein through the addition of proper amounts of carbohydrates was called protein sparing.

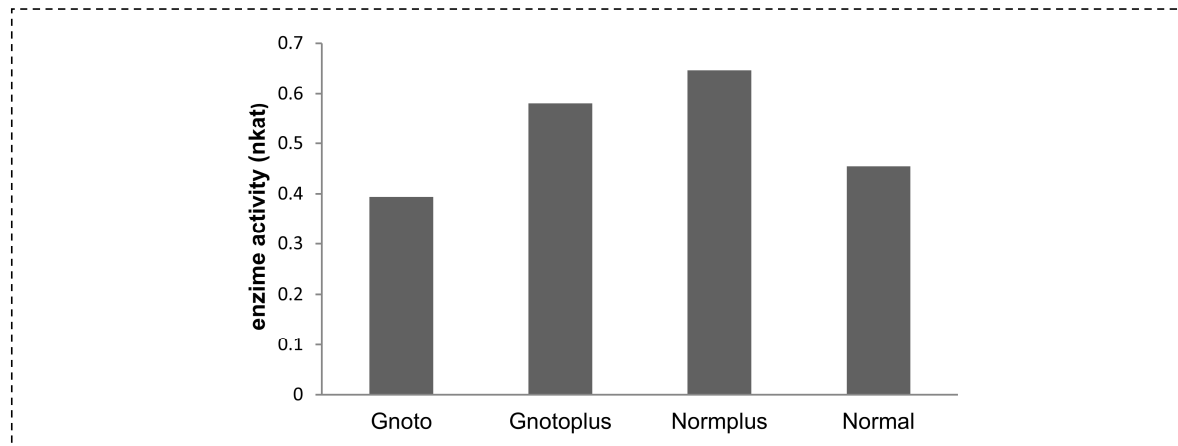


Figure 1. Agarase activity in the gastrointestinal tract of abalone were consumed cake *Gracilaria* standards and supplemented probiotics. Gnotobiotic= feed+antibiotic; Gnotoplus= feed + probiotic + antibiotic

Table 1. Levels Of Agar And Gross Energy In Feed And Feces Abalone

Treatment	Level of agar (%)	Digestible Agar (%)	Gross energy (Kkal/gr)	Digestible energy (Kkal/gr)
Feed	30.84		3077.58	
Gnotobiotik Feces	20.08	34.89	691.45	2386.13
Gnotoplus Feses	16.23	47.37	241.00	2836.58
Normplu Feses	18.15	41.15	479.09	2598.49
Normal Feses	19.04	38.26	487.68	2589.90

Note: Gnotobiotic= feed+antibiotic; Gnotoplus= feed + probiotic + antibiotic; Normalplus= feed+probiotic; Normal= standar feed. Digestible agar = (Agar feed – agar feces)/agar feed). DE = GE feed – GE feces

Table 2. The Number Of Total Bacteria And Agarolytic Bacteria In The Digestive Tract Abalone And Water In Aquarium At 14-Day Maintenance

	Water (cfu/ml)		Gastrointestinal tract (cfu/g)	
	total bacteria	Agarolytic bacteria	total bacteria	agarolytic bacteria
Gnoto	$2,78 \times 10^3$	$1,13 \times 10^2$	$2,50 \times 10^3$	$3,70 \times 10^2$
Gnotoplus	$3,57 \times 10^5$	$2,08 \times 10^5$	$7,40 \times 10^6$	$6,90 \times 10^6$
Normplus	$1,37 \times 10^6$	$4,91 \times 10^5$	$6,10 \times 10^7$	$3,30 \times 10^7$
Normal	$2,57 \times 10^6$	$4,56 \times 10^5$	$9,90 \times 10^7$	$1,20 \times 10^7$

Note: Gnotobiotic= feed+antibiotic; Gnotoplus= feed + probiotic + antibiotic; Normalplus= feed+probiotic; Normal= standar feed.

Table 3. Relative Growth, Biomass Growth And Shell Length Gain Of Abalone For 60 Days Of Maintenance

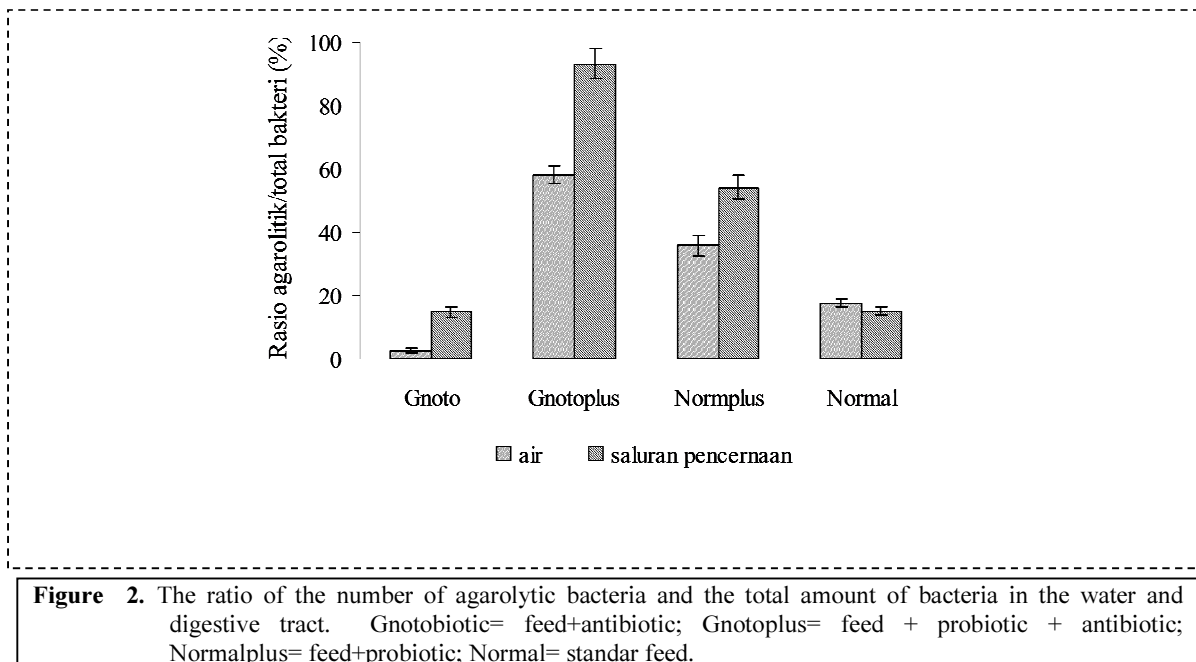
	Relative growth (%)	Biomass growth (g)	Shell length gain (mm)
Gnotobiotic	14.52±1.85	8.11±0.81	0.360±0.060
Gnotoplus	18.53±0.64	10.73±0.23	0.447±0.059
Normplus	21.32±1.61	11.82±0.76	0.507±0.063
Normal	20.42±1.67	10.64±0.65	0.416±0.072

Note: Gnotobiotic= feed+antibiotic; Gnotoplus= feed + probiotic + antibiotic; Normalplus= feed+probiotic; Normal= standar feed. Data are presented as the average of 3 replicates (± SE)

Table 4. Average Of Feed Efficiency And Feed Conversion During The 60-Day Maintenance Abalone

	Feed Efficiency (%)	Feed conversion
Gnotobiotik	3.45±0.28	29.35±2.19
Gnotoplus	4.78±0.25	21.04±1.15
Normplus	4.69±0.39	21.62±1.68
Normal	4.56±0.17	22.02±0.85

Note: Gnotobiotic= feed+antibiotic; Gnotoplus= feed + probiotic + antibiotic; Normalplus= feed+probiotic; Normal= standar feed. Data are presented as the average of 3 replicates (± SE)



CONCLUSION

Introduction agarolytic bacterial isolates contributed to improvement of physiological digestive function characterized by an increase in digestible energy, increased availability of exogenous agarase enzymes in the gastrointestinal tract as well as to improve the growth of abalone abalone.

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