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Selection of Probiotic Bacteria and *In vitro* Evaluation of Alginate as a Prebiotic for Freshwater Lobster (*Cerax quadricarinatus*)

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Abstract: The use of probiotic affects the aquatic animals' immune response and growth performance. This study aimed to isolate bacteria from freshwater lobster's intestines and observe *in vitro* the isolate's ability in utilizing alginate from brown alga (*Sargassum* sp.) as a prebiotic. The bacteria was isolated from the freshwater lobster (*Cerax quadricarinatus*) intestine's and the water of the lobster habitat. The lobster were collected from four stations i.e., Makassar, Bantimurung, Maros and Mandalle. The results of the study showed that after being soaked in a physiological solution with a pH of 2, there were 30 species of bacterial isolates discovered. The antagonistic test of inhibition zone against the pathogenic bacteria *Aeromonas hydrophila* showed the isolates of MDL7, MKS4 and MKS3 might inhibit the growth of *A. hydrophila*. While co-culture antagonistic test revealed that the isolates MDL1, MDL3, MDL6, BTL5, MRS4 and MRS5 could inhibit the growth of the pathogenic bacteria *A. hydrophila*. *In vitro* culture of alginate as prebiotic with the four best isolates as probiotic candidates, namely MDL7, MKS4, BTL5 and MRS5 demonstrated that the probiotic bacteria could grow and utilized prebiotic optimally, especially the MRS5 and BTL5 probiotic bacteria. Therefore, alginate as a prebiotic and MRS5 and BTL5 as probiotic bacteria have a great potential to be a symbiotic.

Key words: Alginate, *Cerax quadricarinatus*, prebiotic, probiotic, *Sargassum* sp.

INTRODUCTION

Application of probiotics for aquaculture benefits the host by improving disease resistance (Nayak, 2010), feed utilization and growth performance. Several important aspects of probiotics in shrimp cultivation have been studied and have shown satisfied results (Liu *et al*., 2010; Shen *et al*., 2010; Zokaeifar *et al*., 2012), for example, the administration of *Pediococcus acidilactici* resulted in a better survival rate in the *Litopenaeus stylirostris* shrimp (Castex *et al*., 2008), a strain of *Bacillus subtilis* is used a potential probiotic in shrimp cultivation (Liu *et al*., 2010; Shen *et al*., 2010), the white shrimp (*Litopenaeus vannamei*) fed with *B. subtilis* E20 showed good growth (Liu *et al*., 2010).

The study using fucoidan extracted from brown alga was conducted by Sweeney *et al*. (2011). The administration of feed containing fucoidan could increase the number of lactobacilli and the molar amount of butyric acid in the caecum and rectum and simultaneously lowered the molar proportion of valeric acid in the caecum and rectum. The increased population of probiotic bacteria showed its optimum role in improving the growth performance and the immune response of the animals. Besides the fucoidan form, the product of brown alga extraction is also found in the form of alginate. Alginate is easier to extract than fucoidan.

The freshwater lobster (*Cerax quadricarinatus)* has good economic value and is relatively easy to cultivate. However, its slow growth rate and its proneness to disease, especially to *Aeromonas hydrophila* bacterial infections, are serious issues in lobster cultivation. The intensive cultivation system, high population densities and limited space causes stress and supports the development of diseases and low growth rates, even causing mass mortality. In order to overcome these problems, a biological control using the application of synbiotics is a plausible alternative.

This study aimed to isolate and select bacteria which were candidates for probiotics from the intestines of lobsters (*C. quadricarinatus)* and the water of the lobster habitat and *in vitro* evaluation of alginate from brown algae (*Sargassum* sp.) as a prebiotic.

MATERIALS AND METHODS

Experiment animals and the prebiotic: The experiment animals were freshwater lobster (*C. quadricarinatus*) which were obtained from lobster farmers in Makassar, Maros, Bantimurung and Mandalle and the water of the lobster habitat in the four locations. The prebiotic investigation on them was alginate extracted from the brown alga (*Sargassum* sp).

Isolation of the probiotic bacteria: The bacteria were isolated from the intestines and placed in a physiological solution (NaCL 0.9%) with a pH of 2 aseptically. Therefore, the only bacteria which could thrive were bacteria which could tolerate a pH of 2, similar to the acidity in the stomach.

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Probiotic bacteria's antagonistic against *A. hydrophila*: The probiotic bacteria's antagonistic tests against *A. hydrophila consist* of inhibition zone and co-culturing tests. The inhibition zone test was done *in vitro* using the Disk Diffusion Method (Jacquelyn, 1999). The probiotic bacteria's antagonistic response toward the bacteria *A. hydrophila* was gauged based on the presence of a bacteria-free zone around the paper disc; the translucent area is measured.

The co-culture test was done by diluting 10^6 CFU/mL of each probiotic candidate bacteria. A volume of 50 µl liquid culture of the probiotic and pathogenic bacteria *A. hydrophila* was grown in a TSB medium. As a control, 50 µL of the pathogenic bacteria with the same density was grown singularly in a TSB medium. After 24 h of incubation, a serial dilution was done to the singular pathogen culture to 10^{-5}, 10^{-6} and 10^{-7} dilutions and the mixed culture (the co-culture between the probiotic bacteria candidate and *A. hydrophila*) to 10^{-1} and 10^{-2} dilutions. A volume of 100 µL of the serial dilution was spread on a PCA medium and incubated at room temperature for 24 h. The number of colonies growing was counted.

***In vitro* evaluation of the alginate as prebiotic:** In this test, the TSB medium used contained the prebiotic alginate at concentrations of 100, 200 and 300 ppm. TSB without alginate was used as a control. As for the probiotic bacteria candidates, four of the best bacteria which fulfilled the requirements were chosen.

An amount of 0.1 mL of probiotic bacteria candidates at a density of 10^6 CFU/mL were put in media tubes containing 10 mL TSB containing the prebiotic at various dosages then incubated for 28 h. The population of bacteria growing was calculated based on the OD value with an optical density of 600 nm at the 0, 8, 18 and 28th h.

RESULTS AND DISCUSSION

Isolation of probiotic bacteria candidates: As a result of the isolation from the four stations, there were 30 kinds of bacterial isolates which were able to develop in the TSA medium at a pH of 2, one of the indicators of probiotic bacteria. There were bacterial 6 isolates from the Makassar station, 4 bacterial isolates from the water where the lobsters were cultivated, 4 isolates from lobster intestines from Bantimurung station, 4 isolates from the water, 5 isolates from lobster intestines from Maros station and 7 bacterial isolates from Mandalle station.

As a requirement for probiotics, the bacteria which are to be selected as probiotic candidates must be able to live in the intestines, can be prepared as live-cell products in an industrial scale and must be stable and survive for long periods of time in storage and in the field (Irianto, 2005). Prebiotic bacteria must be able to withstand lysozymes in the saliva, the acidic conditions in the stomach (pH 2) and acids or bile salts in the small intestines in order to reach the intestines alive.

The survival and activity of the probiotic bacteria are important to its survival in feed during the shelf life and the transition through the acidic conditions of the stomach. In order to be function as probiotic bacteria, the bacteria must also withstand degradation by hydrolytic enzymes and bile salts in the small intestines (Belma and Gulcin, 2009).

The colonization of the intestines by the probiotic bacteria will prevent the growth of pathogenic bacteria through competition and the production of organic acids and anti-microbial substances. Tolerance towards acidic conditions and bile salts show the probiotic microorganism's ability to survive in the digestive tract (Erkkila and Petaja, 2000) with digested food and survive the competition with intestinal micro-biota (Succi *et al*., 2005; Tirloni *et al*., 2014).

Antagonistic inhibition zone test of the probiotic bacteria candidates: The results of the measurements of the inhibition zone of the probiotic bacteria candidate isolates against the bacteria *A. hydrophila* aged 24 h showed that there were 31 kinds of isolates which demonstrated an antagonistic response, exhibited by a bacteria-free zone surrounding the paper disc. Four species of isolates exhibited inhibition zones of more than 1 cm (Table 1).

The antagonistic nature demonstrated by the translucent zone surrounding the paper disc showed the probiotic bacteria candidate's ability to secrete chemical substances which are bactericidal or bacteriostatic, affecting the population of *A. hydrophila*. In general, the ability to inhibit the growth of bacteria is caused by one of the following factors or the combination of them such as the production of antibiotics, bacteriocins, siderophores, lysozymes, protease and or hydrogen peroxide or by affecting the pH of the medium by producing certain organic acids (Verschure *et al*., 2000). On the other hand, according to Pelzcar (1986), anti-microbial substances can be used to inhibit the growth of microbes or to kill microbes through the destruction of cell walls by inhibiting the construction process or by causing lysis of existing cell walls and changing cytoplasmic membrane permeability, causing a leakage of nutritional substances from inside the cell. The damage in the cytoplasmic membrane will inhibit the growth of the cell or even cause cell death.

Bacteriocin found in lactic acid bacteria (BAL) is a secondary metabolic product which has similar properties as antibiotics which are able to inhibit the growth of a few specific bacteria. Bacteriocins are protein compounds; therefore they are synthesized through a common protein bio-synthesis mechanism which involves transcription and translation (Davidson and Hoover, 1993). The anti-microbial properties of bacteriocin are species-specific and the inhibition activity occurs through adsorption on specific or non-specific

Table 1: Diameter of clear zone from the antagonist inhibition zone of the probiotic bacterial candidate against the bacteria *Aeromonas hydrophila*. Amrullah; the selection of probiotic bacteria and *in vitro* evaluation of alginate as a prebiotic for freshwater lobster (*Cerax quadricarinatus*)

Bacterial isolate	Diameter of clear zone (cm)	Bacterial isolated	Diameter of clear zone (cm)
MDL 1	0.75	BTL 2	0.6
MDL 2	0.6	BTL 3	0.6
MDL 3	0.6	BTL 4	0.6
MDL 4	0.6	MKS 1	1.1
MDL 5	0.65	MKS 2	0.65
MDL 6	0.6	MKS 3	1.1
MDL 7	1.65	MKS 4	1.15
VTA 1	0.6	MKS 5	0.8
VTA 2	0.6	MKS 6	0.65
VTA 3	0.6	VTL 1	0.6
VTA 4	0.6	VTL 2	0.6
BTA 1	0.6	VTL 3	0.6
BTA 2	0.6	VTL 4	0.6
BTA 3	0.6	VTL 5	0.6
BTA 4	0.6	VTL 6	0.6
BTL 1	0.6	Control	-

Table 2: Co-culture antagonistic of probiotic bacterial candidate against bacteria *Aeromonas hydrophila*. Amrullah; the selection of probiotic bacteria and *in vitro* evaluation of alginate as a prebiotic for freshwater lobster (*Cerax quadricarinatus*)

	-------- No. of bacterial colonies ---------	
Bacterial isolate	Probiotic	*Aeromonas* sp
MDL 1	++	++
MDL 3	++	++
MDL 4	++	++
MDL 5	++	++
MDL 6	++	15
MDL 7	5	++
BTA 1	125	++
BTA 2	39	++
BTA 3	253	++
BTL 1	++	++
BTL 2	210	95
BTL 3	123	65
BTL 4	135	++
BTL 5	++	2
MRA 1	++	++
MRA 2	35	++
MRA 3	200	++
MRS 1	++	++
MRS 2	64	++
MRS 3	++	++
MRS 4	++	23
MRS 5	++	1
MKS 1	-	++
MKS 2	++	++
MKS 3	35	++
MKS 4	21	++
MKS 5	++	++

Note: - no growth ++uncountable growth

receptors located on the outer surface of the target bacteria's cell. Adsorption is followed by metabolic, biological and morphological changes which lead to the death of the bacteria attacked (Naidu *et al*., 1999).

Co-culture with the probiotic bacteria candidate antagonistic test: The antagonistic test with the co-culture between probiotic bacteria candidate and *A. hydrophila* method was done in a broth media for 24 h. The results of research showed that the probiotic bacteria candidate was dominant on the agar medium and was able to suppress the population of the bacteria *A. hydrophila* (Table 2).

Competition over substances essential to metabolism is an important factor which determines the presence of probiotic bacteria. For example, the probiotic bacteria *Vibrio* sp. could be antagonistic and could absorb iron from pathogenic strains of *Vibrio* sp., because the probiotic Vibrio produces siderophores (Gatesoupe, 1999). Bacteria which produce siderophores have an iron transport protein which is very specific on its outermost membrane, making it capable of utilizing Fe^{3+} which is not soluble in its growth medium. As for the competition for space for adhesion, the earlier the colonization by the potential probiotic in the digestive tract, the better the probiotic's working potential (Bengmark, 1988).

The bacteria's ability in producing a metabolite is highly dependent on the availability of the nutrients which in turn will affect growth. The growth of antibiotic-producing microbes and their production depends on the composition of the medium, especially on the carbon and nitrogen sources and the fermentation condition.

Probiotics which consist of microorganisms or a product are beneficial to the host's health, due to their antagonistic effects on pathogenic bacteria, the improvement to immune responses and feed efficiency and the balance of intestinal micro-flora (Gatesoupe *et al*., 1999).

***In vitro* evaluation of the probiotic and prebiotic:** The results of the study showed that alginate could be utilized by probiotic bacteria as a source of nutrition (prebiotic). This causes the population of probiotic bacteria to increase. The four kinds of bacterial isolates and the alginate dosages tested showed different growth based on the optical density (OD) measurements. All alginate treatments were significantly higher that the control (no alginate added) until the 18th h ($p<0.05$). At the 28th h it became impossible to measure using the spectrophotometer because the OD of the samples were higher than 2.5. As proof that the difference in OD at every hour was caused by the increase in bacterial colony number, the number of bacteria was counted using the plate method. The results of the measurement of OD (Table 3) and the results of the bacterial colony count (Table 4) showed that the increase in OD during the 28 h was caused by the increase in bacterial colony numbers.

Table 3: Growth of the probiotic bacteria candidates with the addition of alginate in 28 h based on the optical density (OD) measured using a spectrophotometer at a 600 nm wavelength. Amrullah; the selection of probiotic bacteria and *in vitro* evaluation of alginate as a prebiotic for freshwater lobster (*Cerax quadricarinatus*)

		Time (h)			
PB	AD (ppm)	0	8	18	28
MKS 4	100	0.043^{a}	0.61600^{ghi}	1.64600^{d}	>2.5000
	200	0.066^{a}	0.64750^{fgh}	1.79500^{cd}	>2.5000
	300	0.071^{a}	0.70450e^{fgh}	1.88650^{bc}	>2.5000
	Control	0.050^{a}	0.15150^{j}	0.63950^{f}	0.92040
MDL 7	100	0.042^{a}	0.76350^{efg}	1.73500^{cd}	>2.5000
	200	0.066^{a}	0.78250^{ef}	1.82700^{c}	>2.5000
	300	0.061^{a}	0.81850^{de}	1.85150^{bc}	>2.5000
	Control	0.078^{a}	0.22350^{j}	0.71250^{ef}	0.96900
MRS 5	100	0.044^{a}	0.92700^{cd}	2.00550^{b}	>2.5000
	200	0.065^{a}	0.95100^{cd}	2.25900^{a}	>2.5000
	300	0.045^{a}	1.06850^{c}	2.39650^{a}	>2.5000
	Control	0.072^{a}	0.58850^{hi}	0.81800^{e}	1.05900
BTL 5	100	0.044^{a}	0.16200^{j}	2.31100^{a}	>2.5000
	200	0.065^{a}	1.25100^{b}	2.36050^{a}	>2.5000
	300	0.064^{a}	1.51350^{a}	2.38100^{a}	>2.5000
	Control	0.060^{a}	0.49550^{i}	0.62150^{f}	0.89600

Values in a row with different superscripts denote a significant difference ($p<0.05$). PB: Probiotic bacteria, AD: Alginate doses

Table 4: Density of probiotic bacteria candidates (CFU/mL) with the addition of alginate in 28 h. Amrullah; the selection of probiotic bacteria and *in vitro* evaluation of alginate as a prebiotic for freshwater lobster (*Cerax quadricarinatus*)

		Time (h)			
PB	AD (ppm)	0	8	18	28
MKS 4	100	2.0×10^{2}	1.8×10^{4}	8.1×10^{6}	4.3×10^{6}
	200	2.5×10^{2}	2.6×10^{4}	9.3×10^{6}	4.4×10^{7}
	300	2.4×10^{2}	3.9×10^{4}	8.6×10^{6}	6.8×10^{7}
	Control	2.3×10^{2}	4.4×10^{3}	7.1×10^{5}	4.3×10^{6}
MDL 7	100	2.0×10^{2}	6.6×10^{4}	7.4×10^{6}	5.7×10^{7}
	200	2.5×10^{2}	4.1×10^{4}	9.3×10^{6}	7.4×10^{7}
	300	2.4×10^{2}	5.2×10^{4}	7.2×10^{6}	6.2×10^{7}
	Control	2.1×10^{2}	3.9×10^{3}	6.8×10^{5}	5.1×10^{6}
MRS 5	100	2.0×10^{2}	6.6×10^{4}	7.4×10^{6}	8.3×10^{7}
	200	2.5×10^{2}	4.1×10^{4}	6.3×10^{7}	6.2×10^{8}
	300	2.4×10^{2}	5.2×10^{4}	7.2×10^{7}	2.3×10^{8}
	Control	3.2×10^{2}	3.6×10^{3}	7.2×10^{5}	7.4×10^{6}
BTL 5	100	2.0×10^{2}	6.6×10^{4}	5.2×10^{7}	7.4×10^{7}
	200	2.5×10^{2}	4.1×10^{3}	8.4×10^{7}	6.2×10^{8}
	300	2.4×10^{2}	5.2×10^{4}	6.3×10^{7}	1.4×10^{8}
	Control	5.2×10^{2}	4.1×10^{3}	3.9×10^{5}	5.8×10^{5}

Values with different letter in the same row are significant different ($p<0.05$). PB: Probiotic bacteria, AD: Alginate doses

From the four isolates tested, it can be seen that the isolates MRS5 and BTL5 could utilize alginate optimally, resulting in significantly higher growth rates compared to those of the other isolates. The main requirement of a prebiotic as material that cannot be hydrolyzed in the digestive tract has been fulfilled by fucoidan which shows that brown alga contains polysaccharides which cannot be yang hydrolyzed by endogenous enzymes in the human intestines, classifying it as food fiber.

The use of alginate found in brown alga as a prebiotic is still limited, especially in fish. Gardiner *et al.* (2008); Gahan *et al.* (2009) and O'Doherty *et al.* (2010) studied the use of fucoidan as a prebiotic and showed that fucoidan and laminarin are polysaccharides found in brown alga such as Laminaria. Other studies also showed that the use of seaweed extract on animals (Gahan *et al.*, 2009) could improve the performance of lactic acid bacteria (Lynch *et al.*, 2010) and the host's immunity (Leonard *et al.*, 2010).

Laminaria hyperborea is a brown alga from the Phaeophyta division which contains mannitol and laminarins as a soluble polysaccharide reserve with sulphated complex polysaccharides (fucoidans) in the cell walls and in the intercellular space. The prebiotic potency of beta-glucan from barley (Lynch *et al.*, 2007) has propelled studies in the use of alga beta-D-glucan as a prebiotic. Therefore, the use of fucoidan could be advanced to synbiotic application. Synbiotics are a combination between probiotic and prebiotic which could selectively stimulate the growth of the bacteria supplemented which in turn will optimize the growth of the probiotic bacteria because of the availability of

nutrients, making nutrition absorption by the fish better and in the end causing the maximum fish growth. Similarly, the fish's immunity would improve. Study results have shown the probiotic bacteria could improve non-specific immune responses.

Conclusion: Based on the study that has been conducted, it is concluded that *in vitro* evaluation of alginate from brown algae (*Sargassum* sp.) as prebiotic demonstrated that the probiotic bacteria could grow and utilized prebiotic optimally, especially the MRS5 and BTL5 probiotic bacteria.

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