The effect of probiotic and vitamin C as feed supplements on the recovery of operculum deformity in barramundi (*Lates calcarifer*) seed

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Abstract. The main constraints of Barramundi seed production are viral disease infection (VNN and Iridoviruses) and a high incidence of deformity on the operculum. The present study aimed to find a way to recover operculum deformity by supplementation of feed with probiotics and vitamin C. The seeds were reared in tank capacity of 200 L at density of 50 ind/tank. The condition of the deformity was signed by a shortened operculum on one side of the seeds. The treatments applied were (A) the seeds without deformity (normal) were fed on the commercial diet, (B) seeds with deformity fed on the commercial diet, and (C) seeds with deformity fed on the commercial diet supplemented with probiotic of *Saccharomyces cerevisiae* at the amount of 100 mL/kg of feed and mixed with Vitamin C at the amount of 2 g / kg of feed. Each treatment was 4 replications and reared for 40 days. The result showed that in treatment (A) deformity was 1%; treatment (B) changes to normal was 11 %, and recovery success was 21 % and still deformity was 68%, while in treatment (C), deformity changed to normal was 20 %, recovery success was 14% and deformity was 66 %. Fish with deformities have low genetic variation values.

1 Introduction

Recently, aquaculture activities have been developed well. Fish, crustaceans, shellfish, and other aquatic animals are excellent sources of protein. Besides the main content of fish is protein, other important compounds on fish is essential nutrients including iodine, vitamin D and calcium as well as healthy omega-3 fatty acid. Aquaculture is one of solution for food security and safety, which is an efficient way of producing protein compared to livestock. In particular, Barramundi (*Lates calcarifer*) culture which has important economic value and it is very strategic for global market. Fish demand of *L. calcarifer* in the world market is

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increasing, especially with form of fillets. Therefore, there is needs to be a balance between barramundi culture and the availability for a large supply of seeds. Seed production capacity is very limited due to mortality and seed health problems. Ideally, to improve the quality of barramundi seeds through feed with nutritional supplement components, including probiotic and vitamin. It is very suitable for growth regulation, immunity, and prevention of abnormality of fish.

Barramundi *L. calcarifer* seeds are available in good quality and in sufficient quantity, which will have a positive impact on the success of the culture. The main constrains often encountered in seed production not only viral infections (VNN and Iridovirus), bacterial infections (Black body and Big Bally diseases), but also high rates of abnormalities or deformities. Morphological abnormalities or deformities are conditions of fish seeds that do not correspond to normal conditions.

According to Berillis [1], it is stated that abnormality that often occurs is skeletal deformities in fish fries (scoliosis, kyphosis, lordosis, jaw malformations) and deformity in the operculum (branchial arches) or shortened operculum. In seed production, the influence of environmental factors (water sources, temperature, microbial balance), natural feed (microalgae, rotifer, and Artemia), formulated feed, and supplement feed to improve the health of fish becomes important. In addition, genetic factors from the broodstock of Barramundi fish that used for seed production also contributed greatly in producing of seed quality. Therefore, good fish hatchery methods should be applied in the hope that abnormalities can be reduced [2]. The economic consequences are important because the abnormality causes a downgrade in product image and biological performance of reared fish, negatively affecting market value and production cost. Thus, efforts to recover fish that have operculum deformities need to be made. This is an option to get healthy seeds and have perfect body morphology without defects.

Fish need vitamins for survival because vitamins act as enzyme cofactors. As a soluble water vitamin, vitamin C helps maintain normal fish growth and physiological function. With the addition of vitamin C it can provide normal growth, cartilage and bone formation, the iron metabolism and hematology, lipid metabolism, reducing stress, increases the immune response, and interacts with other micronutrient compounds, so it is very beneficial for healthy growth and preventing fish abnormalities [3].

Saccharomyces cerevisiae is a type of yeast that is useful for animal feed, but then began to be used for industrial biotechnology purposes. *S. cerevisiae* has been used as a probiotic and immune stimulant to improve the health and productivity of livestock and fish [4, 5, 6] as an immune stimulant, because 1.3 and 1.6 β cell walls of *S. cerevisiae* contain glucan [7]. As a probiotic, this yeast has several mechanisms, namely increasing immune responses, contributing enzymes in feed digestion, and as a source of macro and micro-nutrients [8]. Furthermore, [9] stated that in increasing the digestibility of feed, probiotics will create a balance of intestinal flora and act as a ferment in the fermentation process. With this role, the fish can metabolize nutrient compounds properly, thus morphological growth of the fish including the gill operculum will grow normally without any abnormalities occurring. S cerevisiae contains glucan acid (β nucleic, mannan, nucleotide) [10, 11]. This is in accordance with the statement of [12] that â-glucan can increase phagocytic activity by binding to receptor molecules that are found on the surface of phagocytic cells. The present study aimed to find a way to recover operculum deformity on Barramundi *L. calcarifer* seed by supplementation of feed with probiotic and vitamin C.

2 Methods

2.1 Preparation of test fish *L. calcarifer*

The test fish were Barramundi fish (*L. calcarifer*) produced by controlled larval rearing with one-sided operculum deformity. Total body length of the seeds is 6.38 ± 0.5 cm and body weight of 4.17 ± 0.15 g. The selection of fish should have criteria that should be a healthy condition and active movement. The fish are acclimatized in rearing tank for three days until the fish become good health.

2.2 Feed supplements

The feed supplements that were used were probiotic *S. cerevisiae* and vitamin C. Probiotics were cultured using Bacto-tryptone 0.2% and Yeast Extract 0.1% media. Both mediums were dissolved in seawater and adjusted to pH 7.0. Probiotic *S. cerevisiae* were cultured using a glass flask with a volume of 500 mL. The culture media were sterilized using an autoclave at temperature of 121 ºC for 15 minutes. Inoculation of *S. cerevisiae* was carried out after the culture media had cooled. Incubation time for 48 hours at a temperature room of 23-24 ºC and flask glass culture equipped with aeration.

2.3 Culture of *L. calcarifer* seeds

The culture tank that was used for the study of operculum deformity recovery is a 200 L volume of polycarbonate tanks with a flow-through system. The density of fish in each tank was 50 ind/tank. The treatments applied were (A) the seeds without deformity (normal) were fed on the commercial diet, (B) seeds with deformity fed on the commercial diet; and (C) seeds with deformity fed on the commercial diet supplemented with probiotics of *S. cerevisiae* at amount of 100 mL/kg of feed and mixed with Vitamin C at amount of 2 g / kg of feed. Each treatment was held in 4 replications and reared for 40 days.

The commercial feed that was used is in the form of the micro diet with a size that is adjusted to the mouth opening of fish seeds. Feed supplements (*S. cerevisiae* and Vitamin C) were sprayed on feed at an amount of 100 mL/kg and 2 g/kg feed, respectively, and coated with chitosan. Then, it was air-dried and stored in a refrigerator at 4 ºC before being fed to barramundi fish seeds. Feeding as much as 5% of the weight of biomass with frequency of 3 times/day. During the culture of barramundi fish seeds, the bottom of the tank was cleaned to collect the remains of feed and fish feces through the siphoning technique.

2.4 Genotype analysis of *L. calcarifer* seed

2.4.1 Extraction and clean-up of DNA genome

DNA genomes were prepared by extraction of 20 mg tissues from normal and operculum deformity of Barramundi fish. Tissue samples were crushed in 250 uL solution of 10% Chelex-100 on TE buffer, pH 8.0, and add of 7.5 µL Proteinase Kinase (20 mg/mL). The sample was incubated at a temperature of 55 ºC for 2.5 hours and at 89 ºC for 8 minutes, then centrifuged at 13,000 rpm for 5 minutes to produce the DNA genome. DNA genomes were then cleaned up with a Prep-Ease DNA Clean-up Kit (USB 78758). Clean-up methods followed the manual prepared by Usb Corporation that is by adjusting the DNA binding condition by adding 500 uL N2P buffer into 100 ul DNA genome, binding DNA sample to column, and centrifuging for 1 minute at 13,000 rpm, washing the column with add of 600

µL NT3 buffer, and centrifuge at same as before and dry column with the centrifuge of 2 minutes. The spin column was then moved to the microtube, and NE buffer was used to dilute DNA in the membrane filter on the column and centrifuge for 1 minute. The solution accommodated in the microtube is a pure DNA genome and can be used for PCR amplification.

2.4.2 PCR amplification used RAPD (Random Amplified Polymorphism DNA) method.

The reaction carried out for RAPD analysis by making a master mix with Go taq polymerase kit (Promega) and trying of 4 universal primers with nucleotide sequence of 2AAM2 (CTG CGA CCC AGA GCG), L-21 (GGA TCC GAG GGT GGC GGT TCT), AS-4 (TGT GGG CGC T TCG ACA) and AS-15 (GGC TAA GCG GTC GTT) to obtain polymorphisms in each analyzed fish seed sample. The master mix for the analysis each sample consisted of 5x Buffer (4 uL), 10 mM dNTP (1 uL), 100 picomol Primer (1ul), 25 mM MgCl2 (7 ul), Taq polymerase 0.5 uL and NFW (Nucleic Free Water) of 5.5 ul. The total volume of the master mix is 19 µL and 1 µL of the DNA genome each sample was added to be analyzed. Thermal cycle of PCR amplification begins with initial denaturation of 95 ºC for 5 minutes, denaturation of 95 ºC, annealing of 40 ºC (10 seconds) and extension of 68 ºC for 30 seconds. This cycle was repeated 40 times and continued with a final extension of 72 ºC for 3 minutes and incubation temperature of 12 ºC. DNA amplification results were separated by using electrophoresis with 2% agarose gel in 0.5x TBE (Tris Borat EDTA) buffer and run for 30 minutes. The results of separation were observed with a UV transilluminator gel and documented.

2.5 Data analysis

Data analyses were used is a completely randomized design of 3 treatments with 4 replications. The biological parameters observed included growth, survival, percentage of operculum deformity recovery, and genotypic analysis of barramundi seeds.

3 Results and discussion

The results obtained showed that the growth of the total body length of barramundi seeds for 40 days of rearing was not significantly different ($P>0.05$) among the treatments of A, B, and C. This can be seen from a final total length of 13.66 cm in treatment A (normal seed feed with micro diet), 13.36 cm in treatment B (deformed seed in operculum and feed with micro diet), and 13.69 cm in treatment C (deformed seed in operculum and feed with micro diet added of feed supplements) as presented at Figure 1.

Observation on the body weight growth of barramundi *L. calcarifer* seeds was obtained that weight gain of treatment A (37.79 g) and C (36.96 g) were not significantly different ($P >0.05$), but when compared to B (33.37 g), treatment gave significantly different ($P<0.05$) as presented at Figure 2. It seems that micro diet is only suitable for normal fish seeds without abnormalities. If the fish have abnormalities such as operculum deformity, it is necessary to add additional supplements to the feed.

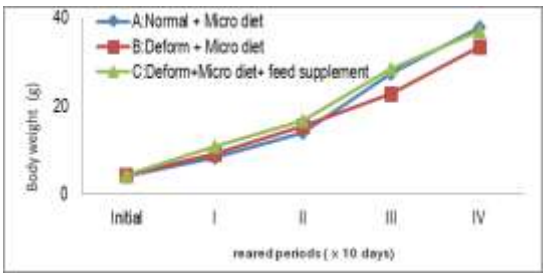

Fig. 1. Total length (cm) of barramundi *L. calcarifer* seeds with normal performance and operculum deformity treated with feed supplementations (probiotic and Vitamin C) and without supplementation.

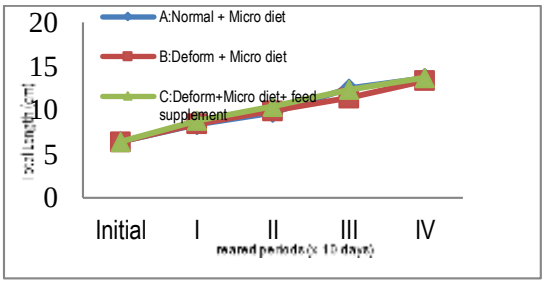

Fig. 2. Body weight (g) of barramundi, *L. calcarifer* seed with normal performance and deformity operculum treated with feed supplementations (probiotic and Vitamin C) and without supplementation.

Feed is one of the most critical factors in fish growth. The growth rate in total length is influenced by the quality of the protein on the feed. This was also expressed by [13], which states that fish growth is closely related to the availability of protein in feed because protein is a source of energy and nutrients needed for fish growth. *S.cerevisiae* contains a high nutritional value which includes protein, fat, vitamins, and minerals that can help increase fish appetite, feed digestibility, and improve the digestive process of food in the fish digestive tract [14] and growth [15, 16]. When a fish consumes a higher amount of feed than usual, the body will use feed to increase the body weight or body length of the fish.

The vitamin content in the artificial feed may act as a catalyst for metabolic processes, especially to increase the survival of fish. Vitamin C is important to improve the resistance system of fish because vitamin C plays a role in maintaining the reduced form of Cu+ ions as a cofactor needed by dopamine beta-hydroxylase enzyme and suppressing the production of noradrenaline and adrenaline in the catecholamine process (stimulating the production of blood glucose, which is used as energy). Furthermore, if vitamins content in the body is at optimal concentration and there are unfavorable environmental conditions, the catecholamine synthesis process can take place properly. Therefore, fish are able to survive when physiological change or stress does not occur [17].

The calculation of the survival rate of Barramundi seeds after 40 days of rearing showed that the survival rates were not significantly different ($P>0.05$). The percentage of survival rate was obtained between 90-97% (Figure 3), and the mortality is mostly caused by cannibalism. The supplementation of Vitamin C has many correlation functions to cellular respiration and enzymes. The role of vitamin C is do oxidation of phenylalanine to tyrosine, reduction of ferric ions to ferrous in the digestive tract, so that iron ions are easily adsorbed, converting folic acid into folinate (in the form of ferric acid active) and plays a role in the formation of fish. Vitamin C is important for fish due to its function in fish body metabolism.

According to Xu et al. [18], vitamin C has a huge role in the physiological process of fish metabolism.

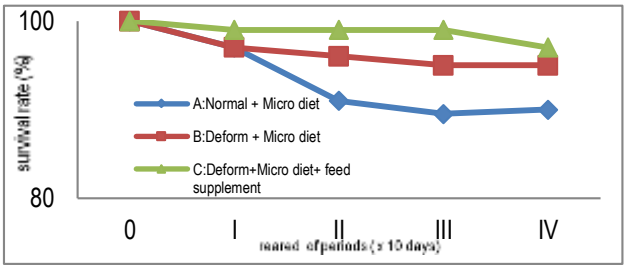

Fig. 3. Survival rate (%) of Barramundi *L. calcarifer* seeds with normal and deformity operculum treated with feed supplementations (probiotic and Vitamin C) and without supplementation.

The criteria for stating that the operculum deformity in barramundi seeds has recovered, is indicated by the performance of the operculum growing completely and closing again and the fish's gills are no longer visible. Meanwhile, if the gill operculum is in the recovery process, the operculum only grows partially during treatment. The growth of the operculum only 50-75% gill closure, whereas in fish that are still deformed even though they have received treatment, the operculum remains open as at the beginning of the treatment, so that the gills can be seen clearly.

From the calculation of the average percentage of barramundi fish seeds that recovered from deformity in treatment B showed that 11% of the operculum had changed to normal and closes again, some still in the recovery process at 21% and still deformed at 68%. In treatment C, where the Barramundi fish seeds fed on feed supplemented on micro diet, the average value of the normal operculum was 20%, while some of seeds were still in the recovery process at 14% and still deform at 66%. These results revealed that by supplemented probiotic and Vitamin-C could recovery deformed operculum of Barramundi *L. calcarifer* seeds.

Result of research showed that the treatment using feed supplements on micro diet (C) resulted in a higher percentage of operculum recovery ($P<0.05$) compared to the treatment using feed without supplementation (B). However, fish that are still in the process of recovery in treatment C tend to be low, considering that barramundi fish have recovered more of their operculum (Table 4). The level of deformity after the end of rearing was not significantly different ($P>0.05$) between treatments B and C. In the control, barramundi fish relatively did not appear deformity during rearing.

Research on deformity in marine fish is often found in the operculum, which reaches 90.1% deformation in hybrid grouper. The same case was also experienced with salmon fry produced by hatcheries with operculum deformity reaching 60% to 90% (19). Fish with open operculum deformity usually have less growth than normal fish even though they can swim normally.

Table 1. Percentage of recovery of operculum deformity in barramundi (*L. calcarifer*) seeds treated with commercial feed and feed supplements (probiotic and Vitamin C).

Treatment	**Percentage (%) of operculum performance**														
	Normal					**Recovery**					**Deform**				
Day	**0**	**10**	**20**	**30**	**40**	**0**	**10**	**20**	**30**	**40**	**0**	**10**	**20**	**30**	**40**
(A)	100	99	99	99	99^{a}	0	0	0	0	0^{a}	0	1	1	1	1^{a}
(B)	0	7	14	10	11^{b}	0	8	15	21	21^{b}	100	85	71	69	68^{b}
(C)	0	12	17	17	20^{c}	0	12	9	15	14^{c}	100	73	74	68	66^{b}

Genotype analyses can be used to express the genotype profile of fry that had normal operculum and deformity performance. The gene variation in the fry is very important to know that deformity is related to genetic variability. The PCR amplification in random regions were using a primer of 2-AAM2, L-21, AS-4, and AS -15. Among the four loci were used to detect the gene polymorphism on normal operculum fry, only locus of L-21, AS-4, and AS 15 gave clear banding patterns and were used for further analyses. Locus of 2 AAM2, L-21, and L-14 gave gene polymorphism on Barramundi fry with operculum deformity. Random amplified polymorphism of DNA sequence on all locus, each resulted in a 2-3 intersection pattern. The DNA amplification resulted in 4-5 composite haplotype DNA random regions shown in Table 2. The total number of composite haplotypes which are owned by each Barramundi fry population, normal and deformity operculum ranged from 5 and 4 composite haplotypes, respectively. The highest composite shown in the population with normal operculum was that almost all of the fry examined were dominated by a composite of DCCA (11 fishes), while on deformity operculum population was dominated by a composite of DCBB (11 fishes).

Table 2. The results of genotype analysis with the RAPD method between normal and operculum deformity of Barramundi *L.calcarifer* fries. There are four loci used, namely 2 AAM2, L-21, AS-4 and AS-15.

Barramundi Seed population	Genotype (Seed sample)	2-AAM2	L-21	AS-4	AS-15
Normal seed	DCCA (11)	D	C	C	A
	DCAA (1)	D	C	A	A
	DCCB (1)	D	C	C	B
	DCBA (1)	D	C	B	A
	DDCA (1)	D	D	C	A
Operculum Deformity seed	DCAB (2)	D	C	A	B
	DCBB (11)	D	C	B	B
	DBBB (1)	D	B	B	B
	CCBB (1)	C	C	B	B

The results of genetic analysis using TFPGA (Tool for Population Genetic Analysis) method showed that heterozygosity on normal fry was higher (0.2333) than operculum deformity fry (0.0889) (Table 3).

Table 3. The results of genetic analysis on normal and operculum deformity of barramundi *L. calcarifer* seeds. There are four loci used, namely 2 AAM2, L-21, AS-4 and AS-15.

Barramundi seed population	Parameters	2-AAM2	L-21	AS-4	AS-15	Average
Normal seed	Number of sample (fish)	15	15	15	15	15
	Heterozygosity observed (Ho)	0.1244	0.1244	0.2400	0.4444	0.2333
	Heterozygosity expected (He)	0.1287	0.1287	0.2483	0.4598	0.2414
Deformity operculum seed	Number of sample (fish)	15	15	15	15	15
	Heterozygosity observed (Ho)	0.1244	0	0.2311	0	0.0889
	Heterozygosity expected (He)	0.1287	0	0.2391	0	0.0920

Analysis of genetic diversity could be done using molecular markers. Molecular markers are traits and that can be inherited and are associated with certain genotypes. Molecular markers include PCR-based Random Amplified Polymorphic DNA (RAPD), which is widely used to identify diversity at the interspecies and intraspecies level. This technique has advantages in its implementation and analysis.

Compared to the Simple Sequence Repeat (SSR) method, the RAPD technique is cheaper, easy to perform, gives quick results, produces a large number of DNA band polymorphisms, and the random primers used are easy to obtain [20]. Heterozygosity shows the potential of an individual could adapt to its environment. The higher the heterozygosity value, many genes that play a role in the fitness of a population [21]. The reduction of genetic diversity in hatchery activities (domestication) is caused by genetic drift due to the number of effective populations or the number of broodstock involved in reproduction being small compared to fish that breed or mate naturally. RAPD-PCR was used to evaluate the effects of irradiation on the DNA of sea bass (*Dicentrarchus labrax*) and sea bream (*Sparusa urata*) [22]. This technique can be used as a basis for screening of irradiated samples based on loss of specific bands for a specific amount of irradiation. The same statement was seen from the genotype analysis of Barramundi seeds with normal operculum and those with deformity. Thus, the RAPD analysis is considered sufficient as an analytical marker in this study.

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