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Dear Eni Kovacs,

Thank you very much for your correction of my manuscript. Now, I sent again of my article that has already repair as your notes.

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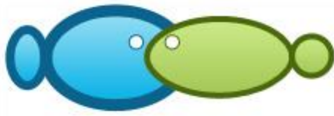

Density and types of probiotic bacteria in filter media with different number of bioballes in culturing media of silver pompano (*Trachinotus blochii*) with recirculation system

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Abstract. Cultivation intensification with a recirculation system tends to have a high stocking density and a large amount of feed, so that feed residue, metabolic waste, and oxygen consumption also increase. To speed up the decomposition of feed residue and metabolic waste, filtering is necessary, namely by means of a bioball. This study aims to analyze the types and numbers of bacteria present in the bioball, to improve water quality and the optimal number of bioballs for bacterial living media. This study used a completely randomized design, 1 factor, 5 levels of treatment and 3 replications. As the level of treatment in this study are; A = No bioball (control), B = 35 bioballs / filter container, C = 45 bioballs / filter container, D = 55 bioballs / filter container and E = 65 bioballs / filter container respectively. The silver pompano fish measuring 10 - 12.55 cm and body weight of 24.3 - 28.9 grams with a density of 1 fish / 4 L (20 fish / 80 L) were cultivated for 56 days. During fish rearing, they are fed with Megami GR2 pellets with a composition of 46% protein, 10% fat, 2% crude fiber and 10% moisture content, 3 times a day adlibitum. As a response to the test are: the type and density of bacteria in the bioball, temperature, pH, dissolved oxygen, total ammonium nitrogen, nitrite and nitrate. The research data were analyzed statistically ANOVA (P<0.05), Newman Keuls's advanced test and multiple regression models using SPSS 17.0. The best treatment was found in the use of 55 bioballs / filter containers with a volume of 9.8 L, which gave a bacterial density of 667.67 ± 1.53 x 105 CFU / mL. The amount of bioball affected the bacterial density by 31.1%, nitrogen absorption by fish 16.5% and filter media by 85.2%. The bacteriocidal types found in the filter media are Acinetobacter sp., Shewanella sp. and Nitratireductor sp. 15 ‰ salinity culturing media using a filter substrate of 55 bioballs / filter containers in the recirculation system gave the best bacterial density and water quality (28.6 - 29.1 0C, pH 7.6 - 7.8, DO 6.3 - 6, 7 mg / L, TAN 0.06 - 1.57 mg / L, nitrite 0.120 - 0.861 mg / L and nitrate 0.93 - 3.07 mg / L).
Key Words: bioball, bacterial density, probiotics, water quality.

Introduction. Silver pompano fish is a marine aquaculture species that is relatively new in Indonesia. However, the demand for this fish continues to increase, especially from the international market. These fish include fish species that have high adaptability, are easy to cultivate and have important economic value. The price of silver pompano for consumption reaches around Rp. 60,000, - / kg at local market and Rp. 200,000, - / kg for export commodities (Mo, 2017). Since 2015 silver pompano has become a leading commodity in marine cultivation fisheries. Production of silver pompano in 2015 reached 1900 tons, and each year it is targeted to increase production to reach 31.5% per year (Prahadi, 2015).

The production target for the silver pompano commodity can be achieved through intensification of cultivation. Intensive cultivation tends to be with a high stocking density, resulting in narrow space for fish, increased competition for oxygen and feed, so that the potential for fish to be stressed is greater, as a result, fish growth will be hampered. High density also accelerates the decline in water quality due to the accumulation of metabolic waste and feed residue, so that it has a major effect on growth.

Naturally nitrogen from fish culture waste produces ammonia (NH_3) from feed residue and fish metabolism, this can result in a buildup of organic matter which causes a

decrease in water quality (Putra and Pamukas, 2011; Prayogo et al., 2012). One of the technologies in fisheries to maintain water quality in order to remain suitable for aquatic organisms and to support the optimization of water utilization is the cultivation of recirculation systems. The recirculation system is able to reduce the level of amonia concentration, to within the range of 31-43% (Djokosetiyanto et al., 2006; Putra and Pamukas, 2011).

According to Lekang (2008) and Fadhil et al. (2010) the use of a recirculation system has advantages including more efficient water use, flexibility in cultivation locations, more hygiene, relatively small space or land requirements, ease of controlling and maintaining cultivated organisms, ease of maintaining water quality, environmentally friendly, safe from pollution. that occurs outside the aquatic environment and can be carried out all the time.

Recirculating Aquaculture System technology can also be used to control dissolved solids adapted to the aquaculture system and filter substrate (Fadhil et al, 2010). The filter substrate serves to filter dissolved solids. The use of the right type and filter material will determine the success of fish rearing in the recirculation system because it will determine the growth of non-pathogenic bacteria on the material. Currently, biological filter substrates are widely used because they are more environmentally friendly.

There are many biological filter materials that can be used, one of which is the bioball. Bioball is spherical, with a diameter of 4 cm, a specific area of ± 230 m^2 / m^3 with a cavity priority of 0.92, made of PVC material (Said, 2002). Bioball is a breeding ground for various bacteria that are needed to process toxins in the water. Bioball functions as a physiological filter which is a growing medium for bacteria that can remove ammonia contained in water (Alfia et al., 2013). The bacteria that grow on the bioball are nitrifying bacteria (Nitrosomonas sp and Nitrobacter sp). Nitrosomonas has a role in oxidizing ammonia to nitrite, while Nitrobacter has a role in oxidizing nitrite to nitrate, this nitrate will become plankton for fish natural food (0-fish, 2012).

Several studies related to improving water quality in aquaculture with recirculation and hydroponic systems have been carried out, such as; Mulyadi and Pamukas (2013) use a recirculation system in nursery and rearing of fish (Mystus nemurus); Alfia et al. (2013) used a recirculation system with a bioball filter in Tilapia (Oreochromis niloticus) culture; Adamu et al. (2014) studied the effect of different feed formulations on the recirculation system prototype container on the histology of the liver and kidneys of male hybrid catfish (Heterobranchus bidorsalis) with female Clarias gariepinus; Nelvia et al. (2015) studied the addition of a bioball to the maintenance media filter on the survival and growth of goldfish (Carassius auratus) fry. Based on the description above, a series of studies on the number and types of bacteria and the improvement of water quality in the cultivation of recirculation systems with different numbers of bioballs are needed. This research is aimed at analyzing the types and numbers of bacteria present in the bioball, improving water quality (temperature, pH, DO, TAN). , nitrite and nitrate) and analyzed the optimal amount of bioball for the live medium of bacteria.

Material and Method

Location and time of research. This research was conducted in January - July 2020, which was carried out in several laboratories, namely: fish maintenance was carried out at the Batam Marine Cultivation Fishery Center (BPBL), Jalan Trans Barelang Jembatan III, Setokok Island, Setokok Village, Bulang District, Batam, water quality analysis at the Laboratory of Fish and Environmental Health Testing at the Batam Marine Aquaculture Fisheries Agency (BPBL), Nitrogen analysis in the Aquaculture Environmental Laboratory, identifying types of bacteria and calculating the number of bacterial densities in the fish and environmental health examiners of the Batam Marine Cultivation Fishery Center (BPBL) PCR (polymerase chain reaction) test at PT. Genetics Science Indonesia, West Jakarta.

Materials and tools. The main ingredients that will be used in this research are as follows: the test animals used in this study are the seeds of pomfret (T. blochii,

Lacepede) originating from the Batam Marine Cultivation Fishery Center (BPBL) Batam, with a size of 10-11 cm. 300 heads. As the test feed, the commercial pellet pellet "GR-2" contains 46% protein, 10% fat, 13% ash content, 2% crude fiber, and 10% moisture content. The test feed was obtained from the Batam Marine Cultivation Center (BPBL). A round bucket with a diameter of 60 cm, a height of 45 cm, a volume of 150 L as a research vessel. Bioball with a diameter of 4 cm, specific area ± 230 m² / m³ with a cavity priority of 0.92, is made of PVC as a filter substrate (Figure 1a). As a filter container is a PVC filter gutter measuring 50 cm x 14 cm x 14 cm.

Experimental design. The research method used in this study was an experimental method completely randomized design, 1 factor, 5 levels of treatment and 3 replications (Steel and Torrie, 1993). As a factor in the study was the difference in the number of bioballs. The treatment level determination in this study is based on the best research results by Nelvia et al., (2015) that the use of 50 bioballs / gutters can improve water quality and provide 100% survival, weight growth 2.605 grams, length growth 1.48 mm on maintenance. goldfish fry (Carassius auratus) in closed recirculation. So that the treatment in this study are; A = No bioball (control) filter, B = 35 bioballs / filter container, C = 45 bioballs / filter container, D = 55 bioballs / filter container and E = 65 bioballs / filter container.

Research procedure. The silver pompano fish measuring 10 - 12.55 cm and body weight of 24.3 - 28.9 grams were obtained from the Batam Marine Cultivation Center (BPBL), adapted for 7 days before being used as test fish. Fish rearing containers use a closed recirculation system. Into each rearing container is filled with sea water with a salinity of 15 ‰. The rearing container is connected to a 50 cm x 14 cm x 14 cm PVC gutter as a filter container, which is placed at the top of the fish-raising containers. Then the water from the filter gutter will flow back through the PVC pipe with a diameter of 2.5 cm to the rearing tub of pomfret fish. Water from the fish rearing container will be flowed into the filter media (gutter) with the number of bioballs according to the treatment (Figure 1b) for each gutter according to the best findings of Nelvia et al., (2015) and using a water pump that has a power of 18 watts. After the water passes through the filter media (gutter), it will be returned to the fish-raising tub through the drain pipe in the filter container. In the maintenance medium, KP-SUPER N (*Nitrosomonas* sp. and *Nitrobacter* sp.) Trademark starter bacteria were added at a dose of 2.5 ml / 200 L / week (Hartini et al., 2013), to accelerate bacterial growth in the bioball.

(a) (b)

Figure 1. a. Bioball used as a filter substrate, b. The design of the Bawal Bintang aquaculture recirculation system.

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The test fish that have been adapted to the maintenance media are then randomly placed into 15 rearing containers. The fish were given commercial feed with 46% protein content, 10% fat, 13% ash content, 2% crude fiber, and 10% moisture content by adsatiation at 07:00, 12.00 and 17.00 WIB.

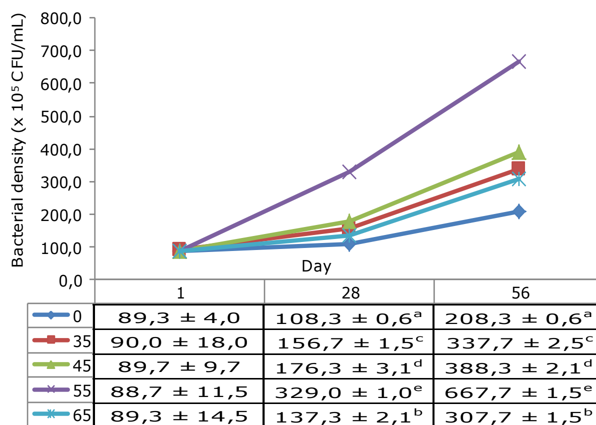
Measured response. The responses measured in this study were: density and type of bacteria in the filter container were observed at the beginning, middle and end of the study (SNI.01-2332.3-2006 and SNI.01-2332.3-2006); temperature, pH and salinity

were observed every day, dissolved oxygen (DO), Total Ammonia Nitrogen (TAN), Nitrite (NO_2) and Nitrate (NO_3) were observed every week. Water quality measurement procedures refer to SNI in the Public Works Service (1990).

Data analysis. Bacterial density data were analyzed according to the RAL model (Steel and Torrie, 1993). To determine the effect of salinity on each measured variable, a diversity analysis was performed using SPSS 17.0 software. If $P < 0.05$, the Newman-Keuls further test was performed to see the differences between treatments. Water quality data were analyzed descriptively using Microsoft Excel application and displayed in tables and graphs. To see the relationship between the number of bioballs and the density of bacteria with water quality, regression analysis was carried out.

Results and Discussion

Density of bacteria in the filter container. The results of bacterial density observations carried out on Plate Count Agar (PCA) media using the Total Plate Count method can be seen in Figure 2.



	1	28	56
0	89,3 ± 4,0	108,3 ± 0,6^{a}	208,3 ± 0,6^{a}
35	90,0 ± 18,0	156,7 ± 1,5^{c}	337,7 ± 2,5^{c}
45	89,7 ± 9,7	176,3 ± 3,1^{d}	388,3 ± 2,1^{d}
55	88,7 ± 11,5	329,0 ± 1,0^{e}	667,7 ± 1,5^{e}
65	89,3 ± 14,5	137,3 ± 2,1^{b}	307,7 ± 1,5^{b}

Figure 2. Bacterial density in all treatments during the study

Figure 2 shows that the bacterial density from day 1 to day 28 in treatment 0, 35, 45 and 65 bioballs / filter containers are relatively similar and insignificant increase, in contrast to treatment 55 bioballs / filter containers a significant increase of bacteria than other treatments. This is due to the fact that the bacteria and silver pompano fish are still adapting to the media in which they live, besides that the nutrients that come from the remaining feed and fish feces are still not sufficient to support the maximum bacterial growth. After the 28th day the number of bacteria in all treatments continued to increase reaching a maximum peak on the 56th day. This is due to the increase in organic matter from fecal matter and fish feed along with the growth of the silver pompano, which is a source of bacterial nutrition, thereby increasing the rate of bacterial development. In accordance with the opinion of Sunarto (2003), dissolved organic matter is needed by living bacteria, then Sidharta (2000) explains that organic material contains carbon, nitrate, phosphate, sulfur, ammonia, and several minerals which are nutrients for bacterial growth. Giving KP SUPER N which is given routinely in a cultivation container causes the bacteria to continue to grow because of the availability of organic materials that can be used optimally. The highest bacterial density was found in the treatment of 55 bioballs / filter containers.

The highest bacterial density was found in the treatment of 55 bioballs / filter containers at the end of the study, namely 667.7 x 10^5 CFU / mL, this is because the

number of bioballs is 55 in a filter container measuring 50 cm x 14 cm x 14 cm for culture media with volume 80 L, is the optimal amount as a living medium for bacteria. This is because the number of bioballs with the size of the filter container is in a balanced state, thus providing an opportunity for oxygen to enter the bioball cavity and bacteria to get sufficient oxygen. In accordance with the opinion of Nut (2009) and Nelvia et al. (2015) which states that the location of the bioball which is not too dense provides an opportunity for oxygen to enter the bioball cavity so that the bacteria attached to the bioball get sufficient oxygen, if the oxygen obtained by the bacteria is sufficient then the bacteria will grow a lot in the bioball cavity and work more optimally.

The lowest bacterial density was found in treatment A (without bioball), which was 208.3 x 10^5 CFU / mL, this was due to the filter without using bioball and without the addition of starter bacteria (KP SUPER N) causing limited live media for bacteria and no additional nutrient availability. for the growth and development of bacteria. The low density of bacteria when using the highest number of bioballs (65 bioballs / filter container) is due to the large number of bioballs for the filter container size of 50 cm x 14 cm x 14 cm, so that the location of the bioballs is very tightly positioned and leaves no empty space. According to Nelvia et al., (2015) the amount of bioball that is too dense in the gutter can cause bacteria in the bioball cavity to not live if there is a lack of oxygen supply, the performance of the bioball filter decreases so that the process of decomposing organic matter by nitrifying bacteria does not run.

The biofilter system is the removal of ammonia, ammonia will accumulate and reach toxic levels if it is not transferred by a nitrification process where the process is first that ammonia is oxidized to toxic nitrite, then to nitrate, which is relatively non-toxic. The above process involves Nitrosomonas and Nitrobacter bacteria. Bacteria grow like a film on the surface of the material (biofilter container) or adhere to organic particles. Biofilter consists of a medium with a large surface for the growth of nitrite bacteria. In the recirculation system, the bacteria plays a role in converting toxic substances (ammonia) to non-toxic substances (nitrates). The performance of bacteria in the biofilter system is marked by an increase in the average BOD value in the culture medium (Nurhidayat et al., 2012). The more bioballs that are used, the more opportunities there are for bacteria to stick, but too much use can also make bacteria die, due to the lack of oxygen received by bacteria (Nelvia et al., 2015).

ANOVA results showed that the number of bioballs had an effect on the density of bacteria on day 28 and 56. The Newman Keuls range study test gave the results of differences in bacterial density between treatments. The results of the regression analysis between the number of bioballs and bacterial density provide the following regression equation:

$$Y = 227.9 + 3,851X$$

Information: Y = bacterial density, X = number of bioballs, r = 0.557 and R2 = 0.311

The results of the regression analysis showed that the number of bioballs had a positive correlation with bacterial density (r = 0.557), this means that the number of bacteria in the filter container increased with the increase in the number of bioballs. The increase in the number of bacteria in the filter container was influenced by the number of bioballs by 31.1% and 68.9%, which is thought to be influenced by the content of organic matter. Nelvia et al (2015) stated that the bioball is a breeding ground for various bacteria needed in the breakdown of organic matter. According to Sunarto (2003) dissolved organic matter is needed by bacteria to live. Furthermore, according to Sidharta, (2000) organic matter contains carbon, nitrate, phosphate, sulfur, ammonia, and several minerals which are nutrients for bacterial growth.

Identification of bacteria in filter containers. Based on the observations, there were 7 pure bacterial isolates. The identification of bacteria against the isolates found in the filter media was carried out by physical, biochemical and sensitivity tests to see which bacteria had the greatest inhibition zone against pathogenic bacteria.

Physics test. The results of observations of color, shape, edge, elevation and gram of the seven isolates found are presented in Table 1.

Table 1. Observation Results of Bacterial Characteristics

No	Isolate	Colony				Cell		Treatment
		Color	**Shape**	**Edge**	**Elevation**	**Gram Type**	**Shape**	
1	A1	Yellow	Circular	Entire	Umbonate	Negative	Stem	P_2U_1
2	A2	Beige	Circular	Entire	Raised	Negative	Stem	P_0U_3
3	A3	White	Circular	Entire	Raised	Negative	Stem	P_3U_2
4	A4	White	Circular	Entire	Raised	Negative	Stem	P_4U_1
5	A5	Beige	Filamentous	Entire	Filiform	Negative	Stem	P_1U_1
6	A6	White	Circular	Entire	Raised	Negative	Stem	P_4U_2
7	A7	White	Circular	Entire	Raised	Negative	Stem	P_3U_3

Table 1 shows that all bacterial isolates have gram-negative, entire edges and are rod-shaped. The dominant bacterial colony was white (4 isolates), 2 isolates were beige and 1 isolate was yellow. Colonies were circular / round (6 isolates) and filamentous (1 isolate). The elevation of the dominant bacterial colony was raised (5 isolates), 1 umbonate isolate and 1 filiform isolate.

Biochemical test. The results of observations of the Catalase, Oxidase, Motility, O / F, Indol and TSIA tests are presented in Table 2.

Table 2.Bacterial Biochemical Test Results

No.	Isolate	Biochemical Characteristics						Treatmen
		Catalase	**Oxidase**	**Motility**	**O/F**	**Indole**	**TSIA**	
1	A1	+	+	+	-	-	A/K	P_2U_1
2	A2	+	+	-	F	-	K/K	P_0U_3
3	A3	+	-	+	O	-	A/K	P_3U_2
4	A4	+	+	+	F	-	A/A	P_4U_1
5	A5	+	+	+	F	+	A/A	P_1U_1
6	A6	+	+	+	F	-	A/A	P_4U_2
7	A7	+	+	-	F	-	A/A	P_3U_3

Information: O = oxidative, F = fermentative, A / K = alkaline acid, K / K = alkaline, A / A = acid

Table 2 shows that all bacterial isolates have positive catalase because these bacteria have a catalase enzyme with a reaction in the form of gas bubbles at the time of dropping H2O2. According to Stoica (2016), catalase is an enzyme that is owned by the majority of bacteria and is involved in the decomposition of hydrogen peroxide into water and oxygen. Hydrogen peroxide and oxygen are used for electron transport in the fermentation of both aerobic and facultative aerobic bacteria.

Oxidase tests A1, A2, A3, A4, A5, A6 and A7 are positive because of the color change on the oxidase paper which indicates oxidase activity, while A3 is negative. Five isolates (A1, A3, A4, A5 and A6) showed motile results in the motility test due to the spread of bacterial growth on the SIM medium and did not grow on the part of the bacteria puncture site. The O / F test results showed no color change in the paraffin-covered media and the color change on the exposed media. Five bacterial isolates (A2, A4, A5, A6 and A7) were observed to be fermentative because the tube without paraffin and the tube filled with paraffin changed color to yellow. According to Fahri (2008) in the O / F test, oxidative organisms occur when a color change is seen in the open media, while fermentative organisms can be indicated by seeing no color change in the closed media.

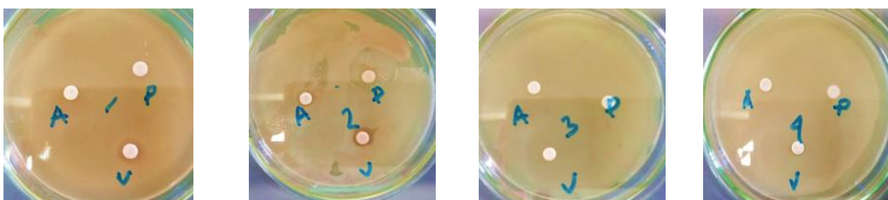
The indole test shows 6 isolates (A1, A2, A3, A4, A6 and A7) give negative results (-) marked with a yellow color on the surface of the media which means that the bacteria

are unable to break down the amino acid tryptophan, and no red ring is formed on the surface of the media. after dropping the kovac reagent on SIM media. According to Acharya (2012), the indole test was carried out to determine the ability of bacteria to break down amino acid tryptophan to form indole compounds. Tryptophan is hydrolyzed by tryptophanase to produce three possible end products, one of which is indole. Indole production is detected by kovac or Ehrlich's reagent which is composed of 4-p-benzaldehyde dimethylamino, this reagent reacts with indole to produce a red compound. A positive reaction is indicated if there is a red color on the surface of the medium which indicates that the bacteria are able to break down the amino acid tryptophan. In the TSIA test, 4 isolates (A4, A5, A6 and A7) showed acidic properties (A / A), 2 isolates (A1 and A3) were acid alkaline and 1 isolate (A2) was alkaline.

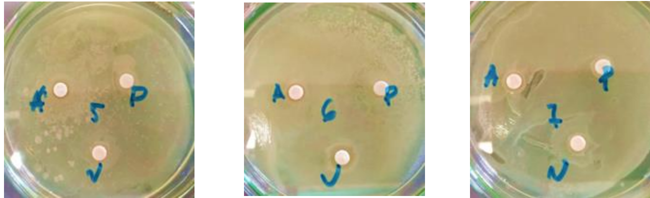
Sensitivity test. The sensitivity test to pathogenic bacteria was carried out after observing the physical and biochemical tests of the seven isolates. This test aims to find probiotic bacteria candidates for PCR testing. The results of the sensitivity test on 7 isolates are presented in Figure 3. Inhibition zones produced by seven bacterial isolates found in different filter containers. The bacteria in isolate A1 were able to inhibit the growth of Vibrio bacteria during the 24-hour incubation period with a temperature of 37 0C. This is indicated by the formation of a clear area around the blank disk, but it is unable to inhibit the growth of Aeromonas and Pseudomonas bacteria. After an incubation period of 48 hours the inhibition zone remains clear, which indicates that these bacteria can kill (bacteriocidal) Vibro bacteria. Similar to the bacteria in isolate A2, a clear zone was formed around the blank disk against Pseudomonas and Vibrio bacteria during the 48-hour incubation period, this indicates that A2 bacteria are bacteriocidal against Pseudomonas and Vibrio, but cannot inhibit the growth of Aeromonas bacteria.

Bacteria in A3 and A4 isolates were unable to inhibit the growth of Aeromonas, Pseudomonas and Vibrio bacteria, it was seen that no inhibition zone was formed on the balnk disk. In A5 and A7 isolates an inhibition zone occurred against Aeromonas, Pseudomonas and Vibrio bacteria during the 24-hour incubation period, but during the 48-hour incubation period, the clear area became cloudy, indicating that these bacteria were only able to inhibit (bacteriostatic) the three pathogenic bacteria . In A6 isolate a clear zone was also formed during the 48-hour incubation period against Aeromonas and Vibrio bacteria, but no inhibition zone was formed against Pseudomonas bacteria. This indicates that the A6 bacteria are able to kill (bacteriocidal) Aeromonas and Vibrio bacteria.

This is in line with the opinion of Farlex (2005) which states that a material is said to be sensitive to bacteria characterized by the presence of an inhibition zone around the blank disk. Furthermore, according to Dwyana and Murniati (2020) this area of inhibition is formed due to the presence of bioactive compounds contained in microbes that produce exoenzymes that function to break down organic matter and also excrete hydrolytic enzymes, proteases, lipases, amylases, and cellulose so that they are able to hydrolyze polysaccharides as a carbon source. and electron donors.



A1 ; Inhibition	A2 ;Inhibition	A3 ; Inhibition	A4 ; Inhibition
A = 0, P = 0 and	A = 0, P = 8,88 mm and	A = 0, P = 0 and	A = 0, P = 0
V = 3,74 mm (bacteriocidal)	V = 3,49 mm (bacteriocidal)	V = 0	and V = 0



A5 ; Inhibition	A6 ; Inhibition	A7 ; Inhibition
A = 4,32 mm,	A = 1,62 mm, P = 0	A = 3,15 mm,
P = 3,87 mm and	and V = 3,8 mm (bacteriocidal)	P = 2,58 mm and
V = 5,24 mm		V = 2,20 mm

Figure 3. Sensitivity test of bacterial isolates against pathogenic bacteria
Description: A: Aeromonas, P: Pseudomonas, V: Vibrio

Madigan et al., (2012), grouped bacteria based on their selective toxicity, antimicrobial compounds had 3 kinds of effects on microbial growth, namely; 1.Bacteriostatic, provides an effect by inhibiting growth but not killing. Bacteriostatic compounds inhibit protein synthesis or bind to ribosomes; 2. Bacteriocidal, gives effect by killing cells but not cell lysis or cell breakdown. 3. Bacteriolytic, causing cells to become lysis or cell breakdown. Furthermore, Dwyana and Murniati (2020) stated that the clear zone formed during the 24-hour incubation period on disc paper during the sensitivity test indicated that the bacteria was bacteriostatic, while 48 hours indicated that the bacteria was classified as bacteriocidal.

According to Jannata (2014) the classification of bacterial growth inhibition responses, based on the inhibition zone consists of 4, namely; weak response (diameter ≤5 mm), medium (diameter 5-10 mm), strong (diameter 10-20 mm), and very strong (≥20 mm). Based on this classification isolates A5, A6 and A7 were classified as having a weak response to Aeromonas, isolate A2 had a moderate response and isolates A5 and A7 had a weak response to Pseudomonas bacteria. Furthermore, A5 isolate had a moderate response to Vibrio bacteria, while isolates A1, A2, A6 and A7 were classified as having a weak response. According to Sugita et al. (1996), this is because these bacteria are able to produce antimicrobial compounds which are a form of competition for nutrients and energy. This mechanism is believed to be able to inhibit the growth of pathogens in the filter container. The inhibiting compounds are very diverse, including siderophores, bacteriocins, lysozymes, proteases, hydrogen peroxidase, and organic acids. According to Rengpipat et al (2000) siderophore is a compound with a low molecular weight (<1500) and is a specific agent to bind ferric ions and can dissolve iron precipitates and change them into the form required for microbial growth. Its ability to form siderophores causes other organisms, especially aquatic animal pathogens, to be unable to obtain these elements and become inhibited.

Based on the results of observations on the inhibition zone diameter and inhibition power, the bacterial isolates selected as candidates for probiotic bacteria were isolates A1, A2 and A6 which were classified as bacteriocidal, then coded MUL_1, MUL_2 and MUL_3. Furthermore, to determine the type of the three bacteria, DNA analysis was carried out using PCR (Polymerase Chain Reaction).

Polymerase Chain Reaction (PCR) test

The results of amplification and visualization of DNA fragments of the 16S rRNA gene. The genomic DNA that had been isolated and extracted from isolates MUL_1, MUL_2 and MUL_3, then amplified the 16S rRNA markers using PCR technique. PCR analysis of the 3 bacteria isolates used primary base 27F forward: AGAGTTTGATCMTGGCTCAG and revers 1492R: TACGGYTA CCTTGTTACGACTT. The analysis results showed positive with the formation of bands with a total molecular weight (bp) of 1000 - 1500 bp. The results of amplified DNA visualization using 27F and 1492R electrophoresed with UV light are presented in Figure 4. The amplified fragment has a wavelength of 1227-1372 bp, after seeing Geneious it is known that isolate MUL_1 produces a single band that is clearly visible with a molecular weight of 1331 bp (base pair), MUL_2 1227 bp and MUL_3 1372 bp according to the primary amplification size 27F and 1492R used by comparison using 1 Kb Ladder DNA. The size of this size is in accordance with the expected size of the bacterial 16S rRNA genes, which is between 1300 - 1500 bp (Seprianto et al., 2017).

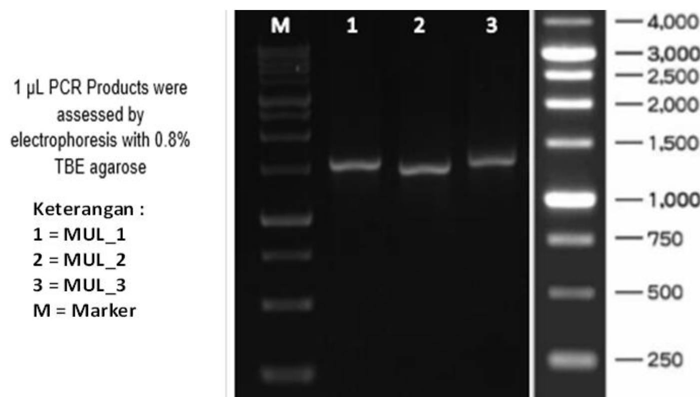


Figure 4. Results of Universal DNA-PCR Amplification on Agarose Gel

The base pairs obtained were combined and trimmed using Bioedit 7.0 software. DNA sequencing analysis gives the following results for the nucleotide and fasta sequences:

A1. The nucleotide sequence and fasta MUL_1

- Nucleotide sequence

```
Sequence Assembly 1331  bp
1     CTTGCGGTTA GCGCACTGCC TTCGGGTAAC CCAACTCCCA TGGTGTGACG GGCGGTGTGT
61    ACAAGGCCCG GGAACGTATT CACCGCGGCA TGCTGATCCG CGATTACTAG CGATTCCAAC
121   TTCATGCACC CGAGTTGCAG AGTGCAATCC GAACTGAGAT GGTTTTTGGA GATTAGCTCG
181   ACCTCGCGGT CTCGCTGCCC ACTGTCACCA CCATTGTAGC ACGTGTGTAG CCCAGCCCGT
241   AAGGGCCATG AGGACTTGAC GTCATCCCCA CCTTCCTCTC GGCTTATCAC CGGCAGTCCC
301   CTTAGAGTGC CCAACTTAAG GCTGGCAACT AAGGGCGAGG GTTGCGCTCG TTGCGGGACT
361   TAACCCAACA TCTCACGACA CGAGCTGACG ACAGCCATGC AGCACCTGTC TTGGGTCCAG
421   CCTAACTGAA GGATACCGTC TCCGGTATCC GCGACCCAGA TGTCAAGGGC TGGTAAGGTT
481   CTGCGCGTTG CTTCGAATTA AACCACATGC TCCACCGCTT GTGCGGGCCC CCGTCAATTC
541   CTTTGAGTTT TAATCTTGCG ACCGTACTCC CCAGGCGGGA AGCTTAATGC GTTAACTGCG
601   CCACCGACAG GTAAACCTGC CGACGGCTAG CTTCCATCGT TTACGGCGTG GACTACCAGG
661   GTATCTAATC CTGTTTGCTC CCCACGCTTT CGCACCTCAG CGTCAGTATC GAGCCAGTGA
721   GCCGCCTTCG CCACTGGTGT TCCTCCGAAT ATCTACGAAT TTCACCTCTA CACTCGGAAT
781   TCCACTCACC TCTCTCGAAC TCTAGATCGG CAGTATTAGA GGCAGTTCCG GGGTTGAGCC
841   CCGGGATTTC ACCCCTAACT GACCGATCCG CCTACGCGCG CTTTACGCCC AGTAATTCCG
901   AACAACGCTA GCCCCCTTCG TATTACCGCG GCTGCTGGCA CGAAGTTAGC CGGGGCTTCT
961   TCTCCGGTTA CCGTCATTAT CTTCACCGGT GAAAGAGCTT TACAACCCTA GGGCCTTCAT
1021  CACTCACGCG GCATGGCTGG ATCAGGCTTG CGCCCATTGT CCAATATTCC CCACTGCTGC
1081  CTCCCGTAGG AGTCTGGGCC GTGTCTCAGT CCCAGTGTGG CTGATCATCC TCTCAGACCA
1141  GCTACTGATC GTCGCCTTGG TGAGCCTTTA CCTCACCAAC TAGCTAATCA GACATGGGCT
1201  CATCTAACTC CGATAAATCT TTCTCCCGAA GGACGTATAC GGTATTAGTT CAAGTTTCCC
1261  TGAGTTATTC CGTAGAGCTA GGTAGATTCC CATGCATTAC TCACCCGTCT GCCGCTCCCC
1321  CGAGGGGGCG C
```

- Fasta

> MUL_1
CTTGCGGTTAGCGCACTGCCTTCGGGTAACCCAACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATT
CACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCAACTTCATGCACCCGAGTTGCAGAGTGCAATCCGAACTGAGATG
GTTTTTGGAGATTAGCTCGACCTCGCGGTCTCGCTGCCCACTGTCACCACCATTGTAGCACGTGTGTAGCCCAGCCCGTAA
GGGCCATGAGGACTTGACGTCATCCCCACCTTCCTCTCGGCTTATCACCGGCAGTCCCCTTAGAGTGCCCAACTTAAGGCT
GGCAACTAAGGGCGAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCAGC
ACCTGTCTTGGGTCCAGCCTAACTGAAGGATACCGTCTCCGGTATCCGCGACCCAGATGTCAAGGGCTGGTAAGGTTCTGC
GCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTTAATCTTGCGACCGTA
CTCCCCAGGCGGGAAGCTTAATGCGTTAACTGCGCCACCGACAGGTAAACCTGCCGACGGCTAGCTTCCATCGTTTACGGC
GTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTCAGCGTCAGTATCGAGCCAGTGAGCCGCCTT
CGCCACTGGTGTTCCTCCGAATATCTACGAATTTCACCTCTACACTCGGAATTCCACTCACCTCTCTCGAACTCTAGATCGGC
AGTATTAGAGGCAGTTCCGGGGTTGAGCCCCGGGATTTCACCCCTAACTGACCGATCCGCCTACGCGCGCTTTACGCCCAG
TAATTCCGAACAACGCTAGCCCCCTTCGTATTACCGCGGCTGCTGGCACGAAGTTAGCCGGGGCTTCTTCTCCGGTTACCG
TCATTATCTTCACCGGTGAAAGAGCTTTACAACCCTAGGGCCTTCATCACTCACGCGGCATGGCTGGATCAGGCTTGCGCC
CATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGATCATCCTCTCAG
ACCAGCTACTGATCGTCGCCTTGGTGAGCCTTTACCTCACCAACTAGCTAATCAGACATGGGCTCATCTAACTCCGATAAAT
CTTTCTCCCGAAGGACGTATACGGTATTAGTTCAAGTTTCCCTGAGTTATTCCGTAGAGCTAGGTAGATTCCCATGCATTACT
CACCCGTCTGCCGCTCCCCCGAGGGGGCGC

A2. The nucleotide sequence and fasta MUL_2

- Nucleotide sequence

```
Sequence Assembly 1227  bp
1     GTTAGACTAC CTACTTCTGG TGCAACAAAC TCCCATGGTG TGACGGGCGG TGTGTACAAG
61    GCCCGGGAAC GTATTCACCG CGGCATTCTG ATCCGCGATT ACTAGCGATT CCGACTTCAT
121   GGAGTCGAGT TGCAGACTCC AATCCGGACT ACGATCGGCT TTTTGAGATT AGCATCCTAT
181   CGCTAGGTAG CAACCCTTTG TACCGACCAT TGTAGCACGT GTGTAGCCCT GGCCGTAAGG
241   GCCATGATGA CTTGACGTCG TCCCCGCCTT CCTCCAGTTT GTCACTGGCA GTATCCTTAA
301   AGTTCCCATC CGAAATGCTG GCAAGTAAGG AAAAGGGTTG CGCTCGTTGC GGGACTTAAC
361   CCAACATCTC ACGACACGAG CTGACGACAG CCATGCAGCA CCTGTATCTA GATTCCCGAA
421   GGCACCAATC CATCTCTGGA AAGTTTCTAG TATGTCAAGG CCAGGTAAGG TTCTTCGCGT
481   TGCATCGAAT TAAACCACAT GCTCCACCGC TTGTGCGGGC CCCCGTCAAT TCATTTGAGT
541   TTTAGTCTTG CGACCGTACT CCCCAGGCGG TCTACTTATC GCGTTAGCTG CGCCACTAAA
601   GCCTCAAAGG CCCCAACGGC TAGTAGACAT CGTKTACGGC ATGGACTACC AGGGTATCTA
661   ATCCTGTTTG CTCCCCATGC TTTCGTACCT CAGCGTCAGT ATTAGGCCAG ATGGCTGCCT
721   TCGCCATCGG TATTCCTCCA GATCTCTACG CATTTCACCG CTACACCTGG AATTCTACCA
781   TCCTCTCCCA TACTCTAGCC ATCCAGTATC GAATGCAATT CCCAAGTTAA GCTCGGGGAT
841   TTCACATTTG ACTTAAATGG CCGCCTACGC ACGCTTTACG CCCAGTAAAT CCGATTAACG
901   CTCGCACCCT CTGTATTACC GCGGCTGCTG GCACAGAGTT AGCCGGTGCT TATTCTGCGA
961   GTAACGTCCA CTATCCAGTA GTATTAATAC TAGTAGCCTC CTCCTCGCTT AAAGTGCTTT
1021  ACAACCATAA GGCCTTCTTC ACACACGCGG CATGGCTGGA TCAGGGTTCC CCCCATTGTC
1081  CAATATTCCC CACTGCTGCC TCCCGTAGGA GTCTGGGCCG TGTCTCAGTC CCAGTGTGGC
1141  GGATCATCCT CTCAGACCCG CTACAGATCG TCGCCTTGGT AGGCCTTTAC CCCACCAACT
1201  AGCTAATCCG ACTTAGGCTC ATCTATT
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- Fasta

> MUL_2
GTTAGACTACCTACTTCTGGTGCAACAAACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGC
GGCATTCTGATCCGCGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTCCAATCCGGACTACGATCGGCTTTT
TGAGATTAGCATCCTATCGCTAGGTAGCAACCCTTTGTACCGACCATTGTAGCACGTGTGTAGCCCTGGCCGTAAGGGCCA
TGATGACTTGACGTCGTCCCCGCCTTCCTCCAGTTTGTCACTGGCAGTATCCTTAAAGTTCCCATCCGAAATGCTGGCAAGT
AAGGAAAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCAGCACCTGTAT
CTAGATTCCCGAAGGCACCAATCCATCTCTGGAAAGTTTCTAGTATGTCAAGGCCAGGTAAGGTTCTTCGCGTTGCATCGAA
TTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAGTCTTGCGACCGTACTCCCCAGGCGGT
CTACTTATCGCGTTAGCTGCGCCACTAAAGCCTCAAAGGCCCCAACGGCTAGTAGACATCGTGTACGGCATGGACTACCAG
GGTATCTAATCCTGTTTGCTCCCCATGCTTTCGTACCTCAGCGTCAGTATTAGGCCAGATGGCTGCCTTCGCCATCGGTATT
CCTCCAGATCTCTACGCATTTCACCGCTACACCTGGAATTCTACCATCCTCTCCCATACTCTAGCCATCCAGTATCGAATGCA
ATTCCCAAGTTAAGCTCGGGGATTTCACATTTGACTTAAATGGCCGCCTACGCACGCTTTACGCCCAGTAAATCCGATTAAC
GCTCGCACCCTCTGTATTACCGCGGCTGCTGGCACAGAGTTAGCCGGTGCTTATTCTGCGAGTAACGTCCACTATCCAGTA
GTATTAATACTAGTAGCCTCCTCCTCGCTTAAAGTGCTTTACAACCATAAGGCCTTCTTCACACACGCGGCATGGCTGGATC

AGGGTTCCCCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCGGA
TCATCCTCTCAGACCCGCTACAGATCGTCGCCTTGGTAGGCCTTTACCCCACCAACTAGCTAATCCGACTTAGGCTCATCTA
TT

A3. The nucleotide sequence and fasta MUL_3

- Nucleotide sequence

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Sequence Assembly 1372  bp
1      AAGGTTAAGC TATCTACTTC TGGTGCAGCC CACTCCCATG GTGTGACGGG CGGTGTGTAC
61     AAGGCCCGGG AACGTATTCA CCGTGGCATT CTGATCCACG ATTACTAGCG ATTCCGACTT
121    CATGGAGTCG AGTTGCAGAC TCCAATCCGG ACTACGACCA GCTTTATGGG ATTAGCTCCA
181    CCTCGCGGCT TCGCAACCCT CTGTACTGAC CATTGTAGCA CGTGTGTAGC CCTACTCGTA
241    AGGGCCATGA TGACTTGACG TCGTCCCCAC CTTCCTCCGG TTTATCACCG GCAGTCTCCC
301    TAAAGTTCCC GGCATGACCC GCTGGCAAGT AAGGATAGGG GTTGCGCTCG TTGCGGGACT
361    TAACCCAACA TTTCACAACA CGAGCTGACG ACAGCCATGC AGCACCTGTC TCACAGTTCC
421    CGAAGGCACT GAAGCATCTC TGCTAAATTC TGTGGATGTC AAGAGTAGGT AAGGTTCTTC
481    GCGTTGCATC GAATTAAACC ACATGCTCCA CCGCTTGTGC GGGCCCCCGT CAATTCATTT
541    GAGTTTTAAC CTTGCGGCCG TACTCCCCAG GCGGTCTACT TAATGCGTTA GCTTGAGAGC
601    CCAGTGTTCA AGACACCAAA CTCCGAGTAG ACATCGTTTA CGGCGTGGAC TACCAGGGTA
661    TCTAATCCTG TTTGCTCCCC ACGCTTTCGT GCCTGAGCGT CAGTCTTTGT CCAGGGGGCC
721    GCCTTCGCCA CCGGTATTCC TCCAGATCTC TACGCATTTC ACCGCTACAC CTGGAATTCT
781    ACCCCCCTCT ACAAGACTCT AGTTTGCCAG TTCGAAATGC GGTTCCCAGG TTGAGCCCGG
841    GGCTTTCACA TCTCGCTTAA CAAACCGCCT GCGCACGCTT TACGCCCAGT AATTCCGATT
901    AACGCTCGCA CCCTCCGTAT TACCGCGGCT GCTGGCACGG AGTTAGCCGG TGCTTCTTCT
961    GCGAGTAACG TCACAGATGT AAGGTATTAA CTTACACCCT TTCCTCCTCG CTGAAAGTGC
1021   TTTACAACCC GAAGGCCTTC TTCACACACG CGGCATGGCT GCATCAGGGT TTCCCCCATT
1081   GTGCAATATT CCCCACTGCT GCCTCCCGTA GGAGTCTGGG CCGTGTCTCA GTCCCAGTGT
1141   GGCTGATCAT CCTCTCAGAC CAGCTAGGGA TCGTCGCCTA GGTGAGCCTT TACCTCACCT
1201   ACTAGCTAAT CCCACCTGGG CTTATCCATC AGCGCAAGGC CCGAAGGTCC CCTGCTTTCC
1261   CCCGTAGGGC GTATGCGGTA TTAGCAGTCG TTTCCAACTG TTATCCCCCA CAAATGGGCA
1321   AATTCCCAGG CATTACTCAC CCGTCCGCCG CTCGTCATCT TCAAAAGCAA GC
```

- Fasta

>MUL_3
AAGGTTAAGCTATCTACTTCTGGTGCAGCCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCAC
CGTGGCATTCTGATCCACGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTCCAATCCGGACTACGACCAGC
TTTATGGGATTAGCTCCACCTCGCGGCTTCGCAACCCTCTGTACTGACCATTGTAGCACGTGTGTAGCCCTACTCGTAAGGG
CCATGATGACTTGACGTCGTCCCCACCTTCCTCCGGTTTATCACCGGCAGTCTCCCTAAAGTTCCCGGCATGACCCGCTGGC
AAGTAAGGATAGGGGTTGCGCTCGTTGCGGGACTTAACCCAACATTTCACAACACGAGCTGACGACAGCCATGCAGCACCT
GTCTCACAGTTCCCGAAGGCACTGAAGCATCTCTGCTAAATTCTGTGGATGTCAAGAGTAGGTAAGGTTCTTCGCGTTGCAT
CGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGG
CGGTCTACTTAATGCGTTAGCTTGAGAGCCCAGTGTTCAAGACACCAAACTCCGAGTAGACATCGTTTACGGCGTGGACTA
CCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGTGCCTGAGCGTCAGTCTTTGTCCAGGGGGCCGCCTTCGCCACCG
GTATTCCTCCAGATCTCTACGCATTTCACCGCTACACCTGGAATTCTACCCCCCTCTACAAGACTCTAGTTTGCCAGTTCGAA
ATGCGGTTCCCAGGTTGAGCCCGGGGCTTTCACATCTCGCTTAACAAACCGCCTGCGCACGCTTTACGCCCAGTAATTCCG
ATTAACGCTCGCACCCTCCGTATTACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGCGAGTAACGTCACAGAT
GTAAGGTATTAACTTACACCCTTTCCTCCTCGCTGAAAGTGCTTTACAACCCGAAGGCCTTCTTCACACACGCGGCATGGCT
GCATCAGGGTTTCCCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTG
GCTGATCATCCTCTCAGACCAGCTAGGGATCGTCGCCTAGGTGAGCCTTTACCTCACCTACTAGCTAATCCCACCTGGGCTT
ATCCATCAGCGCAAGGCCCGAAGGTCCCCTGCTTTCCCCCGTAGGGCGTATGCGGTATTAGCAGTCGTTTCCAACTGTTAT
CCCCCACAAATGGGCAAATTCCCAGGCATTACTCACCCGTCCGCCGCTCGTCATCTTCAAAAGCAAGC

16S rRNA gene sequence alignment with basic local alignment search tool (BLAST). Analysis of BLAST (Basic Local Alignment Search tool) online via: http://blast.ncbi.nlm.nih.gov/Blast.cgi on the results of DNA sequencing was carried out to search for species names, percentages of DNA homology sequenced with existing databases on GenBank. The results of identification of probiotic bacterial isolates from the BLAST results were taken based on the highest homology that has the closest relationship with the bacteria in GenBank. Based on the nucleotides registered with the National Center for Biotechnology Information (NCBI), the species, strains and accession numbers of bacterial isolates identified in this study are presented in Table 3.

Table 3. Results of Tracing 16S rRNA Sequencing of Bacterial Isolates with the BLAST System

Isolate	Species	Strain	Accession Number	Query Coverage	Homology
MUL_1	*Acinetobacter* sp.	MUL37	MT229070	100%	100%
MUL_2	*Shewanella* sp.	MUL31	MT229068	100%	100%
MUL_3	*Nitratireductor* sp.	MUL35	MT229069	100%	100%

The alignment results of 16S rRNA gene sequences with BLAST (Table 3) showed that the bacterial isolate MUL_1 was identified as Acinetobacter sp. strain MUL37 with

100% homology and 100% query coverage; MUL_2 isolate was identified as Shewanella sp. strain MUL31 with 100% homology and 100% query coverage; and bacterial isolate MUL_3 identified as Nitratireductor sp. strain MUL35 with 100% homology and 100% query coverage. The types of bacteria found are thought to have come from the waters of the Batam Sea which are used as a medium for maintaining pomfret, because the starter bacteria added to the media do not contain these types of bacteria.

According to Aditya et al., (2017) the sequence equation between 93-97% can represent the identity of bacteria at the genus level but different at the species level. Furthermore, Hagstrom et al (2000) and Seprianto et al (2017) state that bacteria that have a 16S rRNA sequence equation greater than 97% are the same species, while the equation between 93-97% can represent identity at the genus level but differ at the level species. According to Dancourt et al (2000), if the similarity of the sequences is less than 97%, it is likely a new species because there is no data in the database or the size of the sequencing results compared to the database is too short.

Phylogenetic tree analysis. The analysis of phylogenetic tree formation was carried out by looking at the similarities that exist in the genetic isolates of MUL_1, MUL_2 and MUL_3 bacteria with a database of the results of alignment of nucleotide sequences in GenBank using the Mega X 10.0.5 WIN 64 application. Phylogenetic analysis using the UPGMA distance-based method (Unwight Pair Group Method with Arithmetic Average) or the unweight pair group method with arithmetic mean is the simplest method of all the clustering methods used to build phylogenetic trees. Phylogenetic analysis used 17 sequences with 3 sample sequences and 14 comparison sequences, the alignment process used 1000 bootstraps and Kimura 2 model parameters. Branches that connect the dots (nodes) are taxonomic units, such as species or genes, tree roots are the ancestors (ancestors) for all organisms, the dendogram is presented in Figure 5.

Figure 5 shows the bacteria MUL_1, MUL_2 and MUL_3 are in 1 cluster and separated from the type of control bacteria, this means that between MUL_1 and MUL_2 have close relationships, even tend to be the same species or belong to the same subspecies, while MUL_3 bacteria have a genus. the same one. Judging from the point of branching, the three isolates were closely related to Nitratireductor and Shewanella.

The results of the calculation of genetic distance using Mega X 10.0.5 WIN 64, it was found that the distances between species varied, ranging from 0.00 to 1.6659. The smallest genetic distance was found between bacteria MUL_1 and MUL_2 of 0.2005, while between MUL_1 and MUL_3 was 0.2081. The genetic distance between bacteria MUL_1 and Acinetobacter venetianus (GenBank: NR.042049.1) was 1.2531; between MUL_2 and Shewanella algae (GenBank: NR117771.1) of 1.2955; and between MUL_3 and Nitratireductor aquimarinus (GenBank: 117929.1) of 1.2468. According to Tallei et al. (2016) the smaller the value of the genetic distance between two organisms, the closer the kinship of the two is.

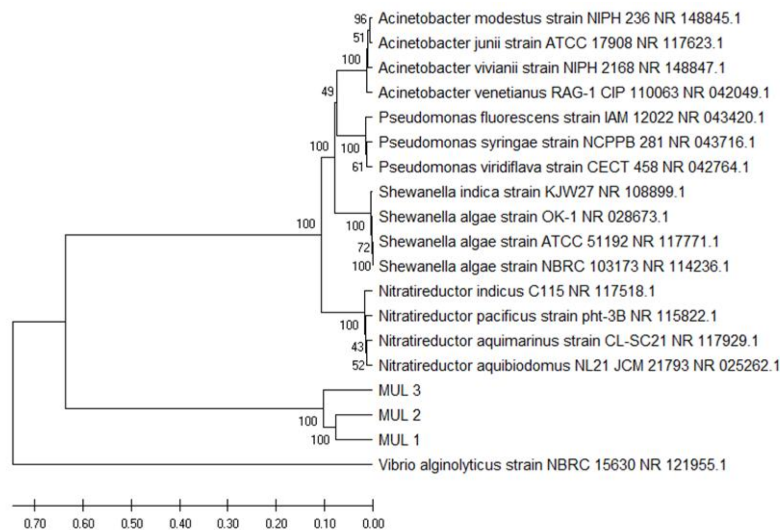

Figure 5. Pylogenetic Tree Dendogram of Isolates MUL_1, MUL_2 and MUL_3 with 14 Comparative Species.

Bauvet and Grimont (1986) stated that Acinetobacter (Isolate MUL_1) belongs to the kingdom Acinetobacter, phylum Proteobacteria, class Gammaproteobacteria, order Pseudomonadales, amylo Moraxellaceae, genus Acinetobacter. In general, these bacteria are rod-shaped, gram-negative, non-motile, catalase positive and oxidase negative, live aerobically, and are opportunistic bacteria. Beleneva and Maslennikova (2004) and Soslau et al (2011) reported that the Acinetobacter bacteria were found to be associated with several invertebrates such as mollusks, sea cucumbers, sea urchins, starfish and crustaceans.

Shewanella bacteria (Isolate MUL_2) belong to the Bacteria kingdom, the Proteobacteria phylum, the Gamma Proteobacteria class, the Alteromonadales order, the Shewanellaceae family and the Shewanella genus. The special characteristics of Shewanellae are their ability to capture electrons in oxygen deficient conditions and their ability to survive in various habitats (Gralnick and Newman, 2007). The genus Shewanella is a gram-negative rod-shaped proteobacteria 2-3 μm long and 0.4-0.7 μm in diameter. These bacteria are facultative anaerobic bacteria that are commonly found in marine sediments and can swim with the help of a single polar flagellum (Venkateswaran et al., 1999).

The genus Shewanella is a genus of metal reducing bacteria. These bacteria are found in marine environments, freshwater, lakes, land or terrestrial, rivers, Arctic and Antarctic oceans, corroded or corroded oil pipes and aquifer environments contaminated with uranium. These bacteria are widely used for bioremediation or environmental cleaning of pollutants such as chlorinated compounds, radionuclides and other environmental pollutants (Venkateswaran et al., 1999).

According to Labbe et al. (2004) Nitratireductor (Isolate MUL_3) belongs to the kingdom Bacteria, phylum Proteobacteria, class Alpha Proteobacteria, order Rhizobiales, family Phyllobacteriaceae and genus Nitratireductor. Characteristics The nitratireductor isolated from sea water in the Indian Ocean and marine sediments in the Pacific Ocean, has the ability to reduce ammonia, rod-shaped, with white bacterial colonies. The optimum temperature for growth is 25-35 ºC and pH 5-12.

Water quality. The results of observations on temperature, pH, dissolved oxygen (DO), TAN, nitrite, (NO2) and nitrate (NO3) in all treatments during the study are presented in Table 4.

Table 4. Average temperature, pH, dissolved oxygen (DO), total ammonia nitrogen (TAN), nitrite (NO2) and nitrate (NO3) in all treatments during the study

Parameters being measured	Jumlah *bioball*/wadah filter				
	0	**35**	**45**	**55**	**65**
Temperature (^{0}C)	28,6-29,1	28,4-29,1	28,7-29,1	28,6-29,1	28,6-29,1
pH	7,5-7,7	7,6-7,7	7,6-7,7	7,6-7,8	7,6-7,7
DO (mg/L)	6,2-6,5	6,2-6,6	6,2-6,6	6,3-6,7	6,2-6,6
Salinity (ppt)	15	15	15	15	15
TAN (mg/L)	0,61-1,58	0,28-1,59	0,18-1,56	0,06-1,57	0,12-1,56
Nitrite (mg/L)	0,6240,953	0,209-0,863	0,170-0,875	0,120-0,861	0,129-0,874
Nitrate (mg/L)	0,820-1,12	0,98-1,40	0,96-2,15	0,93-3,07	0,98-1,42

Table 4 shows that temperature, pH, dissolved oxygen (DO), ammonia (NH3) and nitrite (NO2) are relatively similar in all treatments, except for the nitrate content. The highest nitrate content was found in the 55 bioball / filter container treatment. In general, temperature, pH, DO, TAN, Nitrite and Nitrate are in a good range for the growth of pomfret fish.

SNI 7901.4., (2013) and Ashari et al., (2014) state that the optimal temperature for the growth of star pomfret ranges from 28-32 ° C, pH 6.8 - 8.4. According to Ezraneti et al., (2019) pomfret fish can live optimally at a salinity of 14-19 ppt. SNI 7901.4. (2013) require that dissolved oxygen is good for the maintenance of pomfret fish seeds at least 5 mg / L. Royan et al. (2019) reported that the lethal concentration (LC50) of TAN ranged from 1.10 to 22.8 ppm for invertebrates and from 0.56 to 2.37 ppm for fish within 24 - 96 hours after exposure. The range of nitrite levels for the maintenance of pomfret fish seeds is a maximum of 1 mg / L (SNI 7901.4., 2013).

The highest nitrate concentration was found in the treatment of 55 bioballs / filter containers. The high nitrate content in the 55 bioball / filter container treatment, due to the number of bioballs as many as 55 pieces / filter container with a volume of 9.8 L is the optimal amount for the live media for bacteria, especially bacteria that play a role in the nitrification process, so that the highest bacterial growth was found in the treatment this. The higher the number of bacteria causes the faster the nitrification process to take place, resulting in an increase in the maximum concentration of nitrate in the media. According to Lampert and Sommer (2007) bacteria have proteolytic enzymes that can break down organic nitrogen into nitrates, which are nutrients needed by phytoplankton and other aquatic microflora. Furthermore, Nelvia et al., (2015) obtained the best number of bioballs which were almost the same for the growth of goldfish, namely 50 bioballs / 9.8 L filter containers. Nitrate levels obtained during the study were still in accordance with the quality standards referred to in Government Regulation No. 82 of 2001, namely <20 mg / L. Effendi (2014) states that the nitrate content of 0 - 1 mg / L is included in the oligotrophic (low) category, and 1 - 5 mg / L is in the mesotrophic (moderate) category. According to Ulqodry et al. (2010), normal nitrate content in marine waters generally ranges from 0.01 - 50 mg / L. Furthermore Hartoko (2010) reports that algae, especially phytoplankton, can grow optimally at a nitrate content of 0.009 - 3.5 mg / L.

Relationship between number of bioballs and water quality. The results of the regression analysis between the number of bioballs with temperature, pH, DO, TAN, NO2 and NO3 are presented in Table 5.

Table 5. Linear regression analysis between the number of bioballs with temperature, pH, DO, TAN, NO2 and NO3

No.	Parameter	Linear regression equation	Correlation (r)	R determinant (R^2)
1.	Temperature	Y = 289,72 - 0,038 X	-0,767	0,589

2.	pH	Y = 759,51 + 0,067 X	0,444	0,197
3.	Dissolved oxygen (DO)	Y = 634,56 + 0,196 X	0,650	0,423
4.	TAN	Y = 58,29 – 0,83 X	-0,965	0,931
5.	Nitrite (NO_2)	Y = 587,55 – 8,345 X	-0,949	0,900
6.	Nitrate (NO_3)	Y = 94,73 + 1,75 X	0,664	0,441

Note: the value of r = 0 indicates that there is no correlation between the two variables (number of bioballs and water quality), r> 0 - 0.25 the correlation is very weak, r> 0.25 - 0.5 is sufficient correlation, r> 0.5 - 0.75 strong correlation, r> 0.75 - 0.99 very strong correlation and r = 1 perfect correlation; R2 ≥ 0.5 (50%) = ability to explain the independent variable (number of bioballs) to the dependent variable (water quality) is strong (Sarwono, 2012).

Table 5 shows that the number of bioballs has the greatest effect on the reduction in TAN (R2 = 0.931 or 93.1%) and has a very strong negative linear correlation (r = 0.965), this means that the more the number of bioballs in the filter container, the ammonia concentration will decrease. This is because in the filter media there are several types of nitrifying bacteria derived from the introduced starter bacteria, namely Nitrosomonas sp. and Nitrobacter sp., in addition to the bacteria found in the bioball, namely Acinetobacter sp., Shewanella algae and Nitratireductor aquimarinus. The presence of these bacteria can help accelerate the oxidation process of TAN which is toxic to fish into non-toxic nitrate through the nitrification process, so that the reduction in TAN in the pomfret cultivation medium becomes faster.

Relationship between bacterial density and water quality. The results of regression analysis between bacterial density and temperature, pH, DO, TAN, NO2 and NO3 are presented in Table 6.

Table 6. Linear regression analysis between bacterial density with temperature, pH, DO, TAN, NO2 and NO3

No.	Parameter	Linear regression equation	Correlation (r)	R determinant (R^2)
1.	Temperature	Y = 289.50 – 0,003 X	-0,226	0,475
2.	pH	Y = 756.37 + 0,015 X	0,695	0,484
3.	Dissolved oxygen (DO)	Y = 289.50 + 0,036 X	0,816	0,667
4.	TAN	Y = 59.578 – 0,090 X	-0,725	0,526
5.	Nitrite (NO_2)	Y = 557,73 – 0,796 X	-0,625	0,391
6.	Nitrate (NO_3)	Y = 30,304 + 0,352 X	0,923	0,852

Table 6 shows that bacterial density has the greatest influence on the increase in nitrate concentration in the pomfret rearing medium (R2 = 0.852 or 85.2%). This is due to the presence of Nitrosomonas bacteria, Nitrobacter which are included as a starter in the filter container, and heterotrophic bacteria in the bioball which accelerate the ammonia to turn into nitrate, so that the nitrate concentration increases with increasing bacterial density.

Conclusions. The number of bioballs had an effect (P <0.05) on bacterial density. The best treatment was found in the use of 55 bioballs / filter containers with a volume of 9.8 L, which gave a bacterial density of 667.67 ± 1.53 x 105 CFU / mL. The number of bioballs affected the bacterial density by 31.1. The bacteriocidal types found in the filter media are Acinetobacter, Shewanella and Nitratireductor. The highest number of bioballs (93.1%) affected the decrease in TAN, while the largest bacterial density (85.2%) influenced the increase in Nitrates.

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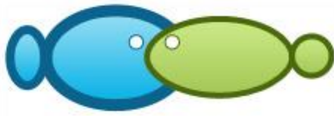

Density and types of probiotic bacteria in filter media with different number of bioballes in culturing media of silver pompano (*Trachinotus blochii*) with recirculation system

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Abstract. Cultivation intensification with a recirculation system tends to have a high stocking density and a large amount of feed, so that feed residue, metabolic waste, and oxygen consumption also increase. To speed up the decomposition of feed residue and metabolic waste, filtering is necessary, namely by means of a bioball. This study aims to analyze the types and numbers of bacteria present in the bioball, to improve water quality and the optimal number of bioballs for bacterial living media. This study used a completely randomized design, 1 factor, 5 levels of treatment and 3 replications. As the level of treatment in this study are; A = without bioball (control), B = 35 bioballs filter container^{-1}, C = 45 bioballs filter container^{-1}, D = 55 bioballs filter container^{-1} and E = 65 bioballs filter container^{-1} respectively. The silver pompano fish measuring 10 - 12.55 cm and body weight of 24.3 - 28.9 grams with a density of 1 fish 4 L^{-1} (20 fish / 80 L) were cultivated for 56 days. During fish rearing, they are fed with Megami GR2 pellets with a composition of 46% protein, 10% fat, 2% crude fiber and 10% moisture content, 3 times a day adlibitum. As a response to the test are: the type and density of bacteria in the bioball, temperature, pH, dissolved oxygen, total ammonium nitrogen, nitrite and nitrate. The research data were analyzed statistically ANOVA ($P<0.05$), Newman Keuls's advanced test and multiple regression models using SPSS 17.0. The best treatment was found in the use of 55 bioballs filter containers^{-1} with a volume of 9.8 L, which gave a bacterial density of 667.67 ± 1.53 x 105 CFU mL^{-1}. The amount of bioball affected the bacterial density by 31.1%, nitrogen absorption by fish 16.5% and filter media by 85.2%. The bacteriocidal types found in the filter media are *Acinetobacter* sp., *Shewanella* sp. and *Nitratireductor* sp. 15 ‰ salinity culturing media using a filter substrate of 55 bioballs filter containers^{-1} in the recirculation system gave the best bacterial density and water quality (28.6 - 29.1 0C, pH 7.6 - 7.8, DO 6.3 - 6, 7 mg L^{-1}, TAN 0.06 - 1.57 mg L^{-1}, nitrite 0.120 - 0.861 mg L^{-1} and nitrate 0.93 - 3.07 mg L^{-1}).
Key Words: bioball, bacterial density, probiotics, water quality.

Introduction. Silver pompano fish is a marine aquaculture species that is relatively new in Indonesia. However, the demand for this fish continues to increase, especially from the international market. These fish include fish species that have high adaptability, are easy to cultivate and have important economic value. The price of silver pompano for consumption reaches around USD 4,25 kg^{-1} at local market and USD 14.144 kg^{-1} for export commodities (Mo 2017). Since 2015 silver pompano has become a leading commodity in marine cultivation fisheries. Production of silver pompano in 2015 reached 1900 tons, and each year it is targeted to increase production to reach 31.5% per year (Prahadi 2015).

The production target for the silver pompano commodity can be achieved through intensification of cultivation. Intensive cultivation tends to be with a high stocking density, resulting in narrow space for fish, increased competition for oxygen and feed, so that the potential for fish to be stressed is greater, as a result, fish growth will be hampered. High density also accelerates the decline in water quality due to the accumulation of metabolic waste and feed residue, so that it has a major effect on growth.

Naturally nitrogen from fish culture waste produces ammonia (NH_3) from feed residue and fish metabolism, this can result in a buildup of organic matter which causes a

decrease in water quality (Putra and Pamukas 2011; Prayogo et al 2012). One of the technologies in fisheries to maintain water quality in order to remain suitable for aquatic organisms and to support the optimization of water utilization is the cultivation of recirculation systems. The recirculation system is able to reduce the level of amonia concentration, to within the range of 31-43% (Djokosetiyanto et al 2006; Putra and Pamukas 2011).

According to Lekang (2008) and Fadhil et al (2010) the use of a recirculation system has advantages including more efficient water use, flexibility in cultivation locations, more hygiene, relatively small space or land requirements, ease of controlling and maintaining cultivated organisms, ease of maintaining water quality, environmentally friendly, safe from pollution. that occurs outside the aquatic environment and can be carried out all the time.

Recirculating Aquaculture System technology can also be used to control dissolved solids adapted to the aquaculture system and filter substrate (Fadhil et al 2010). The filter substrate serves to filter dissolved solids. The use of the right type and filter material will determine the success of fish rearing in the recirculation system because it will determine the growth of non-pathogenic bacteria on the material. Currently, biological filter substrates are widely used because they are more environmentally friendly.

There are many biological filter materials that can be used, one of which is the bioball. Bioball is spherical, with a diameter of 4 cm, a specific area of ± 230 m^2 / m^3 with a cavity priority of 0.92, made of PVC material (Said 2002). Bioball functions as a physiological filter which is a growing medium for bacteria that can remove ammonia contained in water (Alfia et al 2013). The bacteria that grow on the bioball are nitrifying bacteria (*Nitrosomonas* sp and *Nitrobacter* sp). *Nitrosomonas* has a role in oxidizing ammonia to nitrite, while *Nitrobacter* has a role in oxidizing nitrite to nitrate, this nitrate will become plankton for fish natural food (0-fish 2012).

Several studies related to improving water quality in aquaculture with recirculation and hydroponic systems have been carried out, such as; Mulyadi and Pamukas (2013) use a recirculation system in nursery and rearing of fish (*Mystus nemurus*); Alfia et al. (2013) used a recirculation system with a bioball filter in Tilapia (*Oreochromis niloticus*) culture; Adamu et al (2014) studied the effect of different feed formulations on the recirculation system prototype container on the histology of the liver and kidneys of male hybrid catfish (*Heterobranchus bidorsalis*) with female *Clarias gariepinus*; Nelvia et al (2015) studied the addition of a bioball to the maintenance media filter on the survival and growth of goldfish (*Carassius auratus*) fry. Based on the description above, a series of studies on the number and types of bacteria and the improvement of water quality in the cultivation of recirculation systems with different numbers of bioballs are needed. This research is aimed at analyzing the types and numbers of bacteria present in the bioball, improving water quality (temperature, pH, DO, TAN), nitrite and nitrate) and analyzed the optimal amount of bioball for the live medium of bacteria.

Material and Method

Location and time of research. This research was conducted in January - July 2019, which was carried out in several laboratories, namely: rearing fish was carried out at the Batam Marine Cultivation Fishery Center (BPBL) Batam, water quality analysis at the Laboratory of Fish and Environmental Health Testing at the Batam Marine Aquaculture Fisheries Agency (BPBL), identifying types of bacteria and calculating the number of bacterial densities in the Fish and Environmental Health Examiners of the Batam Marine Cultivation Fishery Center (BPBL), PCR (polymerase chain reaction) test at PT. Genetics Science Indonesia, West Jakarta.

Materials and tools. The main ingredients that will be used in this research are as follows: the test animals used in this study are the seeds of silver pompano (*T. blochii*, Lacepede) originating from the Batam Marine Cultivation Fishery Center (BPBL) Batam, with a size of 10-11 cm. 300 heads. As the test feed, the commercial pellet pellet "GR-2" contains 46% protein, 10% fat, 13% ash content, 2% crude fiber, and 10% moisture

content. The test feed was obtained from the Batam Marine Cultivation Center (BPBL). A round bucket with a diameter of 60 cm, a height of 45 cm, a volume of 150 L as a research vessel. Bioball with a diameter of 4 cm, specific area ± 230 m^2 $m^{3\ -1}$ with a cavity priority of 0.92, is made of PVC as a filter substrate (Figure 1a). As a filter container is a PVC filter gutter measuring 50 cm x 14 cm x 14 cm.

Experimental design. The research method used in this study was an experimental method completely randomized design, 1 factor, 5 levels of treatment and 3 replications (Steel and Torrie, 1993). As a factor in the study was the difference in the number of bioballs. The treatment level determination in this study is based on the best research results by Nelvia et al., (2015) that the use of 50 bioballs $gutters^{-1}$ can improve water quality and provide 100% survival rates, growth weight of 2.605 grams, growth length of 1.48 mm of goldfish fry (*Carassius auratus*) in closed recirculation system. So that the treatment in this study are; A = without bioball (control), B = 35 bioballs filter $container^{-1}$, C = 45 bioballs filter container $^{-1,}$ D = 55 bioballs filter $container^{-1}$ and E = 65 bioballs filter $container^{-1}$.

Research procedure. The silver pompano fish measuring 10 - 12.55 cm in length and body weight of 24.3 - 28.9 grams were obtained from the Batam Marine Cultivation Center (BPBL), adapted for 7 days before being used as test fish. Fish rearing containers use a closed recirculation system. Into each rearing container is filled with sea water with a salinity of 15 ‰. The rearing container is connected to a 50 cm x 14 cm x 14 cm PVC gutter as a filter container, which is placed at the top of the fish-raising containers. Then the water from the filter gutter will flow back through the PVC pipe with a diameter of 2.5 cm to the rearing tub of silver pompano fish. Water from the fish rearing container will be flowed into the filter media (gutter) with the number of bioballs according to the treatment (Figure 1b) for each gutter according to the best findings of Nelvia et al., (2015) and using a water pump that has a power of 18 watts. After the water passes through the filter media (gutter), it will be returned to the fish-raising tub through the drain pipe in the filter container. In the rearing medium, KP-SUPER N (*Nitrosomonas* sp. and *Nitrobacter* sp.) Trademark starter bacteria were added at a dose of 2.5 ml 200 $L^{-1}week^{-1}$ (Hartini et al., 2013), to accelerate bacterial growth in the bioball.

(a) (b)

Figure 1. a. Bioball used as a filter substrate, b. The design of the Silver pompano]] aquaculture recirculation system.

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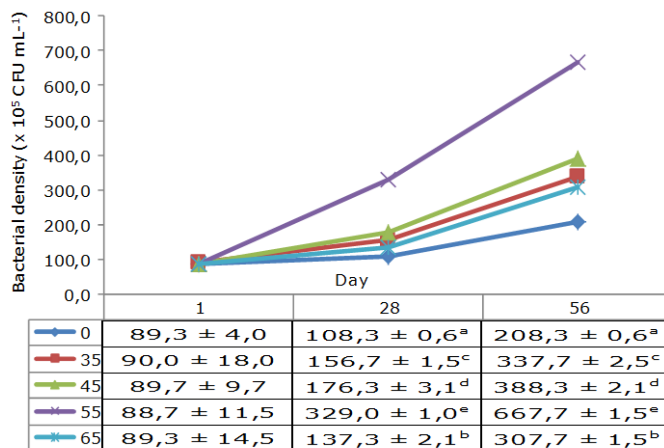
The test fish that have been adapted to the culturing media are then randomly placed into 15 rearing containers. The fish were given commercial feed with 46% protein content, 10% fat, 13% ash content, 2% crude fiber, and 10% moisture content by ad satiation at 07:00, 12.00 and 17.00 WIB.

Measured response. The responses measured in this study were: density and type of bacteria in the filter container were observed at the beginning, middle and end of the study (SNI.01-2332.3-2006 and SNI.01-2332.3-2006); temperature, pH and salinity were observed every day, dissolved oxygen (DO), Total Ammonia Nitrogen (TAN), nitrite (NO_2) and nitrate (NO_3) were observed every week. Water quality measurement procedures refer to SNI in the Public Works Service (1990).

Data analysis. Bacterial density data were analyzed according to the RAL model (Steel and Torrie, 1993). To determine the effect of salinity on each measured variable, a diversity analysis was performed using SPSS 17.0 software. If P <0.05, the Newman-Keuls further test was performed to see the differences between treatments. Water quality data were analyzed descriptively using Microsoft Excel application and displayed in tables and graphs. To see the relationship between the number of bioballs and the density of bacteria with water quality, regression analysis was carried out.

Results and Discussion

Density of bacteria in the filter container. The results of bacterial density observations carried out on Plate Count Agar (PCA) media using the Total Plate Count method can be seen in Figure 2.



	1	28	56
0	89,3 ± 4,0	108,3 ± 0,6^{a}	208,3 ± 0,6^{a}
35	90,0 ± 18,0	156,7 ± 1,5^{c}	337,7 ± 2,5^{c}
45	89,7 ± 9,7	176,3 ± 3,1^{d}	388,3 ± 2,1^{d}
55	88,7 ± 11,5	329,0 ± 1,0^{e}	667,7 ± 1,5^{e}
65	89,3 ± 14,5	137,3 ± 2,1^{b}	307,7 ± 1,5^{b}

Figure 2. Bacterial density in all treatments during the study

Figure 2 shows that the bacterial density from day 1 to day 28 in treatment 0, 35, 45 and 65 bioballs filter containers^{-1} are relatively similar and insignificant increase, in contrast to treatment 55 bioballs filter containers^{-1} a significant increase of bacteria than other treatments. This is due to the fact that the bacteria and silver pompano fish are still adapting to the media in which they live, besides that the nutrients that come from the remaining feed and fish feces are still not sufficient to support the maximum bacterial growth. After the 28th day the number of bacteria in all treatments continued to increase reaching a maximum peak on the 56th day. This is due to the increase in organic matter from fecal matter and fish feed along with the growth of the silver pompano, which is a source of bacterial nutrition, thereby increasing the rate of bacterial development. In accordance with the opinion of Sunarto (2003), dissolved organic matter is needed by living bacteria, then Sidharta (2000) explains that organic material contains carbon, nitrate, phosphate, sulfur, ammonia, and several minerals which are nutrients for bacterial growth. Giving KP SUPER N which is given routinely in a cultivation container causes the bacteria to continue to grow because of the availability of organic materials that can be used optimally. The highest bacterial density was found in the treatment of 55 bioballs filter containers^{-1}.

The highest bacterial density was found in the treatment of 55 bioballs filter containers^{-1} at the end of the study, namely 667.7 x 10^{5} CFU mL^{-1}, this is because the number of bioballs is 55 in a filter container measuring 50 cm x 14 cm x 14 cm for culture media with volume 80 L, is the optimal amount as a living medium for bacteria.

This is because the number of bioballs with the size of the filter container is in a balance, thus providing an opportunity for oxygen to enter the bioball cavity and bacteria to get sufficient oxygen. In accordance with the opinion of Nut (2009) and Nelvia et al (2015) which states that the location of the bioball which is not too dense provides an opportunity for oxygen to enter the bioball cavity so that the bacteria attached to the bioball get sufficient oxygen, if the oxygen obtained by the bacteria is sufficient then the bacteria will grow a lot in the bioball cavity and work more optimally.

The lowest bacterial density was found in treatment A (without bioball), which was 208.3 x 10^5 CFU mL^{-1}, this was due to the filter without using bioball and without the addition of starter bacteria (KP SUPER N) causing limited live media for bacteria and no additional nutrient availability for the growth and development of bacteria. The low density of bacteria when using the highest number of bioballs (65 bioballs filter $container^{-1}$) is due to the large number of bioballs for the filter container size of 50 cm x 14 cm x 14 cm, so that the location of the bioballs is very tightly positioned and leaves no empty space. According to Nelvia et al (2015) the amount of bioball that is too dense in the gutter can cause bacteria in the bioball cavity to not live if there is a lack of oxygen supply, the performance of the bioball filter decreases so that the process of decomposing organic matter by nitrifying bacteria does not run.

The biofilter system is the removal of ammonia, ammonia will accumulate and reach toxic levels if it is not transferred by a nitrification process where the process is first that ammonia is oxidized to toxic nitrite, then to nitrate, which is relatively non-toxic. The above process involves *Nitrosomonas* and *Nitrobacter* bacteria. Bacteria grow like a film on the surface of the material (biofilter container) or adhere to organic particles. Biofilter consists of a medium with a large surface for the growth of nitrite bacteria. In the recirculation system, the bacteria plays a role in converting toxic substances (ammonia) to non-toxic substances (nitrates). The performance of bacteria in the biofilter system is marked by an increase in the average BOD value in the culture medium (Nurhidayat et al 2012). The more bioballs that are used, the more opportunities there are for bacteria to stick, but too much use can also make bacteria die, due to the lack of oxygen received by bacteria (Nelvia et al 2015).

ANOVA results showed that the number of bioballs had an effect on the density of bacteria on day 28 and 56. The Newman Keuls range study test gave the results of differences in bacterial density between treatments. The results of the regression analysis between the number of bioballs and bacterial density provide the following regression equation:

$$Y = 227.9 + 3,851X$$

Information: Y = bacterial density, X = number of bioballs, r = 0.557 and R2 = 0.311

The results of the regression analysis showed that the number of bioballs had a positive correlation with bacterial density (r = 0.557), this means that the number of bacteria in the filter container increased with the increase in the number of bioballs. The increase in the number of bacteria in the filter container was influenced by the number of bioballs by 31.1% and 68.9%, which is thought to be influenced by the content of organic matter. Nelvia et al (2015) stated that the bioball is a breeding ground for various bacteria needed in the breakdown of organic matter. According to Sunarto (2003) dissolved organic matter is needed by bacteria to live. Furthermore, according to Sidharta (2000) organic matter contains carbon, nitrate, phosphate, sulfur, ammonia, and several minerals which are nutrients for bacterial growth.

Identification of bacteria in filter containers. Based on the observations, there were 7 pure bacterial isolates. The identification of bacteria against the isolates found in the filter media was carried out by physical, biochemical and sensitivity tests to see which bacteria had the greatest inhibition zone against pathogenic bacteria.

Physics test. The results of observations of color, shape, edge, elevation and gram of the seven isolates found are presented in Table 1.

Table 1. Observation Results of Bacterial Characteristics

No	Isolate	Colony				Cell		Treatment
		Color	Shape	Edge	Elevation	Gram Type	Shape	
1	A_1	Yellow	Circular	Entire	Umbonate	Negative	Stem	P_2U_1
2	A_2	Beige	Circular	Entire	Raised	Negative	Stem	P_0U_3
3	A_3	White	Circular	Entire	Raised	Negative	Stem	P_3U_2
4	A_4	White	Circular	Entire	Raised	Negative	Stem	P_4U_1
5	A_5	Beige	Filamentous	Entire	Filiform	Negative	Stem	P_1U_1
6	A_6	White	Circular	Entire	Raised	Negative	Stem	P_4U_2
7	A_7	White	Circular	Entire	Raised	Negative	Stem	P_3U_3

Table 1 shows that all bacterial isolates have gram-negative, entire edges and are rod-shaped. The dominant bacterial colony was white (4 isolates), 2 isolates were beige and 1 isolate was yellow. Colonies were circular round^{-1} (6 isolates) and filamentous (1 isolate). The elevation of the dominant bacterial colony was raised (5 isolates), 1 umbonate isolate and 1 filiform isolate.

Biochemical test. The results of observations of the Catalase, Oxidase, Motility, O / F, Indol and TSIA tests are presented in Table 2.

Table 2.Bacterial Biochemical Test Results

No.	Isolate	Biochemical Characteristics						Treatmen
		Catalase	Oxidase	Motility	O/F	Indole	TSIA	
1	A_1	+	+	+	-	-	A/K	P_2U_1
2	A_2	+	+	-	F	-	K/K	P_0U_3
3	A_3	+	-	+	O	-	A/K	P_3U_2
4	A_4	+	+	+	F	-	A/A	P_4U_1
5	A_5	+	+	+	F	+	A/A	P_1U_1
6	A_6	+	+	+	F	-	A/A	P_4U_2
7	A_7	+	+	-	F	-	A/A	P_3U_3

Information: O = oxidative, F = fermentative, A / K = alkaline acid, K / K = alkaline, A / A = acid

Table 2 shows that all bacterial isolates have positive catalase because these bacteria have a catalase enzyme with a reaction in the form of gas bubbles at the time of dropping H_2O_2. According to Stoica (2016) catalase is an enzyme that is owned by the majority of bacteria and is involved in the decomposition of hydrogen peroxide into water and oxygen. Hydrogen peroxide and oxygen are used for electron transport in the fermentation of both aerobic and facultative aerobic bacteria.

Oxidase tests A_1, A_2, A_3, A_4, A_5, A_6 and A_7 are positive because of the color change on the oxidase paper which indicates oxidase activity, while A_3 is negative. Five isolates (A_1, A_3, A_4, A_5 and A_6) showed motile results in the motility test due to the spread of bacterial growth on the SIM medium and did not grow on the part of the bacteria puncture site. The O / F test results showed no color change in the paraffin-covered media and the color change on the exposed media. Five bacterial isolates (A_2, A_4, A_5, A_6 and A_7) were observed to be fermentative because the tube without paraffin and the tube filled with paraffin changed color to yellow. According to Fahri (2008) in the O / F test, oxidative organisms occur when a color change is seen in the open media, while fermentative organisms can be indicated by seeing no color change in the closed media.

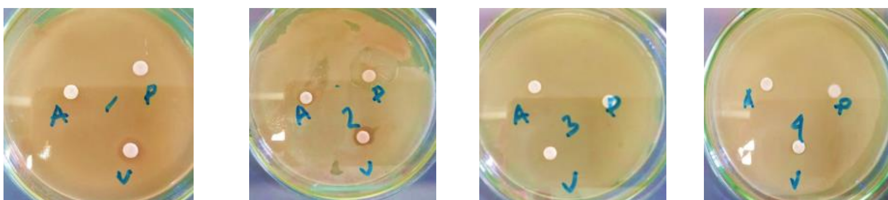
The indole test shows 6 isolates (A_1, A_2, A_3, A_4, A_6 and A_7) give negative results (-) marked with a yellow color on the surface of the media which means that the bacteria are unable to break down the amino acid tryptophan, and no red ring is formed on the surface of the media. after dropping the kovac reagent on SIM media. According to Acharya (2012) the indole test was carried out to determine the ability of bacteria to

break down amino acid tryptophan to form indole compounds. Tryptophan is hydrolyzed by tryptophanase to produce three possible end products, one of which is indole. Indole production is detected by kovac or Ehrlich's reagent which is composed of 4-p-benzaldehyde dimethylamino, this reagent reacts with indole to produce a red compound. A positive reaction is indicated if there is a red color on the surface of the medium which indicates that the bacteria are able to break down the amino acid tryptophan. In the TSIA test, 4 isolates (A_4, A_5, A_6 and A_7) showed acidic properties (A / A), 2 isolates (A_1 and A_3) were acid alkaline and 1 isolate (A_2) was alkaline.

Sensitivity test. The sensitivity test to pathogenic bacteria was carried out after observing the physical and biochemical tests of the seven isolates. This test aims to find probiotic bacteria candidates for PCR testing. The results of the sensitivity test on 7 isolates are presented in Figure 3. Inhibition zones produced by seven bacterial isolates found in different filter containers. The bacteria in isolate A_1 were able to inhibit the growth of *Vibrio* bacteria during the 24-hour incubation period with a temperature of 37 ^{0}C. This is indicated by the formation of a clear area around the blank disk, but it is unable to inhibit the growth of *Aeromonas* and *Pseudomonas* bacteria. After an incubation period of 48 hours the inhibition zone remains clear, which indicates that these bacteria can kill (bacteriocidal) *Vibrio* bacteria. Similar to the bacteria in isolate A_2, a clear zone was formed around the blank disk against *Pseudomonas* and *Vibrio* bacteria during the 48-hour incubation period, this indicates that A_2 bacteria are bacteriocidal against *Pseudomonas* and *Vibrio*, but cannot inhibit the growth of *Aeromonas* bacteria.

Bacteria in A_3 and A_4 isolates were unable to inhibit the growth of Aeromonas, *Pseudomonas* and *Vibrio* bacteria, it was seen that no inhibition zone was formed on the balnk disk. In A_5 and A_7 isolates an inhibition zone occurred against *Aeromonas*, *Pseudomonas* and *Vibrio* bacteria during the 24-hour incubation period, but during the 48-hour incubation period, the clear area became cloudy, indicating that these bacteria were only able to inhibit (bacteriostatic) the three pathogenic bacteria . In A_6 isolate a clear zone was also formed during the 48-hour incubation period against *Aeromonas* and *Vibrio* bacteria, but no inhibition zone was formed against *Pseudomonas* bacteria. This indicates that the A_6 bacteria are able to kill (bacteriocidal) *Aeromonas* and *Vibrio* bacteria.

This is in line with the opinion of Farlex (2005) which states that a material is said to be sensitive to bacteria characterized by the presence of an inhibition zone around the blank disk. Furthermore, according to Dwyana and Murniati (2020) this area of inhibition is formed due to the presence of bioactive compounds contained in microbes that produce exoenzymes that function to break down organic matter and also excrete hydrolytic enzymes, proteases, lipases, amylases, and cellulose so that they are able to hydrolyze polysaccharides as a carbon source and electron donors.



A_1 ; Inhibition A = 0, P = 0 and V = 3,74 mm (bacteriocidal)

A_2 ;Inhibition A = 0, P = 8,88 mm and V = 3,49 mm (bacteriocidal)

A_3 ; Inhibition A = 0, P = 0 and V = 0

A_4 ; Inhibition A = 0, P = 0 and V = 0

A_5 ; Inhibition A = 4,32 mm, P = 3,87 mm and V = 5,24 mm	A_6 ; Inhibition A = 1,62 mm, P = 0 and V = 3,8 mm (bacteriocidal)	A_7 ; Inhibition A = 3,15 mm, P = 2,58 mm and V = 2,20 mm

Figure 3. Sensitivity test of bacterial isolates against pathogenic bacteria
Description: A: *Aeromonas*, P: *Pseudomonas*, V: *Vibrio*

Madigan et al (2012) grouped bacteria based on their selective toxicity, antimicrobial compounds had 3 kinds of effects on microbial growth, namely; 1.Bacteriostatic, provides an effect by inhibiting growth but not killing. Bacteriostatic compounds inhibit protein synthesis or bind to ribosomes; 2. Bacteriocidal, gives effect by killing cells but not cell lysis or cell breakdown. 3. Bacteriolytic, causing cells to become lysis or cell breakdown. Furthermore, Dwyana and Murniati (2020) stated that the clear zone formed during the 24-hour incubation period on disc paper during the sensitivity test indicated that the bacteria was bacteriostatic, while 48 hours indicated that the bacteria was classified as bacteriocidal.

According to Jannata (2014) the classification of bacterial growth inhibition responses, based on the inhibition zone consists of 4, namely; weak response (diameter ≤5 mm), medium (diameter 5-10 mm), strong (diameter 10-20 mm), and very strong (≥20 mm). Based on this classification isolates A_5, A_6 and A_7 were classified as having a weak response to *Aeromonas*, isolate A_2 had a moderate response and isolates A_5 and A_7 had a weak response to *Pseudomonas* bacteria. Furthermore, A_5 isolate had a moderate response to *Vibrio* bacteria, while isolates A_1, A_2, A_6 and A_7 were classified as having a weak response. According to Sugita et al (1996) this is because these bacteria are able to produce antimicrobial compounds which are a form of competition for nutrients and energy. This mechanism is believed to be able to inhibit the growth of pathogens in the filter container. The inhibiting compounds are very diverse, including siderophores, bacteriocins, lysozymes, proteases, hydrogen peroxidase, and organic acids. According to Rengpipat et al (2000) siderophore is a compound with a low molecular weight (<1500) and is a specific agent to bind ferric ions and can dissolve iron precipitates and change them into the form required for microbial growth. Its ability to form siderophores causes other organisms, especially aquatic animal pathogens, to be unable to obtain these elements and become inhibited.

Based on the results of observations on the inhibition zone diameter and inhibition power, the bacterial isolates selected as candidates for probiotic bacteria were isolates A_1, A_2 and A_6 which were classified as bacteriocidal, then coded MUL_1, MUL_2 and MUL_3. Furthermore, to determine the type of the three bacteria, DNA analysis was carried out using PCR (Polymerase Chain Reaction).

Polymerase Chain Reaction (PCR) test

The results of amplification and visualization of DNA fragments of the 16S rRNA gene. The genomic DNA that had been isolated and extracted from isolates MUL_1, MUL_2 and MUL_3, then amplified the 16S rRNA markers using PCR technique. PCR analysis of the 3 bacteria isolates used primary base 27F forward: AGAGTTTGATCMTGGCTCAG and revers 1492R: TACGGYTA CCTTGTTACGACTT. The analysis results showed positive with the formation of bands with a total molecular

weight (bp) of 1000 - 1500 bp. The results of amplified DNA visualization using 27F and 1492R electrophoresed with UV light are presented in Figure 4. The amplified fragment has a wavelength of 1227-1372 bp, after seeing Geneious it is known that isolate MUL_1 produces a single band that is clearly visible with a molecular weight of 1331 bp (base pair), MUL_2 1227 bp and MUL_3 1372 bp according to the primary amplification size 27F and 1492R used by comparison using 1 Kb Ladder DNA. The size of this size is in accordance with the expected size of the bacterial 16S rRNA genes, which is between 1300 - 1500 bp (Seprianto et al., 2017).

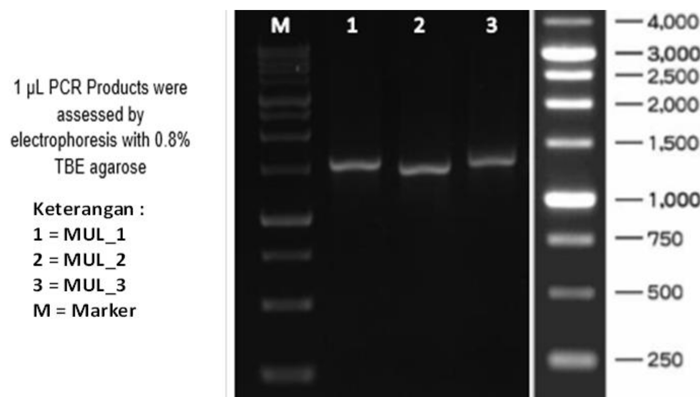


Figure 4. Results of Universal DNA-PCR Amplification on Agarose Gel

The base pairs obtained were combined and trimmed using Bioedit 7.0 software. DNA sequencing analysis gives the following results for the nucleotide and fasta sequences:

A1. The nucleotide sequence and fasta MUL_1

- Nucleotide sequence

```
Sequence Assembly 1331  bp
1     CTTGCGGTTA GCGCACTGCC TTCGGGTAAC CCAACTCCCA TGGTGTGACG GGCGGTGTGT
61    ACAAGGCCCG GGAACGTATT CACCGCGGCA TGCTGATCCG CGATTACTAG CGATTCCAAC
121   TTCATGCACC CGAGTTGCAG AGTGCAATCC GAACTGAGAT GGTTTTTGGA GATTAGCTCG
181   ACCTCGCGGT CTCGCTGCCC ACTGTCACCA CCATTGTAGC ACGTGTGTAG CCCAGCCCGT
241   AAGGGCCATG AGGACTTGAC GTCATCCCCA CCTTCCTCTC GGCTTATCAC CGGCAGTCCC
301   CTTAGAGTGC CCAACTTAAG GCTGGCAACT AAGGGCGAGG GTTGCGCTCG TTGCGGGACT
361   TAACCCAACA TCTCACGACA CGAGCTGACG ACAGCCATGC AGCACCTGTC TTGGGTCCAG
421   CCTAACTGAA GGATACCGTC TCCGGTATCC GCGACCCAGA TGTCAAGGGC TGGTAAGGTT
481   CTGCGCGTTG CTTCGAATTA AACCACATGC TCCACCGCTT GTGCGGGCCC CCGTCAATTC
541   CTTTGAGTTT TAATCTTGCG ACCGTACTCC CCAGGCGGGA AGCTTAATGC GTTAACTGCG
601   CCACCGACAG GTAAACCTGC CGACGGCTAG CTTCCATCGT TTACGGCGTG GACTACCAGG
661   GTATCTAATC CTGTTTGCTC CCCACGCTTT CGCACCTCAG CGTCAGTATC GAGCCAGTGA
721   GCCGCCTTCG CCACTGGTGT TCCTCCGAAT ATCTACGAAT TTCACCTCTA CACTCGGAAT
781   TCCACTCACC TCTCTCGAAC TCTAGATCGG CAGTATTAGA GGCAGTTCCG GGGTTGAGCC
841   CCGGGATTTC ACCCCTAACT GACCGATCCG CCTACGCGCG CTTTACGCCC AGTAATTCCG
901   AACAACGCTA GCCCCCTTCG TATTACCGCG GCTGCTGGCA CGAAGTTAGC CGGGGCTTCT
961   TCTCCGGTTA CCGTCATTAT CTTCACCGGT GAAAGAGCTT TACAACCCTA GGGCCTTCAT
1021  CACTCACGCG GCATGGCTGG ATCAGGCTTG CGCCCATTGT CCAATATTCC CCACTGCTGC
1081  CTCCCGTAGG AGTCTGGGCC GTGTCTCAGT CCCAGTGTGG CTGATCATCC TCTCAGACCA
1141  GCTACTGATC GTCGCCTTGG TGAGCCTTTA CCTCACCAAC TAGCTAATCA GACATGGGCT
1201  CATCTAACTC CGATAAATCT TTCTCCCGAA GGACGTATAC GGTATTAGTT CAAGTTTCCC
1261  TGAGTTATTC CGTAGAGCTA GGTAGATTCC CATGCATTAC TCACCCGTCT GCCGCTCCCC
1321  CGAGGGGGCG C
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- Fasta

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> MUL_1
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CACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCAACTTCATGCACCCGAGTTGCAGAGTGCAATCCGAACTGAGATG
GTTTTTGGAGATTAGCTCGACCTCGCGGTCTCGCTGCCCACTGTCACCACCATTGTAGCACGTGTGTAGCCCAGCCCGTAA
GGGCCATGAGGACTTGACGTCATCCCCACCTTCCTCTCGGCTTATCACCGGCAGTCCCCTTAGAGTGCCCAACTTAAGGCT
GGCAACTAAGGGCGAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCAGC
ACCTGTCTTGGGTCCAGCCTAACTGAAGGATACCGTCTCCGGTATCCGCGACCCAGATGTCAAGGGCTGGTAAGGTTCTGC
GCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTTAATCTTGCGACCGTA
CTCCCCAGGCGGGAAGCTTAATGCGTTAACTGCGCCACCGACAGGTAAACCTGCCGACGGCTAGCTTCCATCGTTTACGGC
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GTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTCAGCGTCAGTATCGAGCCAGTGAGCCGCCTT
CGCCACTGGTGTTCCTCCGAATATCTACGAATTTCACCTCTACACTCGGAATTCCACTCACCTCTCTCGAACTCTAGATCGGC
AGTATTAGAGGCAGTTCCGGGGTTGAGCCCCGGGATTTCACCCCTAACTGACCGATCCGCCTACGCGCGCTTTACGCCCAG
TAATTCCGAACAACGCTAGCCCCCTTCGTATTACCGCGGCTGCTGGCACGAAGTTAGCCGGGGCTTCTTCTCCGGTTACCG
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CATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGATCATCCTCTCAG
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A2. The nucleotide sequence and fasta MUL_2

- Nucleotide sequence

```
Sequence Assembly 1227  bp
1       GTTAGACTAC CTACTTCTGG TGCAACAAAC TCCCATGGTG TGACGGGCGG TGTGTACAAG
61      GCCCGGGAAC GTATTCACCG CGGCATTCTG ATCCGCGATT ACTAGCGATT CCGACTTCAT
121     GGAGTCGAGT TGCAGACTCC AATCCGGACT ACGATCGGCT TTTTGAGATT AGCATCCTAT
181     CGCTAGGTAG CAACCCTTTG TACCGACCAT TGTAGCACGT GTGTAGCCCT GGCCGTAAGG
241     GCCATGATGA CTTGACGTCG TCCCCGCCTT CCTCCAGTTT GTCACTGGCA GTATCCTTAA
301     AGTTCCCATC CGAAATGCTG GCAAGTAAGG AAAAGGGTTG CGCTCGTTGC GGGACTTAAC
361     CCAACATCTC ACGACACGAG CTGACGACAG CCATGCAGCA CCTGTATCTA GATTCCCGAA
421     GGCACCAATC CATCTCTGGA AAGTTTCTAG TATGTCAAGG CCAGGTAAGG TTCTTCGCGT
481     TGCATCGAAT TAAACCACAT GCTCCACCGC TTGTGCGGGC CCCCGTCAAT TCATTTGAGT
541     TTTAGTCTTG CGACCGTACT CCCCAGGCGG TCTACTTATC GCGTTAGCTG CGCCACTAAA
601     GCCTCAAAGG CCCCAACGGC TAGTAGACAT CGTKTACGGC ATGGACTACC AGGGTATCTA
661     ATCCTGTTTG CTCCCCATGC TTTCGTACCT CAGCGTCAGT ATTAGGCCAG ATGGCTGCCT
721     TCGCCATCGG TATTCCTCCA GATCTCTACG CATTTCACCG CTACACCTGG AATTCTACCA
781     TCCTCTCCCA TACTCTAGCC ATCCAGTATC GAATGCAATT CCCAAGTTAA GCTCGGGGAT
841     TTCACATTTG ACTTAAATGG CCGCCTACGC ACGCTTTACG CCCAGTAAAT CCGATTAACG
901     CTCGCACCCT CTGTATTACC GCGGCTGCTG GCACAGAGTT AGCCGGTGCT TATTCTGCGA
961     GTAACGTCCA CTATCCAGTA GTATTAATAC TAGTAGCCTC CTCCTCGCTT AAAGTGCTTT
1021    ACAACCATAA GGCCTTCTTC ACACACGCGG CATGGCTGGA TCAGGGTTCC CCCCATTGTC
1081    CAATATTCCC CACTGCTGCC TCCCGTAGGA GTCTGGGCCG TGTCTCAGTC CCAGTGTGGC
1141    GGATCATCCT CTCAGACCCG CTACAGATCG TCGCCTTGGT AGGCCTTTAC CCCACCAACT
1201    AGCTAATCCG ACTTAGGCTC ATCTATT
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- Fasta

> MUL_2
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TT

A3. The nucleotide sequence and fasta MUL_3

- Nucleotide sequence

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1       AAGGTTAAGC TATCTACTTC TGGTGCAGCC CACTCCCATG GTGTGACGGG CGGTGTGTAC
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121     CATGGAGTCG AGTTGCAGAC TCCAATCCGG ACTACGACCA GCTTTATGGG ATTAGCTCCA
181     CCTCGCGGCT TCGCAACCCT CTGTACTGAC CATTGTAGCA CGTGTGTAGC CCTACTCGTA
241     AGGGCCATGA TGACTTGACG TCGTCCCCAC CTTCCTCCGG TTTATCACCG GCAGTCTCCC
301     TAAAGTTCCC GGCATGACCC GCTGGCAAGT AAGGATAGGG GTTGCGCTCG TTGCGGGACT
361     TAACCCAACA TTTCACAACA CGAGCTGACG ACAGCCATGC AGCACCTGTC TCACAGTTCC
421     CGAAGGCACT GAAGCATCTC TGCTAAATTC TGTGGATGTC AAGAGTAGGT AAGGTTCTTC
481     GCGTTGCATC GAATTAAACC ACATGCTCCA CCGCTTGTGC GGGCCCCCGT CAATTCATTT
541     GAGTTTTAAC CTTGCGGCCG TACTCCCCAG GCGGTCTACT TAATGCGTTA GCTTGAGAGC
601     CCAGTGTTCA AGACACCAAA CTCCGAGTAG ACATCGTTTA CGGCGTGGAC TACCAGGGTA
661     TCTAATCCTG TTTGCTCCCC ACGCTTTCGT GCCTGAGCGT CAGTCTTTGT CCAGGGGGCC
721     GCCTTCGCCA CCGGTATTCC TCCAGATCTC TACGCATTTC ACCGCTACAC CTGGAATTCT
781     ACCCCCCTCT ACAAGACTCT AGTTTGCCAG TTCGAAATGC GGTTCCCAGG TTGAGCCCGG
841     GGCTTTCACA TCTCGCTTAA CAAACCGCCT GCGCACGCTT TACGCCCAGT AATTCCGATT
901     AACGCTCGCA CCCTCCGTAT TACCGCGGCT GCTGGCACGG AGTTAGCCGG TGCTTCTTCT
961     GCGAGTAACG TCACAGATGT AAGGTATTAA CTTACACCCT TTCCTCCTCG CTGAAAGTGC
1021    TTTACAACCC GAAGGCCTTC TTCACACACG CGGCATGGCT GCATCAGGGT TTCCCCCATT
1081    GTGCAATATT CCCCACTGCT GCCTCCCGTA GGAGTCTGGG CCGTGTCTCA GTCCCAGTGT
1141    GGCTGATCAT CCTCTCAGAC CAGCTAGGGA TCGTCGCCTA GGTGAGCCTT TACCTCACCT
1201    ACTAGCTAAT CCCACCTGGG CTTATCCATC AGCGCAAGGC CCGAAGGTCC CCTGCTTTCC
1261    CCCGTAGGGC GTATGCGGTA TTAGCAGTCG TTTCCAACTG TTATCCCCCA CAAATGGGCA
1321    AATTCCCAGG CATTACTCAC CCGTCCGCCG CTCGTCATCT TCAAAAGCAA GC
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- Fasta

>MUL_3
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GTATTCCTCCAGATCTCTACGCATTTCACCGCTACACCTGGAATTCTACCCCCCTCTACAAGACTCTAGTTTGCCAGTTCGAA
ATGCGGTTCCCAGGTTGAGCCCGGGGCTTTCACATCTCGCTTAACAAACCGCCTGCGCACGCTTTACGCCCAGTAATTCCG
ATTAACGCTCGCACCCTCCGTATTACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGCGAGTAACGTCACAGAT
GTAAGGTATTAACTTACACCCTTTCCTCCTCGCTGAAAGTGCTTTACAACCCGAAGGCCTTCTTCACACACGCGGCATGGCT
GCATCAGGGTTTCCCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTG
GCTGATCATCCTCTCAGACCAGCTAGGGATCGTCGCCTAGGTGAGCCTTTACCTCACCTACTAGCTAATCCCACCTGGGCTT
ATCCATCAGCGCAAGGCCCGAAGGTCCCCTGCTTTCCCCCGTAGGGCGTATGCGGTATTAGCAGTCGTTTCCAACTGTTAT
CCCCCACAAATGGGCAAATTCCCAGGCATTACTCACCCGTCCGCCGCTCGTCATCTTCAAAAGCAAGC

16S rRNA gene sequence alignment with basic local alignment search tool (BLAST). Analysis of BLAST (Basic Local Alignment Search tool) online via: http://blast.ncbi.nlm.nih.gov/Blast.cgi on the results of DNA sequencing was carried out to search for species names, percentages of DNA homology sequenced with existing databases on GenBank. The results of identification of probiotic bacterial isolates from the BLAST results were taken based on the highest homology that has the closest relationship with the bacteria in GenBank. Based on the nucleotides registered with the National Center for Biotechnology Information (NCBI), the species, strains and accession numbers of bacterial isolates identified in this study are presented in Table 3.

Table 3. Results of Tracing 16S rRNA Sequencing of Bacterial Isolates with the BLAST System

Isolate	Species	Strain	Accession Number	Query Coverage	Homology
MUL_1	*Acinetobacter* sp.	MUL37	MT229070	100%	100%
MUL_2	*Shewanella* sp.	MUL31	MT229068	100%	100%
MUL_3	*Nitratireductor* sp.	MUL35	MT229069	100%	100%

The alignment results of 16S rRNA gene sequences with BLAST (Table 3) showed that the bacterial isolate MUL_1 was identified as *Acinetobacter* sp. strain MUL37 with 100% homology and 100% query coverage; MUL_2 isolate was identified as *Shewanella* sp. strain MUL31 with 100% homology and 100% query coverage; and bacterial isolate MUL_3 identified as *Nitratireductor* sp. strain MUL35 with 100% homology and 100% query coverage. The types of bacteria found are thought to have come from the waters of the Batam Sea which are used as a medium for culturing silver pompano, because the starter bacteria added to the media do not contain these types of bacteria.

According to Aditya et al (2017) the sequence equation between 93-97% can represent the identity of bacteria at the genus level but different at the species level. Furthermore, Hagstrom et al (2000) and Seprianto et al (2017) state that bacteria that have a 16S rRNA sequence equation greater than 97% are the same species, while the equation between 93-97% can represent identity at the genus level but differ at the level species. According to Dancourt et al (2000) if the similarity of the sequences is less than 97%, it is likely a new species because there is no data in the database or the size of the sequencing results compared to the database is too short.

Phylogenetic tree analysis. The analysis of phylogenetic tree formation was carried out by looking at the similarities that exist in the genetic isolates of MUL_1, MUL_2 and MUL_3 bacteria with a database of the results of alignment of nucleotide sequences in GenBank using the Mega X 10.0.5 WIN 64 application. Phylogenetic analysis using the UPGMA distance-based method (Unwight Pair Group Method with Arithmetic Average) or the unweight pair group method with arithmetic mean is the simplest method of all the clustering methods used to build phylogenetic trees. Phylogenetic analysis used 17

sequences with 3 sample sequences and 14 comparison sequences, the alignment process used 1000 bootstraps and Kimura 2 model parameters. Branches that connect the dots (nodes) are taxonomic units, such as species or genes, tree roots are the ancestors (ancestors) for all organisms, the dendogram is presented in Figure 5.

Figure 5 shows the bacteria MUL_1, MUL_2 and MUL_3 are in 1 cluster and separated from the type of control bacteria, this means that between MUL_1 and MUL_2 have close relationships, even tend to be the same species or belong to the same subspecies, while MUL_3 bacteria have a genus. the same one. Judging from the point of branching, the three isolates were closely related to *Nitratireductor* and *Shewanella*.

The results of the calculation of genetic distance using Mega X 10.0.5 WIN 64, it was found that the distances between species varied, ranging from 0.00 to 1.6659. The smallest genetic distance was found between bacteria MUL_1 and MUL_2 of 0.2005, while between MUL_1 and MUL_3 was 0.2081. The genetic distance between bacteria MUL_1 and *Acinetobacter venetianus* (GenBank: NR.042049.1) was 1.2531; between MUL_2 and *Shewanella algae* (GenBank: NR117771.1) of 1.2955; and between MUL_3 and *Nitratireductor aquimarinus* (GenBank: 117929.1) of 1.2468. According to Tallei et al (2016) the smaller the value of the genetic distance between two organisms, the closer the kinship of the two is.

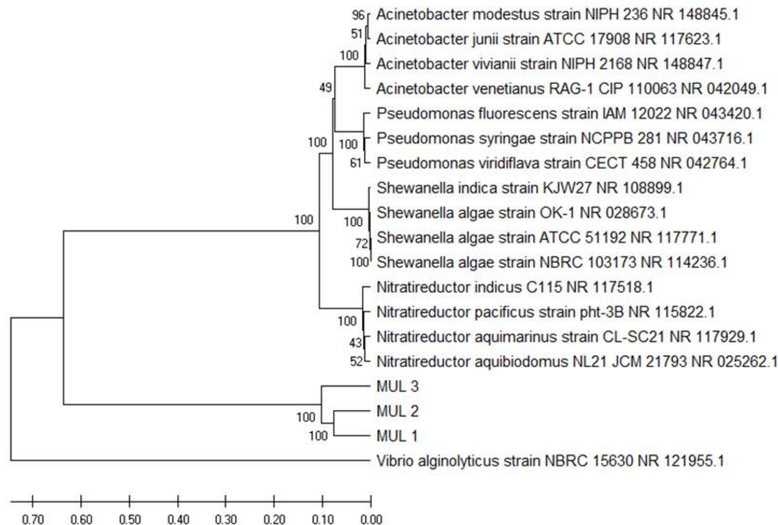

Figure 5. Pylogenetic Tree Dendogram of Isolates MUL_1, MUL_2 and MUL_3 with 16 Comparative Species.

Bauvet and Grimont (1986) stated that Acinetobacter (Isolate MUL_1) belongs to the kingdom Acinetobacter, phylum Proteobacteria, class Gammaproteobacteria, order Pseudomonadales, family Moraxellaceae, genus *Acinetobacter*. In general, these bacteria are rod-shaped, gram-negative, non-motile, catalase positive and oxidase negative, live aerobically, and are opportunistic bacteria. Beleneva and Maslennikova (2004) and Soslau et al (2011) reported that the Acinetobacter bacteria were found to be associated with several invertebrates such as mollusks, sea cucumbers, sea urchins, starfish and crustaceans.

Shewanella bacteria (Isolate MUL_2) belong to the Bacteria kingdom, the Proteobacteria phylum, the Gamma Proteobacteria class, the Alteromonadales order, the family Shewanellaceae and the genus *Shewanella*. The special characteristics of Shewanellae are their ability to capture electrons in oxygen deficient conditions and their

ability to survive in various habitats (Gralnick and Newman, 2007). The genus *Shewanella* is a gram-negative rod-shaped proteobacteria 2-3 μm long and 0.4-0.7 μm in diameter. These bacteria are facultative anaerobic bacteria that are commonly found in marine sediments and can swim with the help of a single polar flagellum (Venkateswaran et al., 1999).The genus *Shewanella* is a genus of metal reducing bacteria. These bacteria are found in marine environments, freshwater, lakes, land or terrestrial, rivers, Arctic and Antarctic oceans, corroded or corroded oil pipes and aquifer environments contaminated with uranium. These bacteria are widely used for bioremediation or environmental cleaning of pollutants such as chlorinated compounds, radionuclides and other environmental pollutants (Venkateswaran et al., 1999).

According to Labbe et al (2004) *Nitratireductor* (Isolate MUL_3) belongs to the kingdom Bacteria, phylum Proteobacteria, class Alpha Proteobacteria, order Rhizobiales, family Phyllobacteriaceae and genus *Nitratireductor*. Characteristics the *Nitratireductor* isolated from sea water in the Indian Ocean and marine sediments in the Pacific Ocean, has the ability to reduce ammonia, rod-shaped, with white bacterial colonies. The optimum temperature for growth is 25-35 ºC and pH 5-12.

Water quality. The results of observations on temperature, pH, dissolved oxygen (DO), TAN, nitrite, (NO_2) and nitrate (NO_3) in all treatments during the study are presented in Table 4.

Table 4. Average temperature, pH, dissolved oxygen (DO), total ammonia nitrogen (TAN), nitrite (NO_2) and nitrate (NO_3) in all treatments during the study

Parameters being measured	**Number of bioball of each filter container**				
	0	**35**	**45**	**55**	**65**
Temperature (^{0}C)	28.6-29.1	28.4-29.1	28.7-29.1	28.6-29.1	28.6-29.1
pH	7.5-7.7	7.6-7.7	7.6-7.7	7.6-7.8	7.6-7.7
DO (mg L^{-1})	6.2-6.5	6.2-6.6	6.2-6.6	6.3-6.7	6.2-6.6
Salinity (g L^{-1})	15	15	15	15	15
TAN (mg L^{-1})	0.61.-1.58	0.28-1.59	0.18-1.56	0.06-1.57	0.12-1.56
Nitrite (mg L^{-1})	0.624-0.953	0.209-0.863	0.170-0.875	0.120-0.861	0.129-0.874
Nitrate (mg L^{-1})	0.820-1.12	0.98-1.40	0.96-2.15	0.93-3.07	0.98-1.42

Table 4 shows that temperature, pH, dissolved oxygen (DO), ammonia (NH_3) and nitrite (NO_2) are relatively similar in all treatments, except for the nitrate content. The highest nitrate content was found in the 55 bioball filter container^{-1}. In general, temperature, pH, DO, TAN, nitrite and nitrate are in a good range for the growth of pomfret fish.

SNI 7901.4 (2013) and Ashari et al (2014) state that the optimal temperature for the growth of silver pompano ranges from 28-32 °C, pH 6.8 - 8.4. According to Ezraneti et al (2019) silver pompano fish can live optimally at a salinity of 14-19 ppt. SNI 7901.4 (2013) require that dissolved oxygen is good for the rearing of silver pompano fish seeds at least 5 mg L^{-1}. Royan et al (2019) reported that the lethal concentration (LC50) of TAN ranged from 1.10 to 22.8 mg L^{-1} for invertebrates and from 0.56 to 2.37 mg L^{-1} for fish within 24 - 96 hours after exposure. The range of nitrite levels for the maintenance of pomfret fish seeds is a maximum of 1 mg L^{-1} (SNI 7901.4., 2013).

The highest nitrate concentration was found in the treatment of 55 bioballs filter containers^{-1}. The high nitrate content in the 55 bioball filter containert^{-1}, due to the number of bioballs as many as 55 pieces filter container^{-1} with a volume of 9.8 L is the optimal amount for the live media for bacteria, especially bacteria that play a role in the nitrification process, so that the highest bacterial growth was found in the treatment. The higher the number of bacteria causes the faster the nitrification process to take place, resulting in an increase in the maximum concentration of nitrate in the media. According to Lampert and Sommer (2007) bacteria have proteolytic enzymes that can break down organic nitrogen into nitrates, which are nutrients needed by phytoplankton and other aquatic microflora. Furthermore, Nelvia et al (2015) obtained the best number of bioballs which were almost the same for the growth of goldfish, namely 50 bioballs 9.8 L^{-1} filter

containers. Nitrate levels obtained during the study were still in accordance with the quality standards referred to in Government Regulation No. 82 of 2001, namely <20 mg L^{-1}. Effendi (2014) states that the nitrate content of 0 - 1 mg L^{-1} is included in the oligotrophic (low) category, and 1 - 5 mg L^{-1} is in the mesotrophic (moderate) category. According to Ulqodry et al (2010) normal nitrate content in marine waters generally ranges from 0.01 - 50 mg L^{-1}. Furthermore Hartoko (2010) reports that algae, especially phytoplankton, can grow optimally at a nitrate content of 0.009 - 3.5 mg L^{-1}.

Relationship between number of bioballs and water quality. The results of the regression analysis between the number of bioballs with temperature, pH, DO, TAN, NO_2 and NO_3 are presented in Table 5.

Table 5. Linear regression analysis between the number of bioballs with temperature, pH, DO, TAN, NO_2 and NO_3

No.	Parameter	Linear regression equation	Correlation (r)	R determinant (R^2)
1.	Temperature	Y = 289.72 – 0.038 X	-0.767	0.589
2.	pH	Y = 759.51 + 0.067 X	0.444	0.197
3.	Dissolved oxygen (DO)	Y = 634.56 + 0.196 X	0.650	0.423
4.	TAN	Y = 58.29 – 0.83 X	-0.965	0.931
5.	Nitrite (NO_2)	Y = 587.55 – 8.345 X	-0.949	0.900
6.	Nitrate (NO_3)	Y = 94.73 + 1.75 X	0.664	0.441

Note: the value of r = 0 indicates that there is no correlation between the two variables (number of bioballs and water quality), r> 0 - 0.25 the correlation is very weak, r> 0.25 - 0.5 is sufficient correlation, r> 0.5 - 0.75 strong correlation, r> 0.75 - 0.99 very strong correlation and r = 1 perfect correlation; $R^2 \geq 0.5$ (50%) = ability to explain the independent variable (number of bioballs) to the dependent variable (water quality) is strong (Sarwono, 2012).

Table 5 shows that the number of bioballs has the greatest effect on the reduction in TAN (R^2 = 0.931 or 93.1%) and has a very strong negative linear correlation (r = 0.965), this means that the more the number of bioballs in the filter container, the ammonia concentration will decrease. This is because in the filter media there are several types of nitrifying bacteria derived from the introduced starter bacteria, namely *Nitrosomonas* sp and *Nitrobacter* sp, in addition to the bacteria found in the bioball, namely *Acinetobacter* sp, *Shewanella algae* and *Nitratireductor aquimarinus.* The presence of these bacteria can help accelerate the oxidation process of TAN which is toxic to fish into non-toxic nitrate through the nitrification process, so that the reduction in TAN in the pomfret cultivation medium becomes faster.

Relationship between bacterial density and water quality. The results of regression analysis between bacterial density and temperature, pH, DO, TAN, NO_2 and NO_3 are presented in Table 6.

Table 6. Linear regression analysis between bacterial density with temperature, pH, DO, TAN, NO_2 and NO_3

No.	Parameter	Linear regression equation	Correlation (r)	R determinant (R^2)
1.	Temperature	Y = 289.50 – 0.003 X	-0.226	0.475
2.	pH	Y = 756.37 + 0.015 X	0.695	0.484
3.	Dissolved oxygen (DO)	Y = 289.50 + 0.036 X	0.816	0.667
4.	TAN	Y = 59.578 – 0.090 X	-0.725	0.526
5.	Nitrite (NO_2)	Y = 557.73 – 0.796 X	-0.625	0.391
6.	Nitrate (NO_3)	Y = 30.304 + 0.352 X	0.923	0.852

Table 6 shows that bacterial density has the greatest influence on the increase in nitrate concentration in the pomfret rearing medium (R^2 = 0.852 or 85.2%). This is due to the presence of *Nitrosomonas* bacteria, *Nitrobacter* which are included as a starter in the filter container, and heterotrophic bacteria in the bioball which accelerate the ammonia to turn into nitrate, so that the nitrate concentration increases with increasing bacterial density.

Conclusions. The number of bioballs had an effect (P <0.05) on bacterial density. The best treatment was found in the use of 55 bioballs filter containers^{-1} with a volume of 9.8 L, which gave a bacterial density of 667.67 ± 1.53 x 105 CFU mL^{-1}. The number of bioballs affected the bacterial density by 31.1. The bacteriocidal types found in the filter media are *Acinetobacter*, *Shewanella* and *Nitratireductor*. The highest number of bioballs (93.1%) affected the decrease in TAN, while the largest bacterial density (85.2%) influenced the increase in nitrates.

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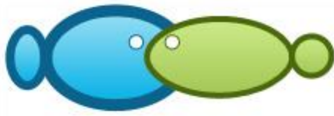

Density and types of probiotic bacterial filter media with different number of bioballs in snubnose pompano (*Trachinotus blochii*) culture with recirculation system

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Abstract. Cultivation intensification with a recirculation system tends to have a high stocking density and a large amount of feed, so that feed residue, metabolic waste, and oxygen consumption also increase. To speed up the decomposition of feed residue and metabolic waste, filtering is necessary, namely by means of a bioball. This study aimed to analyze the types and numbers of bacteria present in the bioball, to improve water quality and the optimal number of bioballs for bacterial living media. The study used a completely randomized design, with 1 factor, 5 levels of treatment and 3 replications. The treatment levels are: A=without bioballs (control), B=35 bioball filters per container, C=45 bioball filters per container, D=55 bioball filters per container and E=65 bioball filters per container. Snubnose pompano (*Trachinotus blochii)* fish specimens measuring 10-12.55 cm and a body weight of 24.3-28.9 g, were cultivated for 56 days, at a stocking density of 1 fish for a water volume of 4 L (a total of 20 fish in a volume of 80 L). During the rearing, fish were fed with Megami GR2 pellets with a composition of 46% protein, 10% fat, 2% crude fiber and 10% moisture content, 3 times a day adlibitum. The type and density of bacteria in the bioball was observed, together with the water quality parameters, such as: temperature, pH, dissolved oxygen, total ammonium nitrogen, nitrite and nitrate. The research data were analyzed statistically using ANOVA ($P<0.05$), Newman Keuls's advanced test and multiple regression models, with the SPSS 17.0 software. The best treatment was found in the use of 55 bioball filters per containers, with a volume of 9.8 L, which gave a bacterial density of 667.67±1.53x105 CFU mL^{-1}. The number of bioballs affected the bacterial density by 31.1%, the nitrogen absorption in fish by 16.5% and the filter media by 85.2%. The bacteriocidal types found in the filter media are *Acinetobacter* sp., *Shewanella* sp. and *Nitratireductor* sp. A salinity of 15‰ in the culturing media with a filter substrate of 55 bioball filters per container in the recirculation system gave the best bacterial density and water quality: 28.6-29.1^{0}C, pH 7.6-7.8, dissolved oxygen (DO) 6.3-6.7 mg L^{-1}, total ammonia nitrogen (TAN) 0.06-1.57 mg L^{-1}, nitrite 0.120-0.861 mg L^{-1} and nitrate 0.93-3.07 mg L^{-1}.
Key Words: bioball, bacterial density, probiotics, water quality.

Introduction. Snubnose pompano (*Trachinotus blochii*) fish is a marine aquaculture species that is relatively new in Indonesia. However, the demand for this kind of fish continues to increase, especially from the international market. *T. blochii* is a highly adaptable fish species, easy to cultivate and with a considerable economic value. Its consumption price reaches USD 4.25 kg^{-1} on the local markets and USD 14.144 kg^{-1} as export commodity (Mo 2017). Since 2015, *T. blochii* has become a leading commodity in marine cultivation fisheries, with a production of 1900 tons in 2015 and the target to increase production with 31.5% $year^{-1}$ (Prahadi 2015).

The production target for the *T. blochii* commodity can be achieved through the cultivation intensification, but the high stocking density results in a higher potential for stressing, due to a narrower space and to an increased competition for oxygen and feed, so that the fish growth will be hampered. High density also accelerates the decline in water quality due to the accumulation of metabolic waste and feed residue, shaving a major effect on growth.

Nitrogen from fish culture waste produces ammonia (NH_3), due to feed residue and fish metabolism, which can result in a buildup of organic matter causing a decrease

in water quality (Putra & Pamukas 2011; Prayogo et al 2012). Recirculation systems are technologies used to maintain water quality in fisheries, in order to remain suitable for aquatic organisms and to support the optimization of water utilization. The recirculation system is able to reduce the level of ammonia concentration, restraining it within the range of 31-43% (Djokosetiyanto et al 2006; Putra & Pamukas 2011).

According to Lekang (2008) and Fadhil et al (2010), the use of a recirculation system has several advantages including: more efficient water use, flexibility in cultivation locations, more hygiene, relatively small space or land requirements, ease of controlling and maintaining cultivated organisms, ease of maintaining water quality, environmentally friendly, pollution prevention and it can be functional permanently, without disturbing the aquatic environment.

Recirculating aquaculture system technology can also be adapted to the aquaculture system and to the filter substrate in order to control the dissolved solids (Fadhil et al 2010). The use of the right type of filter material will determine the success of fish rearing in the recirculation system because it will determine the growth of non-pathogenic bacteria on the material. Currently, biological filter substrates are widely used, being more environmentally friendly.

There are many biological filters that can be used, one of which is the bioball. Bioball is a sphere with a diameter of 4 cm, a specific area of ±230 m^2 m^{-3} with a width of each hole in a bioball of 0.92, made of PVC material (Said 2002). Bioball functions as a physiological filter which is a growing medium for bacteria that can remove ammonia contained in water (Alfia et al 2013). Nelvia et al (2015) reported that the bioball filtered *Nitrosomonas* sp. bacteria, which function to oxidize ammonia to nitrite, and *Nitrobacter* sp., which function to oxidize nitrite to nitrate.

Several studies related to improving the water quality in aquaculture with recirculation and hydroponic systems have been carried out, such as: Mulyadi & Pamukas (2011) used a recirculation system in nursery and rearing of fish (*Mystus nemurus*); Alfia et al (2013) used a recirculation system with a bioball filter in tilapia (*Oreochromis niloticus*) culture; Nelvia et al (2015) studied the addition of a bioball to the maintenance media filter on the survival and growth of goldfish (*Carassius auratus*) fry. Based on the description above, a series of studies on the number and types of bacteria cultured with different numbers of bioballs and on the improvement of water quality in the cultivation and recirculation systems are needed. This research was aimed at analyzing the types and numbers of bacteria present in the bioball, to measure the water quality improvement (temperature, pH, DO, TAN, nitrite and nitrate) and to determine the optimal number of bioball.

Material and Method

Location and time of research. This research was conducted between January and July 2019, in several laboratories, as follows: the fish rearing was carried out at the Batam Marine Cultivation Fishery Center (BPBL), the water quality analysis was carried out at the Laboratory of Fish and Environmental Health Testing of the Batam Marine Aquaculture Fisheries Agency, the bacteria types identification and densities calculation were performed at the Fish and Environmental Health Examiners Lab of the BPBL, the polymerase chain reaction (PCR) test was performed at PT Genetics Science Indonesia, West Jakarta.

Materials and tools. The main ingredients used in this research were 300 seeds of *Trachinotus blochii* (Lacepede) with a size of 10-11 cm, originating from the BPBL, fed with commercial pellets "GR-2" (46% protein, 10% fat, 13% ash content, 2% crude fiber and 10% moisture content). The test feed was obtained from the BPBL. A round bucket with a diameter of 60 cm, a height of 45 cm and a volume of 150 L was used as a research vessel. Bioballs have a diameter of 4 cm, a specific area ±230 m^2 m^{-3} and a witdth of each hole in a bioball of 0.92 mm, and are made of PVC, as a filter substrate (Figure 1a).

Experimental design. The research method used in this study was an experimental method completely randomized design, with 1 factor, 5 levels of treatment and 3 replications (Steel & Torrie 1993). As a factor in the study was the difference in the number of bioballs. The treatment level determination in this study is based on the study of Nelvia et al (2015) on the goldfish fry (*Carassius auratus*) in closed recirculation system, stating that the use of 50 bioballs gutter^{-1} can improve the water quality, providing 100% survival rates and a growth performance up to a weight of 2.605 g and to a length of 1.48 mm. Therefore, the treatment in the present study was established as follows: A=0 (control), B=35, C=45, D=55 and E=65 bioball filters per container.

Research procedure. *T. blochii* fish measuring 10-12.55 cm in length and having a body weight of 24.3-28.9 g were obtained from the BPBL and were adapted for 7 days before being used as test specimens. Fish rearing containers were filled with sea water with a salinity of 15‰, using a closed recirculation system. The rearing container was connected to a 50 x 14 x 14 cm PVC gutter, as a filter container placed at the top of the fish-raising containers. Then the water from the filter gutter flowed back through the PVC pipe with a diameter of 2.5 cm to the rearing tub of *T. blochii*. Water from the fish rearing containers flowed into the filter media (gutters), each gutter containing a number of bioballs corresponding to the tested treatment (Figure 1b) and according to Nelvia et al (2015), by using a water pump with a power of 18 watts. After passing through the filter media (gutter), the water returned to the fish-raising tub through the drain pipe in the filter container. In the rearing medium (KP-SUPER N, with *Nitrosomonas* sp. and *Nitrobacter* sp.), trademark starter bacteria were added at a dose of 2.5 mL 200 L^{-1} week^{-1} (Hartini et al 2013), to accelerate bacterial growth in the bioball.

(a) (b)

Figure 1. (a) Bioball used as a filter substrate, (b) The design of the *Trachinotus blochii* aquaculture recirculation system (original).

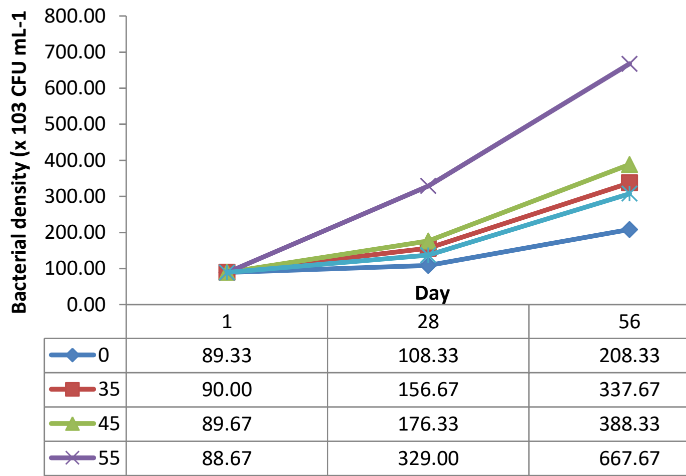
The test specimens that have been adapted to the culturing media were then randomly placed into 15 rearing containers. The fish were given commercial feed at satiation, containing: 46% protein content, 10% fat, 13% ash, 2% crude fiber, and 10% moisture, at 07:00, 12:00 and 17:00 WIB.

Measured response. The response variables measured in this study were: density and type of bacteria in the filter container were observed at the beginning, in the middle and at the end of the study (SNI.01-2332.3-2006 and SNI.01-2332.3-2006); the temperature, pH and salinity were observed every day; the dissolved oxygen (DO), Total Ammonia Nitrogen (TAN), nitrite (NO_2) and nitrate (NO_3) were observed every week. Water quality measurement procedures refer to SNI in the Public Works Service (2013).

Data analysis. Bacterial density data were analyzed according to the RAL model (Steel & Torrie 1993). To determine the effect of salinity on each measured variable, a diversity analysis was performed using the SPSS 17.0 software. If P<0.05, the Newman-Keuls further test was performed to see the differences between treatments. Water quality data were analyzed descriptively using the Microsoft Excel application and displayed in tables and graphs. To see the relationship of the number of bioballs and the density of bacteria with the water quality, a regression analysis was carried out.

Results and Discussion

Density of bacteria in the filter container. The results of bacterial density observations carried out on Plate Count Agar (PCA) media using the Total Plate Count method can be seen in Figure 2.



	1	28	56
0	89.33	108.33	208.33
35	90.00	156.67	337.67
45	89.67	176.33	388.33
55	88.67	329.00	667.67

Figure 2. Bacterial density in all treatments during the study.

Figure 2 shows that the bacterial density from day 1 to day 28 in treatments with 0, 35, 45 and 65 bioball filters per container are relatively similar, in contrast to the treatment with 55 bioball filters per container, which caused a significant increase of bacteria, compared to the other treatments. This is due to the fact that the bacteria and *T. blochii* fish are permanently adapting to the media in which they live and to the fact that the nutrients resulting from the remaining feed and fish feces are still not sufficient to support the maximum bacterial growth. After the 28th day the number of bacteria in all treatments continued to increase, reaching a maximum peak on the 56th day. This is due to the increase in organic matter from fecal matter and fish feed along with the growth of the *T. blochii* specimens, which is a source of bacterial nutrition, thereby increasing the rate of bacterial development. In accordance with the opinion of Sunarto (2003), dissolved organic matter is needed by living bacteria, then Sidharta (2000) explains that organic material contains carbon, nitrate, phosphate, sulfur, ammonia, and several minerals which are nutrients for bacterial growth. Administering KP SUPER N probiotics (routinely added in cultivation containers) causes continuous bacterial growth due to the availability of organic materials that can be used optimally. The highest bacterial density was found in the treatment of 55 bioball filters per container.

The highest bacterial density was found in the treatment with 55 bioball filters per container, at the end of the study, namely 667.7 x 10^5 CFU mL^{-1}, demonstrating the optimality of a configuration consisting of: 55 bioballs for the given size of the filter (50 x 14 x 14 cm) and for the given volume of the culture media (80 L). These parameters are balanced in such a manner that bacteria receive a sufficient amount of oxygen, through the bioball cavity. According to Nelvia et al (2015), the bioball structure (not too compact) provides an opportunity for oxygen to enter the bioball cavity and to reach bacteria. If the oxygen is sufficient, then the bacteria growth determines an optimal functioning of the bioball.

The lowest bacterial density, 208.3 x 10^5 CFU mL^{-1}, was found in treatment A without using bioballs, starter bacteria (KP SUPER N) and additional nutrients, resulting in a limited media for the growth and development of bacteria. The low density of

bacteria when using the highest number of bioball filters (65 container^{-1}) is due a number of bioballs almost exceeding the container capacity. According to Nelvia et al (2015) bioball too densely disposed in the gutter and lacking of oxygen supply can cause extinction of the bacteria in the bioball cavity. Consequently, the performance of the bioball filter decreases, suspending the process of decomposing organic matter by nitrifying bacteria.

The biofilter system removes ammonia. Ammonia accumulates and reaches toxic levels if it is not transferred by a nitrification process where the process, first oxidizing ammonia to toxic nitrite, then to nitrate, which is relatively non-toxic. The above process involves *Nitrosomonas* and *Nitrobacter* bacteria. Bacteria grow like a film on the surface of the material (biofilter container) or adhere to organic particles. Biofilter consists of a medium with a large surface for the growth of nitrite bacteria. In the recirculation system, bacteria play a role in converting toxic substances (ammonia) to non-toxic substances (nitrates). The performance of bacteria in the biofilter system is marked by an increase in the average biochemical oxygen demand (BOD) value in the culture medium (Nurhidayat et al 2012). The more bioballs, the more opportunities for bacteria to stick, when a viable oxygen level is still preserved (Nelvia et al 2015).

ANOVA results showed that the number of bioballs had an effect on the density of bacteria on day 28 and 56. The Newman Keuls range study test identifies the bacterial density differences between treatments. The results of the regression analysis between the number of bioballs and bacterial density provide the following regression equation:

$$Y = 227.9 + 3.851X$$

Where:
Y-bacterial density;
X-number of bioballs;
The calculated r was 0.557 and the R^2 was 0.311.

The results of the regression analysis showed that the number of bioballs had a positive correlation with the bacterial density (r=0.557). The increase in the number of bacteria in the filter container was influenced by the number of bioballs by 31.1 and 68.9%, which is thought to be influenced by the content of organic matter (Stepwise regression analysis). Nelvia et al (2015) stated that the bioball is a breeding ground for various bacteria needed in the breakdown of organic matter. According to Sunarto (2003) dissolved organic matter is needed by bacteria to live. Furthermore, according to Sidharta (2000) organic matter contains carbon, nitrate, phosphate, sulfur, ammonia and several minerals which are nutrients for bacterial growth.

Identification of bacteria in filter containers. Based on the observations, there were 7 pure bacterial isolates. The identification of bacteria against the isolates found in the filter media was carried out by physical, biochemical and sensitivity tests to see which bacteria had the greatest inhibition zone against pathogenic bacteria.

Physics test. The results of observations of color, shape, edge, elevation and gram of the seven isolates found are presented in Table 1.

Table 1

Observation results of bacterial characteristics

No	*Isolate*	*Colony*				*Cell*		*Treatment*
		Color	*Shape*	*Edge*	*Elevation*	*Gram type*	*Shape*	
1	A_1	Yellow	Circular	Entire	Umbonate	Negative	Stem	P_2U_1
2	A_2	Beige	Circular	Entire	Raised	Negative	Stem	P_0U_3
3	A_3	White	Circular	Entire	Raised	Negative	Stem	P_3U_2
4	A_4	White	Circular	Entire	Raised	Negative	Stem	P_4U_1
5	A_5	Beige	Filamentous	Entire	Filiform	Negative	Stem	P_1U_1
6	A_6	White	Circular	Entire	Raised	Negative	Stem	P_4U_2
7	A_7	White	Circular	Entire	Raised	Negative	Stem	P_3U_3

Table 1 shows that all bacterial isolates have gram-negative, entire edges and are rod-shaped. The dominant bacterial colony was white (4 isolates), 2 isolates were beige and 1 isolate was yellow. Colonies were circular or round (6 isolates) and filamentous (1 isolate). The elevation of the dominant bacterial colony was raised (5 isolates), 1 umbonate isolate and 1 filiform isolate.

Biochemical test. The results of observations of the Catalase, Oxidase, Motility, oxidative/fermentation (O/F) glucose, Indole and triple sugar iron agar (TSIA) tests are presented in Table 2.

Table 2

Bacterial biochemical test results

No.	*Isolate*	*Biochemical characteristics*						*Treatment*
		Catalase	*Oxidase*	*Motility*	*O/F*	*Indole*	*TSIA*	
1	A_1	+	+	+	-	-	A/K	P_2U_1
2	A_2	+	+	-	F	-	K/K	P_0U_3
3	A_3	+	-	+	O	-	A/K	P_3U_2
4	A_4	+	+	+	F	-	A/A	P_4U_1
5	A_5	+	+	+	F	+	A/A	P_1U_1
6	A_6	+	+	+	F	-	A/A	P_4U_2
7	A_7	+	+	-	F	-	A/A	P_3U_3

O-oxidative; F-fermentative; A/K-alkaline acid; K/K-alkaline; A/A-acid.

Table 2 shows a positive catalase enzyme test in all bacterial isolates, indicated by the gas bubbles formation during their reaction with the H_2O_2 drops. According to Stoica (2016), catalase is an enzyme contained in the majority of bacteria and it is involved in the decomposition of hydrogen peroxide into water and oxygen. Hydrogen peroxide and oxygen are used for electron transport during fermentation in both aerobic and facultatively anaerobic bacteria.

Oxidase tests A_1, A_2, A_3, A_4, A_5, A_6 and A_7 are positive, as shown by the color change on the oxidase paper, which indicates activity, while A_3 is negative. Five isolates (A_1, A_3, A_4, A_5, A_6) showed positive results in the motility test, due to the spread of bacterial growth on the Sulfide Indole Motility (SIM) medium, and did not grow on the part of the stick site bacteria. The O/F test results showed no color change in the paraffin-covered media and a color change on the exposed media. Five bacterial isolates (A_2, A_4, A_5, A_6, A_7) were observed to be fermentative because the tube without paraffin and the tube filled with paraffin changed their color to yellow. According to Fahri (2008), in the O/F test, oxidative organisms occur at a color change in the open media, while fermentative organisms can be indicated by no color change in the closed media.

The indole test shows 6 isolates (A_1, A_2, A_3, A_4, A_6 and A_7) giving negative results, marked with a yellow color on the surface of the media, which means that the bacteria are unable to break down the amino acid tryptophan, and no red ring after dropping the Kovacs reagent in the sulfur, indole, motility (SIM) media. According to Acharya (2012), the indole test was carried out to determine the ability of bacteria to break down amino acid tryptophan and to form indole compounds. Tryptophan is hydrolyzed by tryptophanase to produce three possible end products, one of which being the indole. Indole production is detected by the Kovacs or Ehrlich's reagent, composed of 4-(dimethylamino)benzaldehyde, which reacts with the indole to produce a red compound. A positive reaction is indicated if there is a red color on the surface of the medium, which indicates that the bacteria are able to break down the amino acid tryptophan. In the TSIA test, 4 isolates (A_4, A_5, A_6 and A_7) showed acidic properties (A/A), 2 isolates (A_1 and A_3) were acid alkaline and 1 isolate (A_2) was alkaline.

Sensitivity test. The sensitivity test to pathogenic bacteria was carried out after observing the physical and biochemical tests of the seven isolates. This test aimed to find probiotic bacteria candidates for polymerase chain reaction (PCR) testing. The results of the sensitivity test on the 7 isolates are presented in Figure 3. Inhibition zones produced

by the seven bacterial isolates were found in different filter containers. Bacteria from isolate A_1 were able to inhibit the growth of *Vibrio* bacteria during the 24-hour incubation period at a temperature of 37^0C, as indicated by the formation of a clear area around the blank disk, but they were unable to inhibit the growth of *Aeromonas* and *Pseudomonas* bacteria. After an incubation period of 48 hours, the inhibition zone remains clear, which indicates that these bacteria can kill (bacteriocidal) *Vibrio* bacteria. Similarly, for the bacteria from isolate A_2, a clear zone was formed around the blank disk, indicating an inhibition of *Pseudomonas* and *Vibrio* bacteria during the 48-hour incubation period, meaning that A_2 bacteria are bacteriocidal against *Pseudomonas* and *Vibrio*, but they cannot inhibit the growth of *Aeromonas* bacteria.

Bacteria in A_3 and A_4 isolates were unable to inhibit the growth of Aeromonas, *Pseudomonas* and *Vibrio* bacteria, as shown by a no inhibition zone formed on the blank disk. In A_5 and A_7 isolates, an inhibition zone occurred against *Aeromonas*, *Pseudomonas* and *Vibrio* bacteria during the 24-hour incubation period, but during the 48-hour incubation period, the clear area became cloudy, indicating that these bacteria were only able to inhibit (bacteriostatic) the three pathogenic bacteria . In A_6 isolate, a clear zone was also formed during the 48-hour incubation period against *Aeromonas* and *Vibrio* bacteria, but no inhibition zone was formed against *Pseudomonas* bacteria. This indicates that the A_6 bacteria are able to kill (bacteriocidal) *Aeromonas* and *Vibrio* bacteria.This is in line with Azaldin et al (2020), who states that sensitivity of a material to bacteria is characterized by the presence of an inhibition zone around the blank disk. Furthermore, according to Dwyana & Murniati (2020), this inhibition area is formed due to bioactive compounds contained in microbes that produce exoenzymes which break down organic matter and secrete hydrolytic enzymes, proteases, lipases, amylases, and cellulose so that they are able to hydrolyze polysaccharides as a carbon source and electron donors.

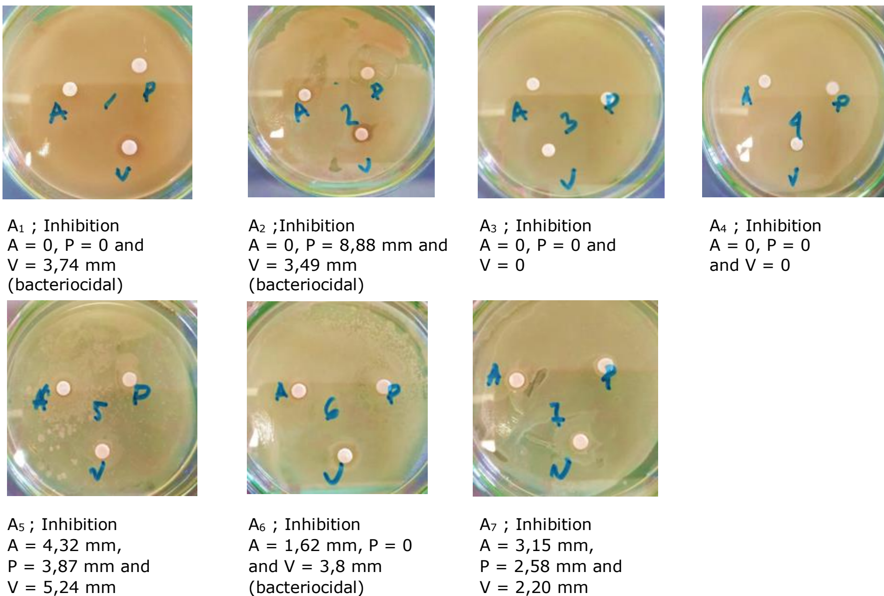


Figure 3. Sensitivity test of bacterial isolates against pathogenic bacteria (A: *Aeromonas*, P: *Pseudomonas*, V: *Vibrio*).

Madigan et al (2012) grouped bacteria based on their selective toxicity. According to them, antimicrobial compounds had 3 kinds of effects on the microbial growth: 1). Bacteriostatic inhibit growth but they do not kill. Bacteriostatic compounds inhibit protein synthesis or bind to ribosomes; 2). Bacteriocidesl kill cells but not cause cell lysis or cell

breakdown. 3). Bacteriolytic cause cells lysis or cell breakdown. Furthermore, Dwyana & Murniati (2020) stated that the sensitivity test on bacteria indicated that these were bacteriostatic after 24 hours of incubation period (clear zone formed on disc paper), while after 48 hours they were classified as bacteriocidal.

According to Jannata (2014), the responses related to the bacterial growth inhibition can be classified based on the inhibition zone diameter, as follows: weak (≤5 mm), medium (5-10 mm), strong (10-20 mm) and very strong (≥20 mm). Based on this scale, isolates A_5, A_6 and A_7 were classified as having a weak response to *Aeromonas*, isolate A_2 had a moderate response and isolates A_5 and A_7 had a weak response to *Pseudomonas* bacteria. Furthermore, A_5 isolate had a moderate response to *Vibrio* bacteria, while isolates A_1, A_2, A_6 and A_7 were classified as having a weak response. According to Sugita et al (1996), these bacteria are able to produce antimicrobial compounds, such as: siderophores, bacteriocins, lysozymes, proteases, hydrogen peroxidase, and organic acids, as a mechanism of competition for nutrients and energy, inhibiting the growth of pathogens in the filter container. According to Rengpipat et al (2000), the siderophore is a compound with a low molecular weight (<1500) and it is also a specific agent for ferric ions binding, which can dissolve iron precipitates and change them into the form required for microbial growth. An organism's ability to form siderophores causes inhibition in other organisms, especially in aquatic animal pathogens, due to their incapacity to obtain the required ferric ions.

Based on the results of observations on the inhibition zone diameter and inhibition power, the bacterial isolates selected as candidates for probiotic bacteria were: A_1, A_2 and A_6, classified as bacteriocidal, then coded MUL_1, MUL_2 and MUL_3. Furthermore, to determine the type of the three bacteria, a DNA analysis was carried out using PCR.

Polymerase chain reaction (PCR) test

The results of amplification and visualization of DNA fragments of the 16S rRNA gene. The genomic DNA had been isolated and extracted from isolates MUL_1, MUL_2 and MUL_3, and then the 16S rRNA markers were amplified using PCR technique. PCR analysis of the 3 bacteria isolates used a 27F forward primer: AGAGTTTGATCMTGGCTCAG and reverse primer 1492R: TACGGYTACCTTGTTACGACTT. The analysis results showed positive with the formation of bands with a total molecular weight (in base pairs - bp) of 1,000–1,500 bp. The results of amplified DNA visualization, using 27F and 1,492R primers electrophoresed with UV light, are presented in Figure 4. The amplified fragment has a molecular weight of 1,227-1,372 bp. Sequence analysis performed with Geneious reveals that isolates produce single clearly visible bands with a molecular weight of 1,331 bp (MUL_1), 1,227 bp (MUL_2) and 1,372 bp (MUL_3), according to the primary amplification size with 27F and 1,492R primers, by comparison using 1 Kb Ladder DNA. The size is in accordance with the expected size of the bacterial 16S rRNA genes, which is between 1,300–1,500 bp (Seprianto et al 2017).

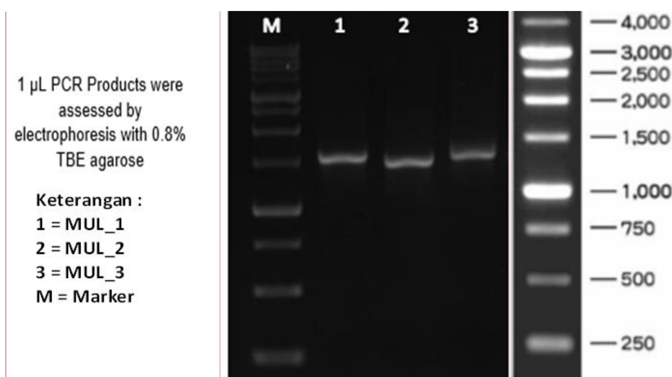


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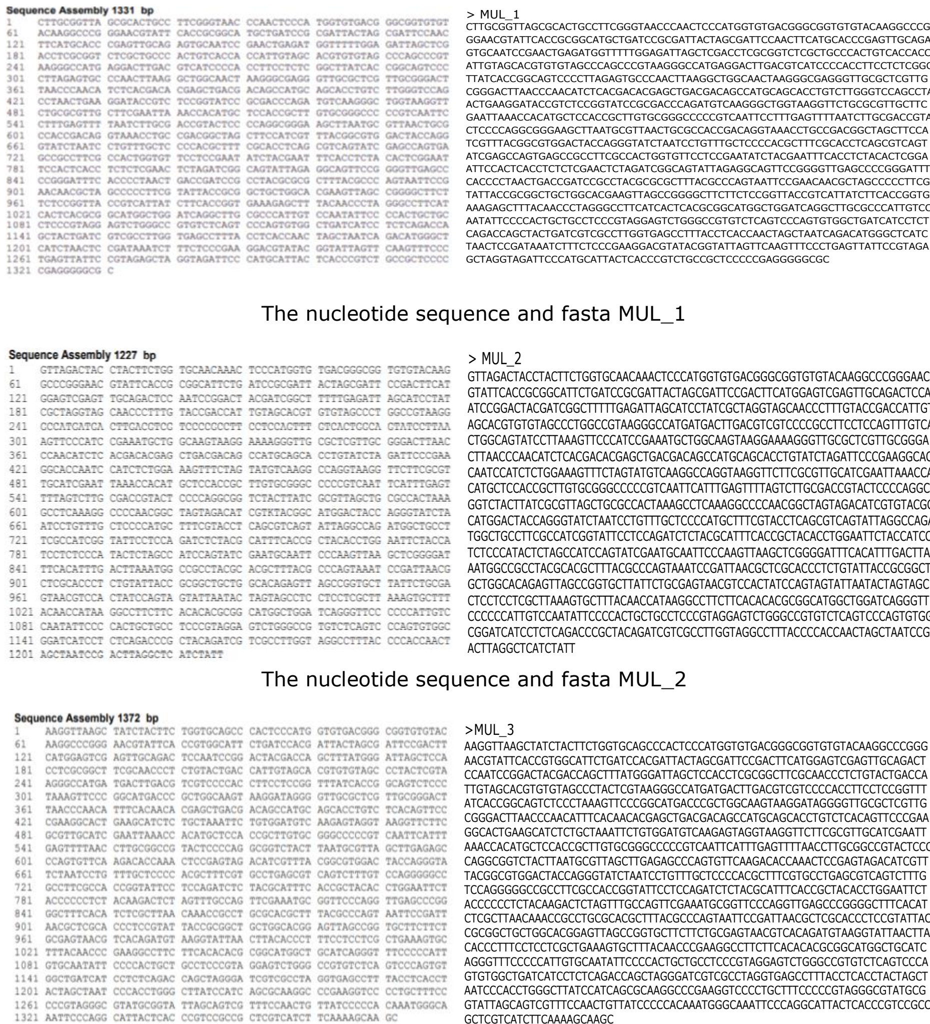
Figure 4. Results of universal DNA-PCR amplification on agarose gel.

The base pairs obtained were combined and trimmed using the Bioedit 7.0 software. DNA sequencing analysis gives the following results for the nucleotide and protein FASTA sequences (Figure 5).



The nucleotide sequence and fasta MUL_1

The nucleotide sequence and fasta MUL_2

The nucleotide sequence and fasta MUL_3

Figure 5. The nucleotide sequence and fasta MUL_1, MUL_2 and MUL_3.

16S rRNA gene sequence alignment with basic local alignment search tool (BLAST). BLAST is accessible online via: http://blast.ncbi.nlm.nih.gov/Blast.cgi and provides the results of the DNA sequencing. A search was carried out for species identification, based on the percentage of the sequenced DNA homology with existing databases on GenBank. The identification results of probiotic bacterial isolates using BLAST correspond to the highest homology (closest relationship) with the bacteria in GenBank. Based on the nucleotides registered with the National Center for Biotechnology

Information (NCBI), the species, strains and accession numbers of bacterial isolates identified in this study are presented in Table 3.

Table 3

Results of tracing the 16S rRNA sequencing of bacterial isolates with the BLAST system

Isolate	*Species*	*Strain*	*Accession number*	*Query coverage*	*Homology*
MUL_1	*Acinetobacter* sp.	MUL37	MT229070	100%	100%
MUL_2	*Shewanella* sp.	MUL31	MT229068	100%	100%
MUL_3	*Nitratireductor* sp.	MUL35	MT229069	100%	100%

The alignment results of 16S rRNA gene sequences with BLAST (Table 3) showed that the bacterial isolate MUL_1 was identified as *Acinetobacter* sp. strain MUL37 with 100% homology and 100% query coverage, MUL_2 isolate was identified as *Shewanella* sp. strain MUL31 with 100% homology and 100% query coverage and bacterial isolate MUL_3 was identified as *Nitratireductor* sp. strain MUL35 with 100% homology and 100% query coverage. The types of bacteria found are thought to have come from the waters of the Batam Sea which are used as a medium for culturing *T. blochii*, because the starter bacteria added to the media do not contain these types of bacteria.

According to Adithiya et al (2017), the sequence equation between 93-97% can represent the identity of bacteria at the genus level but different at the species level. Furthermore, Hagstrom et al (2000) and Seprianto et al (2017) state that bacteria that have a 16S rRNA sequence equation greater than 97% are the same species, while the equation between 93-97% can represent identity at the genus level but differ at the species level. According to Dancourt et al (2000), if the similarity of the sequences is less than 97%, it is likely either a new species, since there is no data in the database, or the the sequencing results are too short to be relevant, when compared to the database.

Phylogenetic tree analysis. The analysis of phylogenetic tree formation was carried out by looking at the similarities that exist in the genetic isolates of MUL_1, MUL_2 and MUL_3 bacteria with a database of the results of alignment of nucleotide sequences in GenBank using the Mega X 10.0.5 WIN 64 application. Phylogenetic trees analysis used the UPGMA (Unweighted Pair Group Method with Arithmetic Average), a clustering method based on the pairwise distance arithmetic means. Phylogenetic analysis used 17 sequences with 3 sample sequences and 14 comparison sequences, the alignment process used 1000 bootstraps and the Kimura 2 model parameters. Branches that connect the dots (nodes) are taxonomic units, such as species or genes and tree roots are the ancestors (ancestors) for all organisms. The dendogram is presented in Figure 5.

Figure 5 shows that the bacteria MUL_1, MUL_2 and MUL_3 are in 1 cluster and that they are separated from the type of control bacteria, which means thatMUL_1 and MUL_2 have close relationships, tending even to be of the same species or to belong to the same subspecies, while MUL_3 bacteria are from the same genus. Based on the the branching point's location, it could be considered that the three isolates were closely related to *Nitratireductor* and *Shewanella*.

According to the calculation results of the genetic distance (using Mega X 10.0.5 WIN 64), it was found that the distances between species varied, ranging from 0.00 to 1.6659. The smallest genetic distance, of 0.2005, was found between bacteria MUL_1 and MUL_2, while between MUL_1 and MUL_3 it was of 0.2081. The genetic distances found were the following: 1.2531 between bacteria MUL_1 and *Acinetobacter venetianus* (GenBank: NR.042049.1), 1.2955 between MUL_2 and *Shewanella algae* (GenBank: NR117771.1) and 1.2468 between MUL_3 and *Nitratireductor aquimarinus* (GenBank: 117929.1). According to Tallei et al (2016), the smaller the genetic distance between two organisms, the closer their kinship.

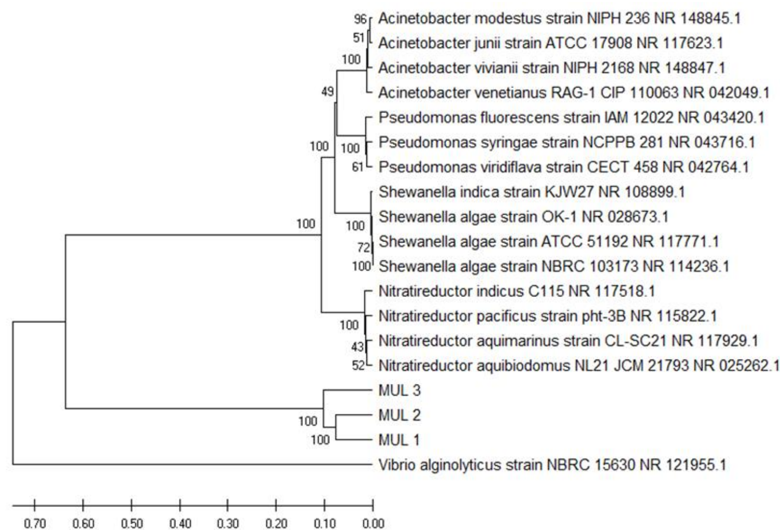


Figure 5. Phylogenetic tree dendrogram of isolates MUL_1, MUL_2 and MUL_3 with 16 comparative species.

Bauvet & Grimont (1986) stated that *Acinetobacter* (Isolate MUL_1) belongs to the kingdom *Acinetobacter*, phylum Proteobacteria, class Gammaproteobacteria, order Pseudomonadales, family Moraxellaceae, genus *Acinetobacter*. In general, these bacteria are rod-shaped, gram-negative, non-motile, catalase positive and oxidase negative, live aerobically, and are opportunistic. Beleneva & Maslennikova (2004) and Soslau et al (2011) reported that the *Acinetobacter* bacteria were found to be associated with several invertebrates such as mollusks, sea cucumbers, sea urchins, starfish and crustaceans.

Shewanella bacteria (Isolate MUL_2) belong to the Bacteria kingdom, the Proteobacteria phylum, the Gamma Proteobacteria class, the Alteromonadales order, the family Shewanellaceae and the genus *Shewanella*. The special characteristics of Shewanellae are their ability to capture electrons in oxygen deficient conditions and their ability to survive in various habitats (Gralnick & Newman 2007). The genus *Shewanella* is a gram-negative rod-shaped proteobacteria, 2-3 μm long and 0.4-0.7 μm in diameter. These bacteria are facultative anaerobic bacteria that are commonly found in marine sediments and can swim with the help of a single polar flagellum (Venkateswaran et al 1999). *Shewanella* is a genus of metal reducing bacteria found in marine environments, freshwater, lakes, land or terrestrial, rivers, Arctic and Antarctic oceans, corroded oil pipes and aquifer environments contaminated with uranium. These bacteria are widely used for bioremediation or environmental cleaning of pollutants such as chlorinated compounds, radionuclides and other environmental pollutants (Venkateswaran et al 1999).

According to Labbe et al (2004), *Nitratireductor* (Isolate MUL_3) belongs to the kingdom Bacteria, phylum Proteobacteria, class Alpha Proteobacteria, order Rhizobiales, family Phyllobacteriaceae and genus *Nitratireductor*. *Nitratireductor* bacteria isolated from sea water in the Indian Ocean and marine sediments in the Pacific Ocean have the ability to reduce ammonia, are rod-shaped and form white bacterial colonies. The optimum temperature for their growth is 25-35ºC and pH 5-12.

Water quality. The results of observations on temperature, pH, dissolved oxygen (DO), TAN, nitrite, (NO_2) and nitrate (NO_3) in all treatments during the study are presented in Table 4.

Table 4

Average temperature, pH, dissolved oxygen (DO), total ammonia nitrogen (TAN), nitrite (NO_2) and nitrate (NO_3) in all treatments during the study

Parameters	Number of bioball of each filter container				
	0	*35*	*45*	*55*	*65*
Temp. (^{0}C)	28.6-29.1	28.4-29.1	28.7-29.1	28.6-29.1	28.6-29.1
pH	7.5-7.7	7.6-7.7	7.6-7.7	7.6-7.8	7.6-7.7
DO (mg L^{-1})	6.2-6.5	6.2-6.6	6.2-6.6	6.3-6.7	6.2-6.6
Salinity (g L^{-1})	15	15	15	15	15
TAN (mg L^{-1})	0.61.-1.58	0.28-1.59	0.18-1.56	0.06-1.57	0.12-1.56
Nitrite (mg L^{-1})	0.624-0.953	0.209-0.863	0.170-0.875	0.120-0.861	0.129-0.874
Nitrate (mg L^{-1})	0.820-1.12	0.98-1.40	0.96-2.15	0.93-3.07	0.98-1.42

Table 4 shows that all water parameters, namely: temperature, pH, dissolved oxygen (DO), ammonia (NH_3) and nitrite (NO_2) are relatively similar in all treatments, except for the nitrate content. The highest nitrate content was found in the treatment with 55 bioball filters per container. In general, temperature, pH, DO, TAN, nitrite and nitrate are in a good range for the growth of *T. blochii*.

SNI 7901.4 (2013) and Ashari et al (2014) state that the optimal temperature for the growth of *T. blochii* ranges from 28 to 32°C, and the pH ranges from 6.8 to 8.4. According to Ezraneti et al (2019), *T. blochii* fish can live optimally at a salinity of 14-19 ppt. SNI 7901.4 (2013) requires a dissolved oxygen level of at least 5 mg L^{-1} for the rearing of *T. blochii* seeds. Royan et al (2019) reported that the lethal concentration (LC50) of TAN ranges from 1.10 to 22.8 mg L^{-1} for invertebrates and from 0.56 to 2.37 mg L^{-1} for fish, within 24-96 hours after exposure. The nitrite level for the maintenance of *T. blochii* seeds is of maximum 1 mg L^{-1} (SNI 7901.4. 2013).

The highest nitrate concentration was found in the treatment with 55 bioball filters containers^{-1} for a volume of 9.8 L, optimal for nitrification bacterial culture media. The higher the number of bacteria, the faster the nitrification process, resulting in an increase in the concentration of nitrate in the media. According to Lampert & Sommer (2007), bacteria have proteolytic enzymes that can break down organic nitrogen into nitrates, which are nutrients needed by phytoplankton and other aquatic microflora. Furthermore, Nelvia et al (2015) obtained the almost the same optimal number of bioballs for the growth of goldfish, namely 50 bioball filters per container of 9.8 L. Nitrate levels obtained during the study were still in accordance with the quality standards referred to in the Government Regulation No. 82 of 2001, namely <20 mg L^{-1}. Effendi (2014) considers a nitrate content of 0-1 mg L^{-1} in the oligotrophic (low) category and a content of 1-5 mg L^{-1} in the mesotrophic (moderate) category. According to Ulqodry et al (2010), normal nitrate content in marine waters generally ranges from 0.01-50 mg L^{-1}. Furthermore, Hartoko (2010) reports that algae, especially phytoplankton, can grow optimally at a nitrate content of 0.009-3.5 mg L^{-1}.

Relationship between number of bioballs and water quality. The results of the regression analysis between the number of bioballs with temperature, pH, DO, TAN, NO_2 and NO_3 are presented in Table 5.

Table 5 shows that the number of bioballs has the greatest effect on the TAN reduction (R^2=0.931 or 93.1%), with a very strong negative linear correlation (r=0.965), meaning that the ammonia concentration decreases by adding bioballs in the filter container. This is because in the filter media there are several types of nitrifying bacteria derived from the introduced starter bacteria, namely *Nitrosomonas* sp. and *Nitrobacter* sp., in addition to the bacteria found in the bioball, namely *Acinetobacter* sp., *Shewanella algae* and *Nitratireductor aquimarinus.* The presence of these bacteria can accelerate the oxidation process of TAN, which is toxic to fish, into non-toxic nitrate through the nitrification process.

Table 5

Linear regression analysis between the number of bioballs with temperature, pH, DO, TAN, NO_2 and NO_3

Parameter	*Linear regression equation*	*Correlation coefficient (r)*	*Coefficient of determination (R^2)*
Temperature	Y=289.72–0.038 X	-0.767	0.589
pH	Y=759.51+0.067 X	0.444	0.197
Dissolved oxygen (DO)	Y=634.56+0.196 X	0.650	0.423
TAN	Y=58.29–0.83 X	-0.965	0.931
Nitrite (NO_2)	Y=587.55–8.345 X	-0.949	0.900
Nitrate (NO_3)	Y= 94.73+1.75 X	0.664	0.441

The value of r=0 indicates that there is no correlation between the two variables (number of bioballs and water quality), r<0.25 indicates a very weak correlation, r>0.25 and <0.5 shows a sufficient correlation, r> 0.5 and <0.75 suggest a strong correlation, r>0.75 and <0.99 demonstrate a very strong correlation and r=1 signifies a perfect correlation; $R^2 \geq 0.5$ (50%) measures the ability to explain the dependent variable (water tanquality) by the independent variable (number of bioballs) (Sarwono 2012).

Relationship between bacterial density and water quality. The results of regression analysis between bacterial density and temperature, pH, DO, TAN, NO_2 and NO_3 are presented in Table 6.

Table 6

Linear regression analysis between bacterial density with temperature, pH, DO, TAN, NO_2 and NO_3

Parameter	*Linear regression equation*	*Correlation coefficient (r)*	*Coefficient of determination (R^2)*
Temperature	Y=289.50–0.003 X	-0.226	0.475
pH	Y=756.37+0.015 X	0.695	0.484
Dissolved oxygen (DO)	Y=289.50+0.036 X	0.816	0.667
TAN	Y=59.578–0.090 X	-0.725	0.526
Nitrite (NO_2)	Y=557.73–0.796 X	-0.625	0.391
Nitrate (NO_3)	Y=30.304+0.352 X	0.923	0.852

Table 6 shows that bacterial density has the greatest influence on the increase in nitrate concentration in the *T. Blochii* rearing medium (R^2=0.852 or 85.2%). This is due to the presence of *Nitrosomonas* bacteria, *Nitrobacter* which are included as a starter in the filter container, and to the heterotrophic bacteria in the bioball, which accelerate the ammonia to turn into nitrate, so that the nitrate concentration increases with the bacterial density.

Conclusions. The number of bioballs had an effect (P<0.05) on the bacterial density. The best treatment was found in the use of 55 bioball filters per container, in a volume of 9.8 L, which gave a bacterial density of 667.67±1.53 x 105 CFU mL^{-1}. The number of bioballs had an influence on the bacterial density by (R^2=31.1%). The bacteriocidal types found in the filter media are *Acinetobacter*, *Shewanella* and *Nitratireductor*. The number of bioballs strongly affected the decrease in TAN (R^2=93.1%), while the bacterial density strongly influenced the increase in nitrates (R^2=85.2%).

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C.I.F.:
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Județul:
Cod IBAN:
Banca:

Nr. crt.	Denumirea produselor sau a serviciilor	U.M.	Cantitatea	Prețul unitar (fără T.V.A.) -lei-	Valoarea -lei-	Valoarea T.V.A. -lei-
0	1	2	3	4	5 (3x4)	6
1	PUBLICATION FEE	at	1	285,71	285,71	14,29

Semnătura și ștampila furnizorului

Date privind expediția:
Numele delegatului e-mail
B.I./C.I. seria ... nr. ... eliberat(ă)
Mijlocul de transport ... nr.
Expedierea s-a făcut în prezența noastră la data de ... ora
Semnăturile

Total din care: accize	285,71	14,29
		X
Semnătura de primire	Total de plată (col.5 + col.6)	300,00 USD

NUME
PRENUME
B.I./C.I.
N.P.

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