The efficacy of probiotic with different storage to decrease the total organic matter, ammonia, and total *Vibrio* on shrimp pond water

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Abstract. Organic matter, ammonia, and total *Vibrio* are some parameters in shrimp culture media that can influence the success of shrimp culture. This study aims to determine the effect of *Pseudomonas* and *Bacillus* probiotic storage duration on decreasing organic matter, ammonia, and Total *Vibrio* in shrimp pond water. This study used a Completely Randomized Design (CRD) with five treatments namely without the addition of probiotics (P0, control), addition of fresh probiotics (P1), addition of probiotics with a storage period of 1 month (P2), addition of probiotics with a storage time of 2 months (P3) and addition of probiotics with a storage period of 3 months (P4). Each treatment repeated four times. The content of total organic matter and *Vibrio* in water measured on day 0 (initial), and third (end), ammonia content was measured every 24 hours during the study (t0-t3). The results showed that the addition of probiotics with a long storage different give in decreasing total organic matter, ammonia, and Total *Vibrio* in shrimp pond water. Addition of probiotics with a storage period of 3 months gave a Total *Vibrio*, organic matter, and ammonia content lower compared to the shrimp pond water without adding probiotics and gave significantly different (p <0.05).

1. Introduction
Market demand for shrimp continue to increase with an average value of 8.42 per cent or 67.514 tonnes per year with a concomitant increase in human population in the world, resulting in the production of shrimp in aquaculture need for intensification in order to increase shrimp production results with a minimum land [1]. Intensive shrimp farming system would also require more inputs especially in seed and feed [2]. But intensive cultivation system is bad for the organism, because it can increase the cultivation of waste in the form of organic matter, residual feed, feces, increasing toxic compounds such as NH₃, and can increase the incidence of diseases in aquaculture organism [3].

Shrimp farming success is determined by factors such as a good environment is the quality of water and the total bacterial pathogens in aquatic cultivation. Water quality, especially levels of total organic matter and ammonia which exceeds the threshold of ≤ 90 mg / l and ≤ 0.1 mg / l [4], is one of two factors causing the failure of shrimp farming. In addition to problems of water quality degradation, shrimp farming failure can also be caused due to disease, especially vibriosis. Austin *et al.* [5] suggests that the factors that typically cause vibriosis disease outbreaks associated with worsening environmental changes and stress on the shrimp. According to Taslihan *et al.* [6], the maximum threshold where the...
cultivation of *Vibrio* in water is $10^4$ CFU/ml, if the presence of *Vibrio* exceed $10^4$ CFU/ml, it can cause disease vibriosis which will then be followed by mass mortality in shrimp aquaculture.

To prevent the deterioration of water quality, it can be done with the administration of probiotic bacteria during the maintenance period of shrimp, which aims to decompose organic matter of the unconsumed feed eaten by shrimp vaname and feces in the waters of aquaculture can be decomposed by bacteria in probiotic [7]. Wang *et al.* [8] explains that the probiotic bacterium produces an enzyme able to break down complex compounds into simpler.

*Pseudomonas* and *Bacillus* bacteria is often used as biodegradation and inhibiting pathogenic bacteria as *Pseudomonas* can secrete proteolytic enzymes, amylase, cellulose, lipase and antibacterial bioactive *Vibrio* [9]. While *Bacillus* is a bacteria that has the advantage that is able to secrete enzymes such extracellular protease, amylase, and lipase which can help accelerate the process of degradation of organic matter in aquatic ecosystems [10]. *Bacillus* decompose the organic matter contained in a body of water to secrete enzymes to decompose complex compounds [11].

A solution is needed to improve the environmental quality of the crop and can increase the production of shrimp farming. One solution that has been developed is to use the help of microorganism decomposers called probiotic bacteria. The microorganisms decomposing or probiotics is a biotechnology product, containing bacteria obtained from nature that have been selected and serves to assist the process of degradation of organic matter in the water [12]. Probiotics is a microscopic organism that is intentionally added to the environment with the aim to improve the environmental quality [13]. Moriarty [14] suggested that the expanded definition of probiotics as live microbial additives that are beneficial to health hydrobionts and can improve productivity. Based on the research results [15] that the use of probiotics can reduce the content of ammonia ($NH_3$) and the total number of *Vibrio*.

Understanding of the benefits of probiotics on water quality has led to an increase in the use of probiotics as an effort to protect water quality in shrimp culture systems. Storage of a probiotic product liquid properly is an important factor considered in order to minimize loss of quality probiotics (viability of bacteria), i.e. by storing in cool air, not exposed to direct sunlight, and store the appropriate environmental conditions characteristic of probiotic bacteria [16]. The desired effect of probiotic bacteria can only provide benefits if the number of bacteria contained sufficient and good quality of the bacteria that, when applied [17].

In this study, the probiotic bacteria content *Pseudomonas diminuta*, *Bacillus subtilis* and *Bacillus mycoides* stored on a certain shelf life. With the hope to determine the shelf life of probiotics that they have a good influence on the field later in the application. The shelf life of *Pseudomonas* and *Bacillus* probiotics which still has a good effectiveness is expected to improve water quality shrimp farming. Expected in its application as probiotics still may have a positive impact on water quality of shrimp culture, thereby increasing the value of survival of shrimp culture, although it has suffered long enough storage. The purpose of this study is to know the effect of different probiotic storage on the decrease of total organic matter, ammonia, and total *Vibrio* in penaeid shrimp pond water.

### 2. Materials and methods

#### 2.1 Place and imte

This study was carried out at the Microbiology and Fish Disease Laboratory, Anatomy and Cultivation Laboratory, Chemistry Laboratory of Fisheries and Marine Faculty, Universitas Airlangga in Surabaya. This study was conducted for five months on December 2018 to April 2019.

#### 2.2 Study material

The material used in this study was vaname shrimp pond water without probiotic, *B. subtilis* isolates with a density of $10^6$ CFU / ml, *B. mycoides* isolates with a density of $10^6$ CFU / ml, *Pseudomonas diminuta* bacterial isolates with a density of $10^6$ CFU / ml, PBS, TSA, TSB, TCBS, molasses, tofu liquid waste, skim milk, glucose, distilled water, 70% alcohol, chlorine, $H_2SO_4$ (6 N), KMnO$_4$ (0,01 N), $H_2C_2O_4$ (0,01 N), and water test kit "Merck" ammonium ($NH_4$).
2.3 Study tools
The tools used in this study was petri discs, ose, matches, bunsen, tray, tube reaction, rack reaction, laminary flow, autoclaves, incubators, drop pipettes, slide objects, micropipett, handtally counters, glass bottle of 220 ml, analytical neraca, microtube 1500 mL, microtip (blue and yellow), spatula, water testkit (pH pens and pH paper), DO meter, refractometer, thermometer, paper labels, markers, aluminum foil, Jerrycan, plastic wrap, magnetic stirrer, vertical laminar flow cabinet, loop ose, petridish, handglove, masks, spectrophotometer, refrigerator, vortex, autoclave, flask, glass measurer, funnel glass, drigalsky, and black background.

2.4 Work Procedures

2.4.1 Sterilizing of Tools for Bacterial Culture
Tools that will be used for bacterial culture must first be washed using soap to be clean. Also the equipment must be sterilized using an autoclave at 121 °C at a pressure of 1 bar(a) for 15 minutes [18]. Tools that cannot withstand high temperatures are sterilized using 70% alcohol.

2.4.2 Bacterial Isolate Preparation
The bacterial isolates used in this study were *Bacillus subtilis*, *Bacillus mycoides*, and *Pseudomonas diminuta* obtained from sediment of Vaname shrimp ponds. Bacteria are cultured on TSA and TSB media.

2.4.3 Calculation of Bacteria
The method of measuring the concentration of *B. subtilis* bacteria, *B. mycoides*, and *Pseudomonas diminuta* using a spectrophotometer. Each isolate was taken as one loop and aseptically cultured on TSB medium, then incubated for 24 hours which was set at 30 °C. After 24 hours the concentration of bacteria measured its density with a spectrophotometer. Before calculating with a spectrophotometer, bacterial isolates were incubated on TSB Carried out using sterile PBS 2x washing on a centrifuge and vortex. Measurements on the spectrophotometer are Carried out with a wavelength of 550 nm [19], then the value of Optical Density (Å) is Obtained from the standard McFarland 0.5; 1; 2; 3; 4; 5; 6; 7; 8 at a 550 nm wavelength the which is then processed using Ms. Excel to find the regression equation so that a regression equation is Obtained with a regression value \( R^2 = 0.9941 \). The value of Optical Density (Å) as a value of “y”, following a regression equation to determine the density of bacteria (x);

\[
y = 0.0009x + 0.0073
\]

2.4.4 Making and Testing Media Test Mix Culture
The probiotics used in this study probiotic bacteria were cultured from *Bacillus subtilis*, *Bacillus mycoides*, and *Pseudomonas diminuta*. The bacteria that will be inoculated on the probiotic medium is 10^5 CFU / ml [20], the which is then incubated for 24 hours, 30 hours and 32 hours at 30°C. The material used in this test is 6% Glucose [21]; 2.5% Molasses [22]; 10% Skim Milk [23; 21]; and Tofu Liquid Waste 10% [21] for the mix culture media and 6% Glucose [21]; 2.5% Molasses [22]; 10% Skim Milk [23; 21]; and 5% soy milk for soymilk media, all media using 15 ppt NaCl solution (1.5%).

2.4.5 Storage Probiotics
Storage of probiotics is placed on the 3 plastic bottles measuring 1 L at a temperature of 25 °C cool and constant [24], with each – each long shelf life of probiotics applied to disk storage a month, two months, and three months before the end of probiotics applied to the vaname shrimp pond water.

2.4.6 Intake Pond Water
Water shrimp for this study was obtained from shrimp culture ponds vaname private property in the Tanggulrejo village, district Manyar, Gresik. Water is used from shrimp pond water of shrimp production vaname age vaname DOC (Days Of Culture) 90 – 100 days. Taking as much as 10 L of pond
water were placed in a 10 L Jerry can which had previously been sterilized using a chlorine solution with a concentration of 2 ppm for 24 hours. Then Jerry cans have been filled with water of vaname shrimp in its transportation to the place of research using ice cooler box with a tube filled there in [25].

2.4.7 Preparation of Case Treatment Research
The container used is sized 220 ml glass bottle. Containers to be used for the treatment of research should be washed first with soap so clean. The containers also should be sterilized using an autoclave at 121 °C with a pressure of 1 bar (a) for 15 minutes [18].

2.4.8 Treatment Research.
Vaname shrimp pond water which is used by 200 ml each vial and added 3% molasses [22]. Efficacy against a decrease in total organic matter, ammonia, and total Vibrio conducted for 4 days (t0-t3). According to [4], probiotics performed on day 0 (t0), a total of 1/1000 or 0.2 ml per 200 ml of vaname shrimp ponds water. Measurement of ammonia concentration is done every 24 hours, namely on day 0 for baseline and day 1 to day 3 (t0 – t3). Measurement of the total organic matter (TOM) and total Vibrio performed on day 0 and day 3 (t0; t3). In this study, no replacement of water and the addition of probiotics.

2.4.9 Parameter Observation
Examination of the parameters in the study include observation of the main parameters that consists of organic material in total (TOM), the levels of ammonia (NH3), the total number of Vibrio, as well as observations supporting parameters consisting of temperature, pH, dissolved oxygen (DO), salinity, and total probiotic bacteria after storage.

1. Main parameter
a. Measurement of Total Organic Matter (TOM)
Measurement of the total organic matter (TOM) in shrimp pond water vaname performed on day 0 and day 3 (t0; t3) at 10:00 am, using KMnO4 titration method to measure (TOM). Pipette 100 ml of the sample put into 300 ml Erlenmeyer and add 3 eggs boiling stones. 0.01 N KMnO4 add a few drops into the specimen to occur pink but prior to standardization KMnO4 solution made according to the SNI 06-6989.22-2004. Add 5 ml of sulfuric acid 6 N-free organic substances. Heat above a hotplate at 105 °C ± 2 °C, when there is the smell of H2S, boiling forwarded a few minutes. Pipette 10 ml of standard solution of KMnO4 0.01 N. Heats up to boil for 10 minutes. Pipette 10 ml of standard solution of oxalic acid 0.01 N. Titration with 0.01 N potassium permanganate until a pink color. Noting the volume of KMnO4 consumption, calculating the organic matter content was calculated using the following calculation formula (SNI 06-6989.22-2004):

$$\text{K MnO}_4 \text{mg/l} = \frac{[(10 \times a) - (10 \times c) / \text{d} \times 31.6 \times 1000]} {\text{xf}}$$

with the understanding:

- a = volume of 0.01 N KMnO4 required in the titration;
- b = normality actual KMnO4;
- c = normality of the oxalic acid;
- d = the volume of sample; and
- f = dilution factor of the sample.

b. Measurement of levels (NH3)
Measurements (NH3) in the water pond shrimp is done every 24 hours for 4 days (t0 – t3) at 09.00 pm, using water test kit ammonium (NH3) with the brand name “Merck”. The trick is to fill vaname shrimp pond water from a glass bottle into a measuring tube filled up to the mark up to 5 ml. After taking 5 ml water sample and then drops reagent as recommended usage, shake until evenly distributed. Compare with the test sample paper color spectrum measurement of nitrite. When comparing the colors keep it
away from direct sunlight. After obtaining ammonium values (mg / l), then the value is converted into ammonia (mg / l) based on the temperature and pH value in the conversion table [26].

**c. Measurements Total Vibrio**
Measurement of total Vibrio on preliminary data shrimp pond water a day to 0 (t0) and day 3 (t3) that is water shrimp from each replicate of each treatment is taken as 1 ml and diluted 10⁻¹, 10⁻², 10⁻³ in PBS solution, then each dilution was taken as many as 100 mL (0.1 ml) to plated on TCBS agar media but previously homogenized with a vortex, but if the calculation results in a dilution of 10⁻³ was still too dense then the dilution can be added. Incubation was performed for 24-48 hours at a temperature (30 °C) and then the number of bacterial colonies growing calculated. Calculation of Vibrio calculated using the spread plate method (TPC) with the following calculation formula [27]:

\[
N_i = \frac{N_o}{V_S \times f_p}
\]

Description: 
- \(N_i\) = number of bacterial cells (CFU / ml)
- \(N_o\) = the number of bacterial colonies growing
- \(V_S\) = volume of sample plated on media
- \(f_p\) = dilution factor

**1. Support parameter**

**a. Density measurements of Probiotic Bacteria Post-Storage**
Measurement of total probiotic bacteria on days 30, 60, 90 probiotic shelf life is taken as 1 ml and diluted 10⁻¹, 10⁻², 10⁻³, 10⁻⁴, 10⁻⁵, 10⁻⁶, 10⁻⁷, 10⁻⁸ in PBS solution, then dilution (10⁻⁶, 10⁻⁷, 10⁻⁸) is taken as many as 100 mL (0.1 ml) to plated on TSA media but previously homogenized. Incubation was performed for 24 hours at a temperature (30 °C) and then the number of bacterial colonies growing calculated. Counting of bacteria was calculated using spread plate method with the following calculation formula [27]:

\[
N_i = \frac{N_o}{V_S \times f_p}
\]

Description: 
- \(N_i\) = number of bacterial cells (CFU / ml)
- \(N_o\) = the number of bacterial colonies growing
- \(V_S\) = volume of sample plated on media
- \(f_p\) = dilution factor

**b. Water Quality Measurement**
Measurement of temperature, pH, dissolved oxygen (DO), and salinity using a thermometer, pH pen, DO meter and refractometer is done every day in the morning and afternoon (09.00 and 15.00) for 4 days (t0 - t3). The study was conducted using an experimental method with five treatments and four repetition. The results from this study were Analyzed by ANOVA (Analysis of Variance).

**3. Results and discussion**

**3.1 Results**
The efficacy of probiotics to Decreased storage of organic matter, ammonia, total Vibrio in this study
Table 1. Levels of total organic matter (TOM) in penaeid shrimp pond water at the end of incubation

| Treatment | Day 0 ± SD | Day 3 ± SD |
|-----------|------------|------------|
| P0        | 39.62 ± 0.00 | 38.26 ± 0.46 |
| P1        | 39.62 ± 0.00 | 25.87 ± 0.38 |
| P2        | 39.62 ± 0.00 | 26.98 ± 0.63 |
| P3        | 39.62 ± 0.00 | 27.12 ± 0.54 |
| P4        | 39.62 ± 0.00 | 29.36 ± 2.22 |

Description: The difference notation letters in the same column shows the results significantly different (P<0.05). P0 = without probiotics; P1 = fresh probiotic; P2 = probiotic shelf life of 1 month; P3 = probiotic shelf life of 2 months; P4 = probiotic shelf life of 3 months.

Table 1 showed the results of decreased levels of organic matter in treatment P1, P2, and P3 showed results that are not significantly different (P>0.05), but significantly different (P<0.05) on treatment P0 and P4. Treatment P1 has the smallest value (25.871 mg/L) and for P0 has the biggest value (38.269 mg/L). This shows that the addition of probiotics can degrade organic matter in the water.

Table 2. Levels of ammonia in penaeid shrimp pond water during the incubation period of 3 days

| Treatment | Day 0 ± SD | Day 1 ± SD | Day 2 ± SD | Day 3 ± SD |
|-----------|------------|------------|------------|------------|
| P0        | 0.100 ± 0.00 | 0.110 ± 0.00 | 0.173 ± 0.00 | 0.173 ± 0.00 |
| P1        | 0.100 ± 0.00 | 0.048 ± 0.00 | 0.046 ± 0.00 | 0.047 ± 0.00 |
| P2        | 0.100 ± 0.00 | 0.054 ± 0.00 | 0.054 ± 0.00 | 0.055 ± 0.00 |
| P3        | 0.100 ± 0.00 | 0.057 ± 0.00 | 0.056 ± 0.00 | 0.056 ± 0.00 |
| P4        | 0.100 ± 0.00 | 0.060 ± 0.00 | 0.058 ± 0.00 | 0.058 ± 0.00 |

Description: The difference notation letters in the same column shows the results significantly different (P<0.05). P0 = without probiotics; P1 = fresh probiotic; P2 = probiotic shelf life of 1 month; P3 = probiotic shelf life of 2 months; P4 = probiotic shelf life of 3 months.

Table 2 showed the results of the 3rd day of ammonia levels in all treatments tend to be stable. This shows that the addition of probiotics can reduce the ammonia in the water. Day 3 levels of ammonia in the treatment P1 significantly different (P<0.05) on treatment P0, P2, P3, and P4. P2 is not significantly different (P>0.05) on P3, but significantly different (P<0.05) on P0 and P4.

Table 3. Total Vibrio in penaeid shrimp pond water at the end of the incubation period

| Treatment | Day 0 ± SD | Day 3 ± SD |
|-----------|------------|------------|
| P0        | 40 x 10^4 ± 0.00 | 35.7 x 10^3 ± 0.526 |
| P1        | 40 x 10^4 ± 0.00 | 18 ± 0.500 |
| P2        | 40 x 10^4 ± 0.00 | 2.21 x 10^3 ± 0.261 |
| P3        | 40 x 10^4 ± 0.00 | 3.95 x 10^4 ± 0.863 |
| P4        | 40 x 10^4 ± 0.00 | 10.5 x 10^4 ± 0.892 |

Description: The difference notation letters in the same column shows the results significantly different (P<0.05). P0 = without probiotics; P1 = fresh probiotic; P2 = probiotic shelf life of 1 month; P3 = probiotic shelf life of 2 months; P4 = probiotic shelf life of 3 months.

Table 3 showed the results of decrease in total Vibrio day 3 in the treatment of P2, P3, and P4 show results that are not significantly different (P>0.05), but significantly different (P<0.05) treatment about P0 and P1. Treatment P1 has the smallest value (18 CFU/ml) and for P0 has the biggest value (35.7 x 10^3 CFU/ml). Total Vibrio relatively decreased in all treatments except at P0 as a control treatment.

This shows that the addition of probiotics can reduce the total Vibrio in maintenance media vaname shrimp.
Table 4. Measurement of pH and water temperature in the penaeid shrimp ponds during the incubation period (t0 - t3)

| Treatment | pH Range  | Temperature (°C) |
|-----------|-----------|------------------|
| P0        | 6.18 – 7.41 | 25 – 28         |
| P1        | 6.04 – 7.20 | 25 – 28         |
| P2        | 6.10 – 7.20 | 25 – 28         |
| P3        | 6.04 – 7.23 | 25 – 28         |
| P4        | 6.06 – 7.21 | 25 – 28         |

Table 5. Probiotic Bacteria Test Results Post-Storage by TPC at TSA (24 hours)

| Storage Period | Total Probiotic Bacteria Before | After |
|----------------|--------------------------------|-------|
| 0 Month / Fresh| 60 x 10^8 CFU / ml              | 60 x 10^8 CFU / ml |
| 1 months       | 60 x 10^8 CFU / ml              | 2 x 10^8 CFU / ml  |
| 2 months       | 60 x 10^8 CFU / ml              | 3 x 10^8 CFU / ml  |
| 3 months       | 60 x 10^8 CFU / ml              | 5 x 10^8 CFU / ml  |

Tables 4 and 5 show the results of water quality parameters before, during and end of the study did not show any significant variations. The number of bacteria after storage also decreased storage results but between the treatment one month, two months, and three months of storage there is no significant difference in bacterial reduction.

3.2 Discussion

Organic material in the control treatment (P0) has the highest value (38.269 mg / L) compared to other treatments (P1, P2, P3, and P4). This is because in treatment P0 there was no addition of probiotic bacteria so that the process of degradation of organic matter was only carried out by natural bacteria that had been present in the shrimp pond water before. [28], argues that naturally organic matter accumulated in the waters will be degraded by natural bacteria found in shrimp ponds aerobically and will produce ammonia (NH3), nitrite (NO2-) and hydrogen sulfide (H2S) compounds. Unlike the treatment of P1, P2, P3, and P4 which was added by probiotic bacteria with a density of 10^6 CFU / ml, the comparison of probiotic administration of 1 ml of probiotics to 1000 ml of media for maintenance of penaeid shrimp containing probiotic bacteria Bacillus subtilis, Bacillus mycoides, and Pseudomonas diminuta.

Bacillus subtilis, Bacillus mycoides, and Pseudomonas diminuta are probiotic bacteria that can degrade organic matter into simpler waters. The process of degradation of organic matter by Bacillus subtilis and Bacillus mycoides is assisted by extracellular enzymes produced in two bacteria such as amylase, protease, lipase and cellulose enzymes [10 ; 29]. Whereas Pseudomonas diminuta produces extracellular enzymes such as amylase, protease, lipase and lignocellulose enzymes to degrade [30], but does not produce cellulose enzymes.

At the end of incubation the treatment with the addition of fresh probiotics (P1) had the best results. This situation is caused by the good performance of probiotic bacteria in hydrolyzing or degrading organic matter in waters to be a simpler compound to be used by probiotic bacteria, as well as in P2 and P3 treatments having the same results as treatment P1. Statistical tests on treatments P2 and P3 also showed results not significantly different (P>0.05) against P1. This shows that the performance of probiotics on decreasing organic matter in probiotic storage until a two-month storage period still has the same good results as fresh probiotics (P1).

The decline in organic matter in this study is in accordance with the statements of [31], who stated that probiotics with Bacillus sp. and Pseudomonas sp. able to degrade organic matter from the rest of the feed and feces quickly so that there is no excessive accumulation at the bottom of the cultivation pond. The degradation process of organic matter occurs because bacteria can metabolize organic substances through an enzymatic system to produce CO2, H2O, and energy. Energy is used by bacteria
for synthesis, motility, and respiration [32]. Also explained by [33], that heterotrophic bacteria in their growth require organic compounds. According to [34] also stated that heterotrophic bacteria are a group of bacteria which in their growth require C-organic as a source of carbon and an energy source.

Based on Indonesian national standards the optimal range of total organic matter content for shrimp is <90 mg / L [4], further strengthened by the statement of [35] which states that the range of organic matter that is still good for vaname shrimp cultivation is <55 mg / L. This shows that the treatments P0, P1, P2, P3, and P4 have the value of total organic matter which is still optimal for shrimp farming. However, for treatment P0 has the optimal total organic matter value because the measurement of the initial organic material has a range that is still optimal because the shrimp maintenance water used in this study originated from traditional vaname shrimp maintenance ponds aged 100 days so that the organic matter content is low. However, when compared with treatments P1, P2, P3, and P4, the organic matter content decreases organic matter from 39.620 mg / L to 25.871 mg / L; 26.989 mg / L; 27.129 mg / L; 29.366 mg / L. This is due to the help of the process of degradation of organic matter by probiotic bacteria, so that natural bacteria in vaname shrimp ponds do not work alone in degrading organic matter.

Ammonia is the result of excretion and degradation of microorganisms that are toxic to aquaculture animals [36]. Ammonia in the control treatment (P0) at the end of the incubation period had the highest value (0.173 mg / L) compared to other treatments (P1, P2, P3, and P4). This is because in the treatment of P0 no probiotic bacteria were added which aimed to reduce ammonia levels, besides that the increase in ammonia in the P0 treatment occurred because naturally the organic material accumulated in the waters will be degraded by natural bacteria on the pond water aerobically and will produce compounds ammonia (NH3), nitrite (NO2-) and hydrogen sulphide (H2S) [28]. The control treatment (P0) is estimated to have only a few ammonia reducing bacteria from shrimp farms.

The decrease in ammonia in treatments P1, P2, P3, and P4 occurred because of the influence of the addition of probiotic bacteria, Bacillus subtilis and Bacillus mycoides is a probiotic bacteria that can degrade complex compounds of organic matter into simple compounds that can be utilized directly by probiotic bacteria. The degradation process by Bacillus subtilis and Bacillus mycoides requires energy sources obtained from the process of reducing ammonia contained in water [37], a similar matter was also stated by [38] which states that during the decomposition process of organic matter, degrading bacteria also need ammonia as their energy source. Ammonia is a source of nitrogen for probiotic bacteria. Bacillus subtilis and Bacillus mycoides play an important role in the process of reducing ammonia or ammonium (NH3 / NH4+) in this treatment, beginning with the process of nitrification of ammonia compounds (NH3) into nitrite (NO2-) compounds [39], then nitrite oxidation reaction which results in the change of nitrite (NO2-) compounds into nitrate (NO3-) compounds. Nitrate compounds (NO3-) will be reduced to nitrite compounds (NO2-) which then change shape to nitrogen monoxide (NO), then nitrogen monoxide (NO) also changes to dinitrogen monoxide (N2O) and then the –O- element in N2O will release thus leaving N2 compounds or commonly called natural nitrogen gas [39 ; 40 ; 41]. This nitrogen (N2) compound will be utilized by bacteria in these waters.

Treatment P1 is the treatment that has the best results in decreasing ammonia levels compared to other treatments, but in P2, P3 and P4 treatments also still have good results. This result is due to treatment P1 using fresh probiotic bacteria so that there is no storage time. Fresh probiotics have a greater number of bacteria with better cell metabolic capacity [42] than P2, P3, and P4. Bacillus subtilis and Bacillus mycoides are bacteria that play a role in reducing ammonia (NH3). The Bacillus genus is a bacterium that can form spores in extreme conditions such as storage treatment carried out in P2, P3, and P4 treatments. Bacterial spores are a form of bacterial defence from unfavourable conditions to stay alive. This is what causes the results in P2 and P3 treatment to be just as good after Duncan's statistics were tested.

The results of the study related to the decrease in ammonia levels showed that the ammonia content during incubation ranged from 0.046 to 0.173 mg / L. According to [43] the optimum ammonia level in shrimp maintenance water is 0.05 - 0.1 mg / L. Reinforced with Indonesian national standards, which states that ammonia levels for shrimp maintenance should not exceed 0.1 mg / L [4]. This shows that
treatment P1, P2, P3, and P4 at the end of the incubation period have ammonia levels that are good for the maintenance of shrimp.

The total *Vibrio* bacteria in the control treatment (P0) was higher than the total *Vibrio* bacteria in the other four treatments. This situation shows that the addition of probiotic bacteria in treatments P1, P2, P3, and P4 can reduce the number of *Vibrio* bacteria on maintenance media, whereas in treatment P0 there is no addition of probiotic bacteria. This is in accordance with the opinion of [44] who reported that bacteria originating from shrimp growing culture media developed as probiotics have the potential and can suppress the growth of pathogenic bacteria such as *Vibrio*.

*Vibrio* total in the control treatment (P0) had the highest amount (5.552 log CFU / ml) compared to other treatments (P1, P2, P3, and P4). This is due to the role of probiotic bacteria (*B. subtilis, B. mycoides, and P. diminuta*) in treatments P1, P2, P3, and P4 to suppress the *Vibrio* bacteria in which the probiotic bacteria are able to produce extracellular enzymes such as lipase, cellulose, and lignocellulose which can lyse the cell wall of *Vibrio* bacteria which is composed of lipids and polysaccharides (lipopolysaccharide) so that it can reduce the number of *Vibrio* in the waters.

In addition, *Pseudomonas diminuta* is known to also produce antibacterial compounds that can kill *Vibrio* bacteria in waters, the compound is called 2,4-diacetylphloroglucinol with a pathogenic inhibiting mechanism that inhibits the absorption of carbon and nitrogen in pathogenic bacteria [45 ; 46], pyocyanine (1-hydroxy-5-methyl phenazine) by inducing superoxide dismutase (MnSOD) containing manganese causes an increase in the production of O₂⁻ and H₂O₂ [47 ; 48], and siderophore compounds [49] works by chelating specific iron (Fe³⁺) in waters so that it is not available for the development of pathogenic bacteria. While the control treatment (P0) of *Vibrio* decrease can be caused due to bacterial cell death, but *Vibrio* bacteria which is still high in this treatment is most likely caused by poor environmental conditions that support the life of *Vibrio* bacteria in the waters.

Based on the results of this study, the decrease in *Vibrio* bacteria in treatment P1 (1.250 log CFU / ml) decreased the number of *Vibrio*’s which was the greatest when compared to treatments P2, P3, and P4 which tended to experience almost the same decline. This is most likely due to the treatment of P2, P3, and P4 which have the same result of bacterial dominance, *Bacillus subtilis and Bacillus mycoides*, because these two bacteria can form spores in quite extreme conditions such as storage treatment, while *Pseudomonas diminuta* has decreased drastically the amount of treatment is because these bacteria cannot form spores to survive extreme conditions. So this is what causes no difference in fact the results between treatments P2, P3, and P4.

In contrast to treatment P1 (1.250 log CFU / ml) which is able to reduce the number of *Vibrio* in shrimp pond water optimally. The cause is in this action using fresh probiotics which is the amount and quality of the *Bacillus subtilis, Bacillus mycoides, and Pseudomonas diminuta* bacteria in the best conditions. Thus based on these results it is known that *Pseudomonas diminuta* with its antibacterial compound plays an important role in the process of suppressing the number of *Vibrio* bacteria in shrimp pond water. This is in accordance with what was stated by [50] in his study which showed that the use of *Bacillus* sp. as a probiotic candidate it has been shown that *Bacillus* sp. can reduce the total number of *Vibrio*. Same is the case with *Pseudomonas* sp. which is one of the best bacteria for shrimp ponds because it has the ability to produce antibacterial against *Vibrio* to suppress the number of *Vibrio* in the waters [9 ; 45].

The results showed that the total *Vibrio* in the waters during the incubation period ranged from 1.250 – 5.552 log CFU / ml. According to [6], the maximum threshold of *Vibrio*’s presence in aquaculture waters is 10⁴ CFU / ml or 4 log CFU / ml. This shows that treatment P1 at the end of the study had a total *Vibrio* below the threshold. The treatment of P2 and P3 has a lower total *Vibrio* than P4, but the total *Vibrio* is still above the threshold, so to be able to reduce the total *Vibrio* it is necessary to re-add probiotics after 2-3 days of initial administration as recommended by Indonesian national standards regarding probiotics.

Water quality parameters before, during and at the end of the study did not indicate a large variation. The water temperature ranges from 25 – 28 °C, dissolved oxygen is 0.19 – 4.38 ppm, salinity is 15 ppt and pH is 6.04 – 7.41. Water temperature and pH are factors that greatly affect the value of ammonia in other treatments (P1, P2, P3, and P4). This situation shows that the addition of probiotic bacteria in treatments P1, P2, P3, and P4 can reduce the number of *Vibrio* bacteria on maintenance media, whereas in treatment P0 there is no addition of probiotic bacteria. This is in accordance with the opinion of [44] who reported that bacteria originating from shrimp growing culture media developed as probiotics have the potential and can suppress the growth of pathogenic bacteria such as *Vibrio*.

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the waters, because the higher the temperature and the higher pH of a waters, it can be ascertained that these waters have a higher ammonia value compared to waters that have lower temperatures and within the normal pH range [26].

The tendency of low pH in treatments P1, P2, P3, and P4 when compared with the control treatment (P0) is caused by the activity of probiotic bacteria which produce more metabolites, one of which is carbon dioxide (CO2). CO2 concentration greatly affects pH in waters, which causes these waters to tend to be acidic. In contrast to the control treatment which tends to have a normal pH because of the lack of bacterial activity in it. Giving probiotics in aquaculture waters will not cause the waters to become very acidic due to the activity of probiotic bacteria. This is because the ability of bacteria to be a buffer in the waters, namely by fixing CO2 in the waters to be used as a carbon source by aquatic bacteria [51].

In this study the dissolved oxygen value from day 0 to day 3 continued to decline, this was due to the absence of oxygen aeration in the research treatment. In addition oxygen is also used by probiotic bacteria in degrading organic matter, ammonia, and for producing antibacterial compounds [28].

4. Conclusion
Results of this study is probiotics that has been stored for 3 months still has the ability to degrade organic matter, ammonia, and total Vibrio in penaeid shrimp pond water, such as fresh probiotics. Fresh probiotics (P1) have the best results among all the treatments. However, in the certain parameters of the results show that treatment P1, P2, P3, and P4 have the same results or not significantly different from treatment P1 (fresh probiotics).

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