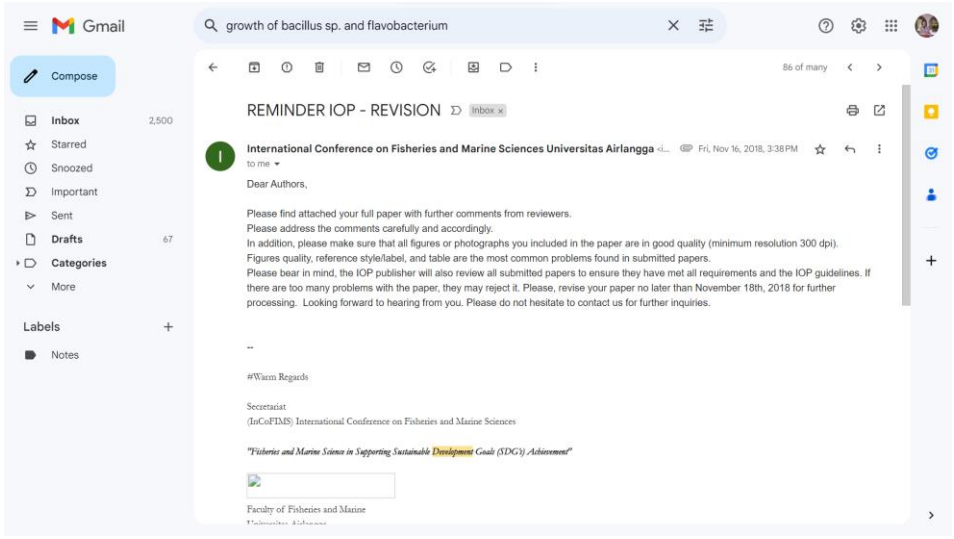
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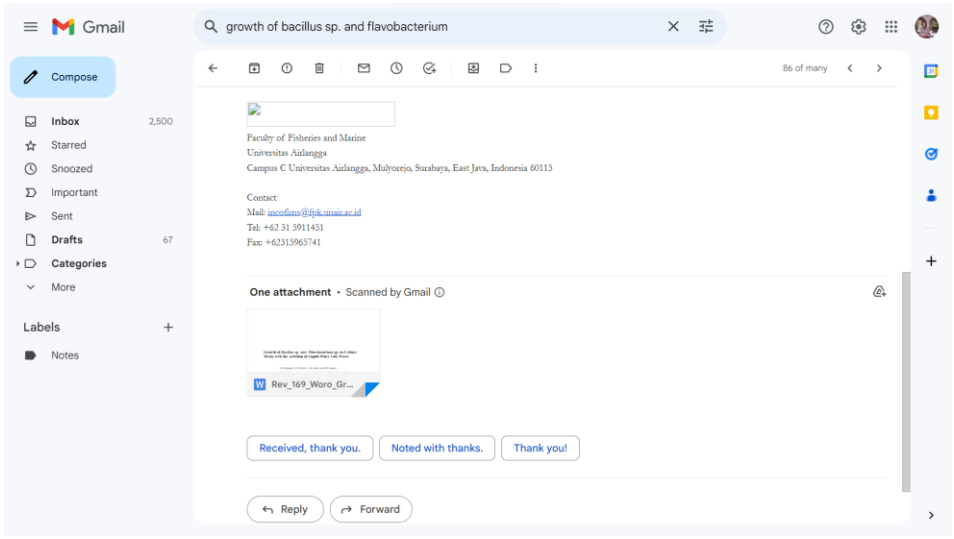
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Growth of *Bacillus* sp. and *Flavobacterium* sp. on Culture Media with the Addition of Liquid Whey Tofu Waste

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Abstract. The purpose of this study was to determine the growth of *Bacillus* sp. and *Flavobacterium* sp. which were cultured together on the culture medium with the addition of liquid whey tofu waste. This study used a Completely Randomized Design (CRD) with four treatments and five repetitions. The treatment is given by adding liquid whey tofu waste to culture media with different doses, 0% (control), 10%, 20%, and 30%. The parameters observed in this study were the growth of *Bacillus* sp. and *Flavobacterium* sp. The results showed the highest growth of *Bacillus* sp. and *Flavobacterium* sp reached 48 hours in the treatment with the addition of 10% liquid whey tofu waste with an average amount of 12.08×10^9 CFU / ml and 18.25×10^8 CFU / ml respectively and significantly different with the control ($P < 0.05$). The specific growth rate of *Bacillus* sp. and *Flavobacterium* sp. in the media with the addition of 10% liquid whey tofu waste were 0.11/hour and 0.13/hour respectively and higher than the control. The conclusion of this study is the addition of liquid whey tofu can increase the growth and the addition of 10% provides the best growth of bacteria

Keywords: *Flavobacterium* sp., *Bacillus* sp., Liquid Whey Tofu waste, Skim Milk, Growth.

1. Introduction

The main problem that must be faced by farmers is the failure of production to be one of the factors causing losses in shrimp farming. Prevention of diseases continues to be carried out but must still pay attention to the balance in the aquatic ecosystem. Control of the spread of diseases in shrimp culture in an environmentally friendly manner (biological control) can be done using competitors for pathogenic bacteria [9].

The use of biological control is one of the better disease control strategies so that sustainable aquaculture can be created [13]. Competitor bacteria that will be used as biological control can be isolated from the bacterial habitat derived so that the bacteria are more adaptable and developing to suppress pathogenic bacteria [11].

Flavobacterium sp. produces antibacterial compounds that can suppress the growth of other bacteria [23,18]. Other bacteria that can be used in fisheries biotechnology are *Bacillus* sp. This is because *Bacillus* sp. having the ability to not produce toxins for shrimp that are maintained, easily grown, does not require expensive substrates, has the ability to survive at high temperatures [17], so it is widely used as a biocontrol agent (28). *Bacillus* sp. can also produce antibiotics that can inhibit *Vibrio parahaemolyticus* [8] and several types of enzymes that have antagonism properties such as carein enzymes produced by *Bacillus cereus* [19], the thuricin enzyme produced by *Bacillus thuringiensis* (1) and bacillocin enzyme produced by *Bacillus*

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subtilis [10]. Based on the ability of *Flavobacterium* sp. and *Bacillus* sp. to inhibit the growth of pathogenic bacteria, it is necessary to conduct bacterial culture or propagation for the availability of stock inoculants and applications in the field.

Bacterial culture is a way to multiply bacterial cells using a culture medium consisting of a mixture of nutrients [8; 28]. Growth media must meet the nutritional requirements needed by a microorganism, including carbon, nitrogen, vitamins, water, non-metallic elements such as sulfur and phosphorus and metal elements such as Zn, K, Cu, Mn, Mg, and Fe [5,28]. The bacterial culture media commonly used to grow bacteria is Tryptic Soy Broth (TSB). This is because the content contained in TSB such as casein, soybean peptone, dextrose, and sodium chloride can support the life of bacteria, but the price of TSB is less economical so solutions must be found that can reduce the use of this material. Based on the composition in the TSB, it is necessary to find alternatives media that can be used for bacterial culture.

Some media that can be used as bacterial culture media are molasses, ~~caolin~~kaolin and skim milk. Skim milk contains high enough protein, which is 35% [26]. This protein content is broken down by bacteria into nitrogen elements which will be used by bacteria as a source of nutrients for cell metabolism. While liquid whey tofu waste is the result of waste from tofu processing which is wasted and cannot be consumed by humans so it is necessary to make efforts to reduce its impact on the environment. Liquid whey tofu waste that is often disposed of directly without processing in advance so as to produce a foul odor and pollute the environment [16].

Liquid whey tofu waste also contains phosphate compounds (P2O) which is 228, 85% [3]. Phosphate is a mineral that can support bacterial growth. This is because phosphate will be broken down by bacteria into phosphorus (P) and used by bacteria for cell formation so that the available phosphorus will affect the growth of bacteria [6]. Based on this background the underlying research needs to be carried out on both materials to be used as bacterial culture media.

The purpose of this study was to determine the growth of *Flavobacterium* sp. and *Bacillus* sp. grown in skim milk medium with the addition of liquid whey tofu waste and knowing the best concentration of liquid waste that is able to support the growth of *Flavobacterium* sp. and *Bacillus* sp.

2. Materials and methods

2.1. Place and time

This study was conducted from July to August 2017 at the Faculty of Fisheries and Marine Education Laboratory, Universitas Airlangga, Surabaya and the Institute of Tropical Diseases at Universitas Airlangga.

2.2 Tools and materials

The materials used in this study include skim milk, *Flavobacterium* sp. and *Bacillus* sp. and liquid whey tofu waste. While the tools used are an autoclave, vortex, hand glove, hot magnetic stirrer, measuring cup, refrigerator, and analytical scale.

2.3 Research design

This research was conducted using a completely randomized design (CRD) with four treatments and five replications. The treatments used in this study are:

P0: 10% TSB + 10% Skim milk + 6% Glucose + 0% Liquid whey tofu waste
P1: 10% TSB + 10% Skim milk + 6% Glucose + 10% Liquid whey tofu waste
P2: 10% TSB + 10% Skim milk + 6% Glucose + 20% Liquid whey tofu waste
P3: 10% TSB + 10% Skim milk + 6% Glucose + 30% Liquid whey tofu waste

All treatments were given 1.5% NaCl solution until the volume of the culture medium reached 10 ml.

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2.4 Research Preparation

2.4.1 Making TSB Media

The preparation of TSB was carried out by dissolving 30 g in 1 liter of distilled water, then heated on a hot plate until homogeneous (19). If the media is homogeneously sterilized using autoclave at 121ºC for 15 minutes with a pressure of 1 atm.

2.4.2 Making Skim Milk Media

Dissolve 100 grams of skim milk powder in 1 liter of distilled water then heat it on the hot plate until it is homogeneous. Homogeneous skim milk is sterilized using the water blanching method at 70ºC for 10 minutes.

2.4.3. Preparation Liquid Whey Tofu Waste

Liquid whey tofu waste is obtained from the tofu factory, Pacar Keling, Surabaya. Liquid whey tofu waste used is liquid waste generated in the tofu-making process. Liquid whey tofu waste is filtered using filter paper to separate the tofu waste with the water, then sterilized using autoclave with a temperature of 121ºC for 15 minutes at a pressure of 1 atm.

2.4.4 . *Media Mixing for Bacillus* sp. *and Flavobacterium sp.*

Flavobacterium sp. and *Bacillus* sp. carried out on a test tube with a volume of 20 ml with media filling only half of the total volume (10 ml). Media mixing is done in the following ways:

P0 : 1 ml Media TSB + 1 ml skim milk + 0,6 ml ~~glukosa~~glucose + 7,4 ml NaCl solution 15 ppt
P1 : 1 ml Media TSB + 1 ml skim milk + 0,6 ml ~~glukosa~~glucose + 1 ml limbah cair tahu + 6,4 NaCl solution 15 ppt
P2 : 1 ml Media TSB + 1 ml skim milk + 0,6 ml ~~glukosa~~glucose + 2 ml limbah cair tahu + 5,4 ml NaCl solution 15 ppt
P3 : 1 ml Media TSB + 1 ml skim milk + 0,6 ml ~~glukosa~~glucose + 3 ml limbah cair tahu + 4,4 ml NaCl solution 15 ppt

2.4.5 . ~~Inculation~~Inoculation and Incubation of Flavobacterium sp. and Bacillus sp.

Flavobacterium sp. and *Bacillus* sp. taken from TSB media which has been calculated using spectrophotometer with a wavelength according to Suminto [27] is 550 nm. *Flavobacterium* sp. and *Bacillus* sp. each was taken as much as 1 ml and put into each treatment. The results of inoculation were incubated for 48 hours at 30-35ºC.

2.4.6 . *Calculation of Bacterial Growth Amounts*

Calculation of bacterial growth in this study was carried out by counting bacterial colonies using the Total Plate Count (TPC) method. Based on the preliminary research the calculation was done at dilutions of 10-7 and 10-8. The number of bacteria was calculated during the incubation period at 1, 6, 12, 18, 24, 30, 36, 42, and 48 hours [24]. The formula for calculating bacteria using the TPC method according to Waluyo [29] is as follows:

$$\text{Number of Bacteria (CFU per ml)} = \text{number of colonies per dish} \times \frac{1}{\text{Dilution factor}}$$

2.4.7 Specific Growth Rate of Bacteria

According to Maier (2009), specific bacterial growth rates were calculated using the formula:

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$$\mu = \frac{(\ln Nt - \ln No)}{t_n - t_o}$$

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μ = Specific growth rate
N = Number of bacteria at t_n
No = Number of bacteria at t_o
t_n = time at the n
t_o = time at the early

2.4.8 Data analysis

Data from the number of *Flavobacterium* sp. and *Bacillus* sp. calculated using the TPC method and then analyzed using ANOVA (Analysis of variance) to determine the effect of the treatment given. If from the analysis it is known that the treatment shows a significantly different effect, then continued by Duncan's test to find out the difference between treatments.

3 Results and Discussion

3.1 Results

Growth data of *Bacillus* sp. those cultured in skim milk media with the addition of liquid whey tofu waste are presented in Table 1.

Table 1. Average number of cells *Bacillus* sp.

Treatment	The hour- (CFU/ml)									
	0	1	6	12	18	24	30	36	42	48
P0	10^5	$17,92 \times 10^7$a	$28,19 \times 10^7$a	$62,89 \times 10^7$a	$68,00 \times 10^7$a	$31,65 \times 10^8$a	$39,89 \times 10^8$a	$42,64 \times 10^8$a	$50,08 \times 10^8$a	$52,40 \times 10^8$a
P1	10^5	$17,05 \times 10^7$a	$27,25 \times 10^7$a	$46,35 \times 10^7$a	$98,25 \times 10^7$a	$59,24 \times 10^8$a	$64,84 \times 10^8$a	$78,46 \times 10^8$b	$96,81 \times 10^8$b	$12,08 \times 10^9$b
P2	10^5	$7,85 \times 10^7$a	$18,73 \times 10^7$a	32,00x107a	$63,46 \times 10^7$a	$26,52 \times 10^8$a	$33,77 \times 10^8$a	$43,01 \times 10^8$a	$60,66 \times 10^8$a	$10,21 \times 10^9$b
P3	10^5	$6,68 \times 10^7$a	$13,98 \times 10^7$a	$65,21 \times 10^7$a	$168,7 \times 10^7$a	$50,27 \times 10^8$a	$64,70 \times 10^8$a	$71,58 \times 10^8$b	$95,58 \times 10^8$b	$10,54 \times 10^9$b

Description: P0: 0% liquid whey tofu waste, P1: 10% liquid whey tofu waste, P2: 20% liquid whey tofu waste, P3: 30% liquid whey tofu waste. Notations shown with different superscript letters in the same column show significant differences ($p < 0.05$).

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Bacillus sp. cultured in the medium with the addition of 10% liquid whey tofu waste (P1) showed a higher growth increase compared to 20% and 30%. The treatment of P1 began to increase from the 18th hour and reached the exponential optimum point at the 48th hour with the number of bacterial cells reaching 12.08×10^9 CFU/ml.

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Treatment media with the addition of 20% liquid whey tofu waste (P2) and in the treatment with the addition of 30% liquid whey tofu waste (P3) showed an increase in the number of bacteria and lower than P1. The growth of *Bacillus* sp. in P2 starting from the first hour to 48 hours with the number of bacteria reaching 10.21×10^9 CFU / ml and in the P3, the number of *Bacillus* sp. reached the exponential optimum point at 48 hours with a bacterial cell count of 10.54×10^9CFU / ml.
In this study the number of cells of *Flavobacterium* sp. less than *Bacillus* sp. Data from the growth of *Flavobacterium* sp. cultured in skim milk media with the addition of liquid whey tofu waste can be seen in Table 2.

Table 2 Average number of ~~cells~~ *Flavobacterium* sp. cells

Treatment	The hour- (CFU/ml)									
	0	1	6	12	18	24	30	36	42	48
P0	10^5	0,52x10^7a	3,25x10^7a	6,67x10^7a	14,67x10^7a	37,11x10^7a	81,23x10^7a	93,91 x10^7a	10,86 x10^8a	12,01x10^8a
P1	10^5	0,28x10^7a	2,55x10^7a	5,72x10^7a	21,26x10^7a	88,00x10^7b	13,85 x10^8b	15,20 x10^8b	16,36 x10^8b	18,25x10^8b
P2	10^5	0,52x10^7a	4,68x10^7a	11,81x10^7a	21,58x10^7a	64,36x10^7ab	11,46x10^8ab	13,71x10^8b	14,16x10^8ab	14,45x108ab
P3	10^5	0,44x10^7a	6,16x10^7a	15,74x10^7a	21,03x10^7a	49,00x10^7ab	95,14x10^7ab	11,55x10^8ab	12,36x10^8ab	13,13x10^8a

Description: P0: 0% liquid whey tofu waste, P1: 10% liquid whey tofu waste, P2: 20% liquid whey tofu waste, P3: 30% liquid whey tofu waste. Notations shown with different superscript letters in the same column show significant differences ($p < 0.05$).

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The growth of *Flavobacterium* sp. in the medium with the addition of 10% liquid whey tofu waste (P1), bacterial growth tends to be stable and increased well. This is shown in the first-hour observation, *Flavobacterium* sp. has a smaller growth increase than other treatments. The growth of *Flavobacterium* sp. began to a significant increase in the 18th hour to reach the optimum point of the exponential phase at 48 hours with the number of bacteria 18.25x10^8 CFU / ml.

The treatment of P2 with the addition of liquid waste to know 20% increase in the number of bacterial cells continues to increase from the first hour and reach the optimum point of the exponential phase at 48 hours with the number of bacterial cells 14.45x108 CFU / ml. The treatment of P3 has a growth in the number of bacterial cells tend to be slower compared to P0, P1, and P2. The optimum point of the exponential phase is *Flavobacterium* sp. in the P3, occurred at 48 hours with a number of bacterial cells as much as 13.13x10^9 CFU / ml.

Bacillus sp. (Table 1) and *Flavobacterium* sp. (Table 2), showed that both bacteria in the treatment grown in liquid whey tofu waste culture media (P1, P2, and P3) showed a higher growth increase than control (P0). In P0. P1, P2 and P3, *Bacillus* sp. and *Flavobacterium* sp. shows the optimum point of the growth phase was at 48 hours. The number of cells *Bacillus* sp. and *Flavobacterium* sp. the highest was achieved in treatment P1 with the number of *Bacillus* sp cells as much as 12.08x10^9 CFU / ml and *Flavobacterium* sp. as much as 18.25x10^8 CFU/ml.

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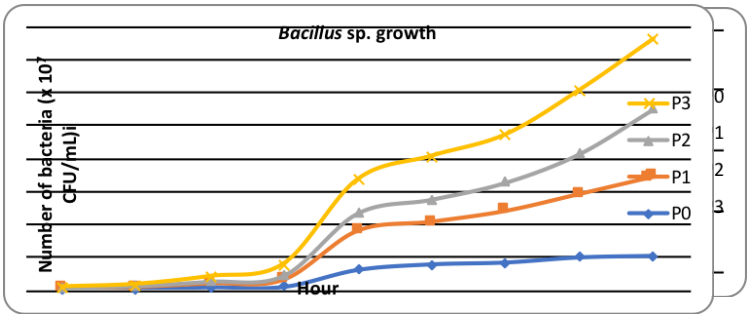

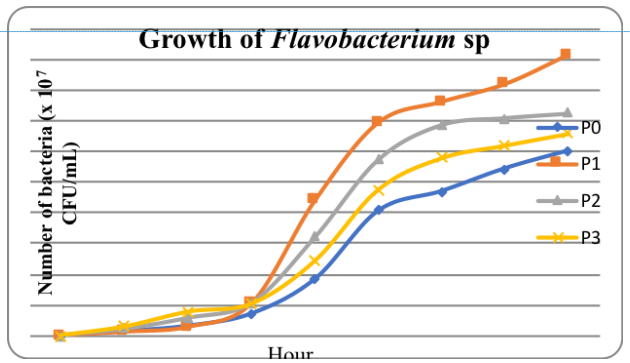

Figure 1. Growth of *Bacillus* sp. ~~d~~and *Flavobacterium* sp.

Based on Figure 1, it appears that *Bacillus* sp. grown in the medium of adding liquid whey tofu waste have a phase of adaptation (lag) at 0 to 1 hour. While the logarithmic growth phase in both bacteria began after 6 to 36 hours. The growth of *Flavobacterium* sp. which is grown in the media with the addition of liquid waste undergoes a phase of adaptation (lag) at 0 to before 6 hours and increased in an exponential phase from the 6th to 36th hours.

The growth rate of both bacteria grown in the medium of adding liquid whey tofu waste can be seen in Table 3.

Table 3. Specific growth of *Bacillus* sp. ~~d~~and *Flavobacterium* sp at exponential phase

Bacteria	Treatment			
	P0	P1	P2	P3
Bacillus sp.	0.09	0.11	0.10	0.13
Flavobacterium sp.	0.11	0.13	0.11	0.09

Both bacteria ~~were~~ cultured on a culture media with the addition of 10% tofu wastewater hav~~cing~~ better growth velocity compared to the growth rate of bacteria cultured on the treatment medium with the addition of liquid waste~~,~~ 0% and 20%.

Bacillus sp. in P1 has a specific growth rate of 0.11. This specific growth rate is higher than the P0 treatment which was 0.09, and P2 was 0.10. While the *Flavobacterium* sp. in~~-~~ P1 culture~~d in~~ had a specific growth rate of 0.13. This specific growth rate was greater than the P0 0.11, P2 0.11 and P3 was 0.09.

3.2 Discussion

Bacterial culture media is a material consisting of a mixture of nutrients or nutrients used to grow microorganisms. Types of media based on their composition consist of natural media, semi-synthesis media and synthesis media. Natural media is a medium composed of natural ingredients [8]. The culture media for *Bacillus* sp. and *Flavobacterium* sp. growth in this study using natural media by adding liquid whey tofu waste. Liquid whey tofu waste contains N 0.27%; P2O5 228.85%; K2O 0.29%; 1.68% protein [3].

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Bacillus sp. those cultured in skim milk media with the addition of 10% (P1), 20% (P2) and 30% (P3) liquid whey tofu waste at 12 hours showed a different growth and growth rate with the control treatment (P0). The number ~~cells~~ of *Bacillus* sp. cells cultured at P1, P2 and P3 was higher than in P0 (control). The height number of *Bacillus* sp. at P1, P2 and P3 because in this media there were the addition of liquid whey tofu waste. Some bacteria such as *Enterobacter gergoviae* and *Bacillus* sp.~~,~~ are known to grow in tofu waste water media [2]. In this study the addition of liquid whey tofu waste given to culture media can also ~~increased~~increase the growth of *Bacillus* sp.

Bacillus sp. cultured on the addition of 10% tofu wastewater (P1) showed a higher number of cells (12.08 × 10^9 CFU~~-/-~~ml) and more stable than those cultured in 20% liquid whey tofu waste (P2) and 30% (P3) . This is presumably because the Nitrogen (N) and Phosphorus (P) content contained in liquid waste knows that it can meet the needs of N and P in *Bacillus* sp. While the addition of 20% and 30% liquid whey tofu waste contains excess N and P, so the *Bacillus* sp. is already unable to absorb the available N and P. *Bacillus thuringiensis* grown in a medium containing sufficient amino acids can increase cell growth, if too much amino acid is present in the media it can cause low bacterial growth [33].
The element P (phosphorus) plays an important role in the formation of nucleic acids and phospholipids in bacteria (32). The element P in liquid whey tofu waste is P2O5 (phosphate), which is 228.85 ppm [3]. Phosphate is needed by bacteria as a component of ATP, nucleic acids and a number of coenzymes such as NAD, NADP and flavin. Lack of P (phosphorus) and excess P in a culture medium can slow down the bacterial growth process [7; 32].

Addition of 10% (P1), 20% (P2) and 30% (P3) of liquid whey tofu waste in this study also affected the growth of *Flavobacterium* sp, this can be seen in *Flavobacterium* sp. those who were cultured in all three treatments had higher cell numbers and growth rates than controls (P0). *Flavobacterium* sp. in P1 the number of cells is higher compared to P2 and P3, which is as much as 18.25 × 10^8 CFU / ml. *Flavobacterium* sp. those cultured in addition 20% (P2) and 30% liquid whey tofu waste (P3) showed lower growth, it was thought that the concentration of nutrients was too much or excessive so that the cells of *Flavobacterium* sp. unable to utilize nutrients in the media. Media containing a lot of nutrients causes its concentration to rise and become hypertonic. Hypertonic solutions (high nutrient levels) can inhibit bacterial growth because they cause plasmolysis in bacterial cells [2].

Bacillus sp. and *Flavobacterium* sp. which is grown together in the medium of skim milk with the addition of liquid whey tofu waste shows a different growth. The specific growth rate of *Bacillus* sp. with addition of liquid whey tofu waste antill 30% dose given higher than P0 (~~kontrol~~control), while on *Flavobacterium* sp. only at 10% dose (Table 3). *Bacillus* sp. growth was higher than *Flavobacterium* sp. The difference in growth between *Bacillus* sp. and *Flavobacterium* sp. can be seen in Table 1 and Table 2. Differences in the number of bacteria between *Bacillus* sp. with *Flavobacterium* sp. those who were cultured together were thought to be due to the nature of *Flavobacterium* sp. non-fermentative [23], while *Bacillus* sp. fermentative [30].

The properties of *Bacillus* sp. which are classified as fermentative bacteria make these bacteria more able to utilize the nutrients in liquid waste to know both in aerobic and anaerobic conditions. *Bacillus* sp. is anaerobic and facultative anaerobic bacterium capable of growing in liquid whey tofu waste because it is capable of producing extracellular enzymes decomposing cellulose and hemicellulose, so it is commonly found in liquid whey tofu waste [20].

Bacillus sp. is a fermentative bacteria, which is a group of bacteria that can produce energy in anaerobic conditions [30]. The use of nutrients in the media is carried out by *Bacillus* through oxidation-reduction reactions to produce the energy needed for its metabolism [29]. The results of this study indicate that although there are differences in the number of bacteria between *Bacillus* sp. and *Flavobacterium* sp., ~~but~~

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these two bacteria can grow together in one medium. This can be seen from the growth of both bacteria which continues to increase until 48 hours.

Bacterial culture in the medium of adding liquid whey tofu waste has an effect on the growth rate of bacteria. This is because liquid whey tofu waste contains phosphate needed by bacteria to grow and develop.

4 Conclusion

Flavobacterium sp. and *Bacillus* sp. cultured in skim milk media with the addition of liquid whey tofu waste can grow together in the same medium. Growth of *Flavobacterium* sp. and *Bacillus* sp. the optimal occurs with the addition of 10% liquid whey tofu waste and growth of *Bacillus* sp. occurs higher than *Flavobacterium* sp. The optimal point of the exponential phase occurs at the 48th hour with the number of *Bacillus* sp. 12.08×10^9 CFU / ml and *Flavobacterium* sp. 18.25×10^8 CFU / ml.

5 Suggestion

Suggestions that can be given from the results of this study need to do further research on the culture of *Bacillus* sp. and *Flavobacterium* sp. in bulk and can be used as basic information in the use of these bacteria as probiotic candidate bacteria.

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