In vitro adhesion of *Bacillus* sp. and *Enterobacter* sp. probiotics on intestinal epithelial cells of red tilapia (*Oreochromis* sp.) and the application effects on the fish growth and survival rate

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Abstract. This research aimed to determine the adhesion of *Bacillus* sp. (PCP1) and *Enterobacter* sp. (JC10) on intestinal epithelial cells of red tilapia (*Oreochromis* sp.) and the effect of the probiotics application in feed on fish growth, survival rate, and feed conversion ratio. In vitro adhesion test was performed by using $10^8$ cells/ml of bacteria, and $10^5$ cells/ml of epithelial cells for 1 hour of incubation. The probiotics were added to the fish pellet with the dose of $5 \times 10^4$ CFU/g of feed with four treatments, including probiotic application every three days, seven days, without probiotic, and commercial probiotic application every three days. Each treatment consists of three replications. Red tilapia is maintained for 30 days in fiberglass ponds. The feed is given two times per day with a dose of 5% of the biomass. The adhesion experiment results showed that *Bacillus* sp. (PCP1) and *Enterobacter* sp. (JC10) have adherence abilities higher than the commercial probiotics. The application of probiotics in tilapia for one month did not affect the fish growth, survival rate, and feed conversion ratio ($P > 0.05$). Probiotic application in a longer period is needed to be addressed.

1. Introduction

Tilapia is one of the main freshwater fish commodities that is easy to cultivate, grows relatively fast, and has a high tolerance for the environment [1][2]. Tilapia production in 2014 was 999,695 tons, while in 2016 it reached 1.14 million tons and in 2017 it was 1.15 million tons or an increase of 3.6% [3].

Probiotics are live microbes which when administered in sufficient quantities can have a beneficial effect on the health of the host and can improve the balance of microbes in the digestive tract [4]. Provision of probiotics in feed has been shown to modify the composition of the gut microbiota [5], improve feed digestibility [6], feed efficiency [7], growth and immunity of tilapia [7][8]. Probiotics are biofriendly agents that can improve intestinal health, growth, and fish production through activation of nutrient absorption and metabolism [9].

Probiotics can work well if they have good adherence capabilities and can colonize the digestive tract [10]. *Bacillus* sp. (PCP1) and *Enterobacter* sp. (JC10) are two candidate fish probiotic bacteria isolated from the digestive tract of fish in Jepara, Central Java [11][12][14]. Both bacteria have strong proteolytic enzymes, are acid resistant, resistant to antibiotics [11]. The optimal dose of probiotics given to tilapia is a reference for this research. The dose of probiotics on red tilapia which was able to increase growth and low FCR was $10^4$ CFU/g of feed [12]. Therefore, this study will look at the adhesion ability of the bacteria used and the effect of the frequency of giving probiotics to improve the digestive system as indicated by the growth of tilapia. The selected probiotic bacteria are expected to increase the growth of tilapia through the right frequency of administration.
2. Material and Methods

2.1 Bacterial Adhesion Test on Epithelial Cells

The sample size of tilapia used was 35–40 g. Tilapia samples had previously been fasted for 2 days, with the aim that bacteria in the intestines could be minimized. Intestinal epithelial cells of tilapia were obtained by dissecting fish, cut, and placed in a sterile petri dish. The inner surface of the fish intestinal epithelial cells was scraped using a sterile spatula and suspended in DMEM (Dulbecco’s Modified Eagle Medium) which was added with 10% filtered serum and added 100 u/ml penicillin and 100 mg/ml streptomycin. Intestinal epithelial cells were then centrifuged at 1000 rpm for 5 minutes. The supernatant was then discarded and DMEM was added. Epithelial cells were counted using a hemocytometer to obtain an epithelial cell density of 10^5 cells/ml DMEM[12]. Then, a solution of epithelial cells that had a density of 10^5 cells/ml DMEM was plated onto a microplate of 125 l with 3 replications for each bacterium tested, then the epithelial cells that had been plated onto a microplate were incubated overnight in a cell incubator (30°C; 5% CO₂).

After the epithelial cells were incubated, they were then tested by the staining method using crystal violet. The supernatant fluid was discarded to remove nonadherent epithelial cells. Isolated probiotic bacteria with a density of 10^8 cells/ml were added with 100 l of microplate wells. Then the microplate was incubated at room temperature for 1 hour to attach the bacteria to the microplate well. The supernatant liquid was discarded so that the bacteria that did not stick were also wasted. Then the microplate was fixed at 60°C for 60 minutes with a Dry Block Heater (dry). Then, each filled well was stained with 0.1% crystal violet at 100 l per well and allowed to stand for 45 minutes. Each well was then washed twice with 100 l of PBS to remove excess stains. Then 100 l of citrate buffer was added (20 mmol/l; pH 4) as a solvent and incubated for 45 minutes. The solution was read for absorbance using a microplate reader with the Rapid Test application at a wavelength of 630 nm. The negative control used was epithelial cells that were not given bacteria but were still given the same treatment, then the positive control used was probiotics.

2.2 Application of the probiotic bacteria to Fish Through Feed

Preparation of probiotics was carried out at the Fish and Environmental Health Laboratory of the Department of Fisheries, Faculty of Agriculture, Gadjah Mada University. The design of this study used a completely randomized design (CRD) to find out which treatment was different among all treatments based on the value of variance. There were four treatments and each had three replications. Tilapia are reared using fiber tubs. Before being applied to the treatment, the fish were acclimatized first. After the acclimatization process, treatment can be given. The treatment given is as follows:

P1: feed without the addition of probiotics (negative control)
P2: feed with the addition of probiotics at a dose of 5x10⁴ CFU/g every 3 days
P3: feed with the addition of probiotics at a dose of 5x10⁴ CFU/g every 6 days
P4: feed with the addition of probiotics at a dose of 5x10⁴ CFU/g Raja Catfish (control positive) every 3 days

Fish rearing tank measuring 50x50x60 cm. The number of tubs used is 12 tubs. Before use, the tub was brushed using the Baycline solution and then rinsed clean. The tub that has been rinsed is then dried in the sun. After drying, then the tub is arranged and filled with water. Each rearing tank is aerated in addition to dissolved oxygen in the water.

![Figure 1. Experimental tanks layout.](image-url)
Fish are stocked after the tub water is filled and no tub is leaking. In each tank is stocked with a density of 32 fish. The size of the fish used in this study was 8-10 cm. After the acclimatization process, the fish was ready to be treated by giving probiotics with a feed dose of 5% of the biomass. Feed is given twice a day, every morning at 09.00 WIB and in the afternoon at 15.00 WIB.

2.3 Probiotic preparation

The bacteria used were *Bacillus* sp., *Enterobacter* sp., and Raja Catfish. Preparations made before mixing probiotics are calculating the density of bacteria using the Mc method. Farland. Bacteria were cultured in test tubes containing 10 ml of Tryptone Soya Broth (TSB) medium for 24 hours. The culture results were taken as much as 1 ml with a micropipette and put into a cuvette. The cuvette that has been filled with the results of the 24-hour bacterial culture is inserted into the spectrophotometer. The blank used is TSB. The wavelength used is 625 nm. After knowing the absorbance value, then the value is entered into the Mc function. Farland. The function of the Mc Farland equation used is $y = 20.955x - 3.6222$, where $y$ is the value of bacterial density and $x$ is the absorbance value [14]. The culture results are also kept as stock. The stock was stored in a microtube with a volume of 200 l consisting of 100 l of culture and 100 l of TSB Glycerol, the stock was then stored in the freezer.

The dose of probiotics used during the study was $5 \times 10^4$ CFU/g feed. This dose has been studied by [2] which states that this dose is the best dose compared to other doses ($10^5$, $10^6$ CFU/g) or without probiotics. The application of probiotic bacteria is carried out according to the needs of probiotic bacteria in the feed. The application was carried out by culturing the stock stored in the freezer on TSB medium for 24 hours. Then the volume of bacteria needed is adjusted based on the amount of feed needed.

2.4 Mixing probiotics in feed

Mixing probiotics in feed begins with weighing the feed according to the needs of each tank. Feed is put in plastic. Furthermore, cultured probiotics are then adjusted to their needs and mixed with PBS into a sprayer. The control treatment was only given PBS without probiotics. The amount of PBS is 10% of the weight of the feed. Under the statement of previous study [16][12], the water content in the feed ranges from 10-12%. Probiotics are then sprayed onto the feed and stirred evenly to make it homogeneous. After the feed is stirred evenly, the feed is ready to be given to the test fish.

2.5 Observation of growth, survival, total production, feed conversion ratio (FCR), and water quality

Observations of growth, synthesis, total production, and PCR were carried out based on previous study [12].

2.6 Data Analysis

Analysis of adhesion data was carried out by Analysis of Variance (ANOVA) with an accuracy of 95% ($\alpha = 5\%$), if there was a significant difference, then continued with the Tukey test to see the real difference between test treatments and adherence ability seen descriptively compared to controls. Data analysis of growth, survival (SR), total production, and Feed Conversion Ratio (FCR) were analyzed using Analysis of Variance (ANOVA) with an accuracy of 95% ($\alpha = 5\%$) if there was a significant difference then proceed with the DMRT test (Duncan Multiple Range Test) to determine the differences between treatments. Water quality parameters were analyzed descriptively by comparing them with the literature.

3. Results and Discussion

3.1 Bacterial adhesion to epithelial cells

Based on the data obtained in the adhesion test, the absorbance results of bacterial adhesion to tilapia epithelial cells can be seen in Figure 2. The highest absorbance value was given to the treatment with JC10 bacteria with an absorbance value of 0.2172, then followed by the administration of PCP1 bacteria with an absorbance value of 0.2027, Raja Catfish commercial probiotics with an absorbance value of 0.1834, and probiotics (positive control) with an absorbance value of 0.1720. The lowest absorbance value was in negative control or cells without bacteria with an absorbance value of 0.1717. The results of the ANOVA test showed a significant difference and then Tukey further tested it to see the real difference between the test treatments. Based on Tukey's further test, the most significant treatment for the control was JC10.
3.2 Application of probiotics through the feed of tilapia (Oreochromis sp.)

3.2.1 Fish growth performance

The results of the calculation of the bacterial culture of *Bacillus* sp. (PCP1), *Enterobacter* sp. (JC10), and Raja Catfish (positive control) which were used to be mixed in the feed of this study, respectively, namely $2.2 \times 10^8$ cells/ml, $1.4 \times 10^9$ cells/ml and $7.7 \times 10^8$. The bacteria were then diluted and sprayed on the feed at a dose of $5 \times 10^4$ CFU/g feed. Feed that has been sprayed with probiotics is ready to be given to fish on the same day.

Fish growth can be seen in Figure 2. Absolute weight growth in feed treatment with probiotics every 6 days weighing 17.65 g, then followed by treatment without probiotics (control) weighing 16.42 g, treatment with Raja Lele probiotics (positive control) weighing 14.9 g, and treatment with probiotics every 3 days weighing 13.87 g. The results of the ANOVA test showed that all treatments were not significantly different. These results indicate that the administration of probiotics does not have a significant effect on the absolute weight growth of tilapia. The same can be seen in the specific weight growth parameters. Specific weight growth in the treatment of giving probiotics every 6 days with a percentage of 2.53%/day, then followed by treatment without administration with a percentage of 2.46% /day, the treatment of giving commercial probiotics Raja Lele (positive control) was 2.42% / day and in the treatment of giving probiotics every 3 days with a percentage of 2.28%/day. The results of the ANOVA test showed that all treatments were not significantly different. These results showed that the administration of probiotics did not have a significant effect on the growth of specific weight in tilapia.

Absolute length growth in treatment with probiotics every 6 days with a length of 2.7 cm, then followed by treatment with probiotics every 3 days with a length of 2.46 cm, treatment without probiotic administration (negative control) with a length of 2.44 cm, and treatment with King Lele probiotic (positive control) with a length of 2.43 cm. The results of the ANOVA test showed that all treatments were not significantly different. These results indicate that the administration of probiotics does not have a significant effect on the absolute length growth of tilapia. The same thing can be seen in the specific length growth. Specific length growth in the treatment of giving probiotics every 6 days with a percentage of 0.85%/day, then followed by the treatment of giving probiotics every 3 days with a percentage of 0.81%/day, giving Raja Lele probiotics (positive control) with a percentage of 0.8% /day and treatment without probiotics (negative control) with a percentage of 0.78%/day. The results of the ANOVA test showed that all treatments were not significantly different. These results showed that the administration of probiotics did not have a significant effect on the specific length growth of tilapia.

![Figure 2](image-url)

**Figure 2.** Invitro absorbance of adherence bacteria and the epithelial cells of tilapia.
Figure 3. The final weight of red tilapia fed with probiotics in different feeding frequencies.

3.2.2. Fish survival rate

Based on the data obtained during rearing, the survival rate of tilapia can be seen in Figure 4. The survival rate in the treatment of probiotic administration every 6 days with a percentage of 91.67%, then followed by the treatment of Raja Lele commercial probiotics (positive control) with a percentage of 78.12%, in the treatment without giving probiotics (negative control) with a percentage of 76.04%, and the treatment giving probiotics every 3 days with a percentage of 66.67%. The results of the ANOVA test showed that all treatments were not significantly different. These results indicate that the administration of probiotics did not have a significant effect on the survival of tilapia.

Figure 4. The survival rate of red tilapia fed probiotic feed in different feeding frequencies.

3.2.3. Total Production of fish biomass

Based on the data obtained during rearing, the FCR of tilapia can be seen in Figure 5. Total production in the treatment of giving probiotics every 6 days was 875 g, then followed by treatment without probiotics (negative control) of 837.5 g, the treatment of giving probiotic Raja Catfish (positive control) of 726.96 g, and the provision of probiotics every 3 days was 703.83 g. The results of the ANOVA test showed that all treatments were not significantly different. These results indicate that the administration of probiotics does not have a significant effect on the total production of tilapia.
Figure 5. Total fish biomass production of red tilapia fed with probiotics in different feeding frequencies.

3.2.4. Food Conversion Ratio / FCR

Based on the data obtained during rearing, the FCR of tilapia can be seen in Figure 6. FCR in the treatment of giving probiotics every 3 days and the administration of commercial probiotic Raja Lele (positive control) was 1.56, then followed by the treatment of giving probiotics every 6 days of 1.42 and the treatment without probiotics was 1.29. The results of the ANOVA test showed that all treatments were not significantly different. These results indicate that the administration of probiotics is not able to reduce the value of FCR in tilapia.

Figure 6. The feed conversion ratio of red tilapia fed with probiotics in different feeding frequencies.

3.2.5. Water quality

Water quality parameters measured during maintenance were DO, temperature, and pH. The following table shows the results of measuring water quality parameters.

| Parameters | Control (-) | Probiotics 3 days interval | Probiotics 6 days interval | Control Probiotics (+) 3 days interval |
|------------|-------------|-----------------------------|-----------------------------|----------------------------------------|
| DO (ppm)   | 3.93 – 6.06 | 3.94 – 6.27                 | 4.32 – 6.03                 | 3.52 – 5.89                            |
| Suhu (°C)  | 27.3 – 28.5 | 27.6 – 28.6                 | 27.6 – 28.8                 | 27.2 – 28.1                            |
| pH         | 7.39 – 8.25 | 7.52 – 8.11                 | 7.41 – 8.11                 | 7.52 – 8.21                            |

Bacterial adhesion is an important criterion in the selection of probiotic bacteria. Based on the adhesion absorbance graph obtained, *Bacillus* sp. (PCP1) and *Enterobacter* sp. (JC10) had higher
adhesion than probiotics (positive control) and negative control (cells without bacteria). This indicates that the probiotic bacterial isolates used can adhere to the epithelial cells/mucus of tilapia. *Enterobacter* sp. (JC 10) showed higher adhesion results than others. This is because the JC10 bacterial isolate used is sourced from direct fish digestion so that many live in intestinal epithelial cells and have a synergistic relationship in adhesion to fish intestinal epithelial cells. However, the ability of these bacteria to stick can not significantly affect the growth of tilapia. Comparison of absorbance between adherent bacteria and bacteria administered in vitro (10^8 cells/ml) for *Bacillus* sp. about 1/3,5 and *Enterobacter* sp. about 1/2 that sticks out of a given bacteria. If in vitro there is sticking power but not 100%, then the application with a dose of 10^4 CFU/gram of feed is also suspected of not sticking to 100%. In the adhesion system, the main mechanisms of action of probiotics include increasing the protective function of the epithelium, increasing adhesion to intestinal cells, inhibiting pathogens by occupying adhesion sites, producing antibacterial substances, and regulating immune function[17]. According to a previous study, the attachment of bacteria to intestinal epithelial cells is specific and reversible (permanent) and is the first step in the colonization process for bacteria [18]. It is also suggested that the mechanism of bacterial inhibition occurs through competition for attachment sites and nutrients needed by pathogenic bacteria to grow[19]. Finally, another factor to be considered in the future is the survival ability of the probiotics in the fish intestine which also plays important role on the function in enhance fish growth [26].

Probiotics could improve growth performance, feed efficiency, and minimize mortality. Application of probiotics with various frequencies, which is every 3 and 6 days for one month in the present study did not affect the growth, survival, total production, and FCR of fish. The present results are similar to previous experiments using *Bacillus* sp. in tilapia diet at a dose of 10^4 CFU/gram feed. Enhancement of the fish growth by the probiotics could not be seen on day 30 but day 60 [25]. Hence, it is suspected that the observation period in the present study was too short [22][23]. Accordingly, probiotics’ effects on fish growth performances need to be investigated over a prolonged period. of the present study growth will occur if there is an excess of energy after the available energy has been used for metabolism, digestion, and activities [20]. Based on cellulolytic and proteolytic enzymatic tests we conducted in our previous study, it was confirmed that the *Bacillus* sp. and *Enterobacter* sp. have strong enzyme activity [11][12][14]. Results of the present study indicate that bacterial activity is decreasing. The value of enzyme activity is less than 1.5 which indicates that the bacteria have weak enzyme activity. It is suspected that the weaker enzyme activity affects the growth rate of fish so that growth is not significant. According to other studies, probiotics bacteria work by produce several enzymes that are beneficial for digestion[6][21]. Some of the digestive enzymes in the feed include amylase, protease, cellulase, and lipase. The bacterial probiotics could produce enzymes such as amylase, protease, lipase, and cellulase that help hydrolyze stored feed nutrients (complex molecules), such as carbohydrates, proteins, and fats into simpler molecules to facilitate the process of digestion and absorption of feed in the digestive tract. The effect of probiotics on growth also depends on stocking density, feed composition, probiotic concentration, feeding, duration, type, and source of probiotics[24]. The factor that influences the success of probiotic products in increasing growth and feeds efficiency in fish is the. In addition, growth is also influenced by internal factors and external factors. Internal factors largely depend on the condition of the fish’s body, for example, the fish’s ability to utilize the remaining energy and protein after eating.

4. Conclusion

*Bacillus* sp. (PCP 1) and *Enterobacter* sp. (JC 10) can attach to the intestinal epithelial cells of red tilapia in vitro, but the effects of the probiotics provision in the fish feed with three and six days intervals on the fish survival rate and growth performance could not be seen at a month of the examinations.

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References

[1] Setiawati, M, M A Suprayudi 2003 *Jurnal Akuakultur Indonesia* 2(1): 27-30
[2] Monalisa, S S, I Minggawati 2010 *Journal of Tropical Fisheries* 5(2): 527-531.
[3] Kementrian Kelautan dan Perikanan 2017 Pusat Data, Statistik dan Informasi: Kementrian Kelautan dan Perikanan, Jakarta
[4] Nayak, S K 2010 *Fish and Shellfish Immunology* 29:2-14
[5] Merrifield, D L, D Arkadios, F Andrew, J D Simon, T M B Remi, B Jarl, C Mathieu, and R Einar 2010 *Journal Aquaculture* 302: 1-18
[6] Setiawati, J A, Y T Tarsim, Adiputra, and S Hudaidah 2013 *E-Jurnal Rekayasa dan Teknologi Budidaya Perairan I* (2): 151-162
[7] Iribarren, D, P Dagá, M T Moreira, and G Feijoo 2012 *Aquacult Int.* 20:779-789
[8] Wang Y B, Li J R., and Lin J 2008 Probiotics in aquaculture: challenges and outlook *Aquaculture* 281: 1-4
[9] Asaduzzaman, Md, I Shumpei, A Sumi, Md. K Abdul, K G Subrata, M A K Nurul, and B A M Ambok 2018 *Aquaculture Report* 9:53-61
[10] Yuniastuti, A 2015 Probiotik Unness Press Semarang
[11] Atitus, I N 2018 Fakultas Pertanian Universitas Gadjah Mada Skripsi
[12] Sulistyai, H E 2019 Fakultas Pertanian Universitas Gadjah Mada Skripsi
[13] Khusnan and Salasia S I 2006 *Jurnal Sain Vesetiner* 24: 102 – 108
[14] Dhinarso, P 2020 Fakultas Pertanian Universitas Gadjah Mada Skripsi
[15] Agusti, E 2020 *Journal of Koenig*
[16] Afrianto, E, dan Liviaway, E 2005 Pakan Ikan Kanisius Yogyakarta
[17] Rijker, G T, S Bengmark, P Enck, D Haller, U Herz, and M Kalliomaki 2010 *J. Nutr.* 140 (3):671
[18] Rohim, A and Soebijanto 2002 Bina Rupa Aksara Jakarta
[19] Widanarni 2008 *Jurnal Akuakultur Indonesia* 7(2):179-188
[20] Pratiwi, Rostika R, and Diah Hayati Y 2011 *Jurnal Akuatika* 2(2)
[21] Devi, S, T S Raza I, and R Wulandari 2019 *Intek Akuakultur* 3(1) : 80-91
[22] Putra, A N 2010 Institut Pertanian Bogor Tesis
[23] Kurniawan, A P, Suminto, A H C Haditomo 2019 *Jurnal Sains Akuakultur Tropis* 3(1) : 82-92
[24] Welker, T L and Lim, C 2013 *J. Aquac. Res. Development* 1-8
[25] Reda, R M, and K M Selim 2014 *Aquac. Int.* 23: 203–217
[26] Mirna, and S Wahana 2020 *Jurnal Agrokompleks* 9(1): 16-25