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Study of Growth Curve of *Lactobacillus plantarum* FNCC 0026 and Its Antibacterial Activity

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Abstract

Background*: Lactobacillus plantarum is one of the lactic acid bacteria (LAB) with strong antibacterial activity. However, these bacteria show different growth for each strain. The turbidimetric bacterial growth curve approach is the most accurate, fastest, and most reproducible method for obtaining an overview of the bacterial life cycle. In addition, we also examined the antibacterial activity of each observation of the growth curve.* ***Objective****: The aim of the study was to determine the optimal incubation time with the highest biomass concentration and antibacterial activity of the Lactobacillus plantarum FNCC 0026.* ***Method:*** *Observations of optical density (OD) values were performed simultaneously on 10 points of Lactobacillus plantarum FNCC 0026 and performed every 12 hours. Antibacterial activity tested against Staphylococcus aureus ATCC 25923 and Escherichia coli ATCC 8739.* ***Result:*** *The Lactobacillus plantarum FNCC 0026 fermentation broth showed the highest OD value and antibacterial activity after 30 hours of incubation. The maximum diameter of the inhibition zone against Staphylococcus aureus ATCC 25923 and Escherichia coli 25923 were 17.08 ± 0.51 mm and 16.83 ± 0.54 mm.* ***Conclusion****: The results showed that the antibacterial activity had a linear relationship with the concentration of bacteria. In the Lactobacillus plantarum FNCC 0026, the optimum cultivation time is in the lag phase (24 – 30 hours).*

Keywords*: growth curve, Lactobacillus plantarum FNCC 0026, antibacterial activity*

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INTRODUCTION

In the recent years, food is not only intended to overcome hunger and fulfill the body`s nutrition, but also to prevent chronic diseases and improve the quality of life. These terms are called functional foods and Nutraceuticals. Functional foods, according to the International Food Information Council (IFIC), can be defined as "foods that provide health benefits beyond basic nutrition". Nutraceuticals, on the other hand, are more accurately described as foods (or parts of foods) that provide medicinal or health benefits, including prevention and/or treatment of disease (Wang & Li, 2015). One of the functional foods with health benefits is probiotics.

Probiotics are live microorganisms that, when present in sufficient quantity, provide a benefit to the host. Most probiotics belong to the lactic acid bacteria (LAB) group (Daba & Elkhateeb, 2020). The LAB consists of a diverse group of Gram-positive, catalase-negative bacteria that produce lactic acid as their main product (Seddik et al., 2017). One of the LABs that has started to be widely applied in commercial products is *Lactobacillus plantarum*, which has great abilities in the fermentation process of various products (Seddik *et al.*, 2017).

Lactobacillus plantarum is considered a versatile bacterium because of their activities, including antioxidant, anticancer, anti-inflammatory, anti-proliferative, anti-obesity, anti-diabetic, antibacterial, and high environmental adaptability. The *Lactobacillus plantarum* is used as an antibacterial agent against *Bacillus cereus, Escherichia coli*, *Proteus vulgaris*, *Enterococcus fecalis* (Shim et al., 2016), *Staphylococcus aureus* (Layus et al., 2020). Furthermore, these bacteria inhibit the growth of antibiotic-resistant bacteria such as MRSA (Kumar et al*.*, 2017), ESBL *E.coli*, and Pseudomonas aeroginosa (Layus et al., 2020).

In addition, *Lactobacillus plantarum* has an anti-adherent effect on bacteria, reducing the ability of pathogenic bacteria to adhere to host surfaces and preventing biofilm formation, which is important for the persistence and resilience of bacterial infections. It is also believed that the antibacterial activity of *Lactobacillus plantarum* is caused by the presence of organic acids and hydrogen peroxide and bacteriocins called plantaricin (Kumar, et al. 2017). Since the bactericidal activity of this peptide depends on the binding mechanism of the pathogenic mannose phosphotransferase permease (Man-PTS) to its MptC and MptD subunits. When these bacteriocins insert into the target cell membrane, they irreversibly open endogenous channels, allowing toxic ions to diffuse across the membrane and kill the target cell (Hernández-González et al., 2021).

The antibacterial activity of probiotics is greatly influenced by their viability. In general, probiotics show optimal activity when the viable cell count is at a concentration of approximately 106-109 colony-forming units (CFU)/mL, therefore the incubation time of the bacteria should be considered to reach the highest concentration (Slizewska & Chlebicx - Wojcik, 2020). The OD value of microorganisms is generally observed at a wavelength of 580 nm. However, the microbial growth of probiotics is strain-dependent, each bacterium has a different cell growth rate (Rezvani et al., 2017). Several approaches can be used to determine the rate of cell growth. Statistical approaches using bacterial growth parameters are highly accurate. The plating method is widely used, but this method cannot provide actual results and is time-consuming because it takes incubation time to determine the number of living cells. Other methods, such as turbidity measurements by OD measurements, by which real-time knowledge of bacterial populations, were used in this study (Rahman et al., 2017).

Therefore, the main objective of this study was to study the growth rate of *Lactobacillus plantarum* FNCC 0026 fermentation broth using OD measurements at different wavelengths and its antibacterial activity of *Lactobacillus plantarum* FNCC 0026 fermentation broth so that the optimum incubation time with the highest biomass can be determined.

MATERIAL AND METHODS

Materials

Materials used for this research are *Lactobacillus plantarum* FNCC 0026 culture from Pusat Studi Pangan dan Gizi Universitas Gajah Mada Yogyakarta; De Man, Rogosa, Sharp (MRS) broth medium (MERCK Millipore, USA), Nutrient agar medium (MERCK Millipore, USA); *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 8739 were provided by RSUD Dr. Soetomo.

Methods

Microbial preparation

One öse of *Lactobacillus plantarum* was mixed aseptically with 10.0 mL of MRS broth. Then the bacterial suspension in MRS broth was shaken using a rotary shaker at 150 rpm and 37°C during the test time.

Growth curve study of *Lactobacillus plantarum* FNCC 0026

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The OD of 1.5 ml bacterial suspension was measured using a UV-Vis spectrophotometer at the same time every day after incubation. Suspension of *Lactobacillus plantarum* was measured at five different wavelengths (540, 570, 600, 630, and 660 nm). Observations were recorded simultaneously every 12 hours for 5 days after inoculation (Rahman et al., 2017).

Optimization of bacterial inhibitory test media

Optimization of test media was performed by combining two media, MRS broth and nutrient agar (NA), under three conditions. The combination concentrations used in MRS-NA medium were 75%:25% (w/v); 50%:50% (w/v); and 25%; 75% (w/v) respectively. Each combination was tested for probiotic inhibition against two test bacteria. The selected media combination is based on the growth of test organisms on the media and the presence of clear zones around the wells.

Antibacterial activity of *Lactobacillus plantarum* FNCC 0026

The bioassay was performed by the well agar diffusion method using selected modification (M) media. Test media were prepared by the pour method. 10-12 mL of melted M media (45-50 °C) was poured into an empty sterile Petri disc, allowing to solidify and used as a base layer media. The seed layer was prepared by adding 3-5 µL of 25% T bacterial inoculum into 8 mL of melted M media, vortex well, pouring over the surface and allowing it to solidify. The wells were made with a 7mm diameter drill bit. The reservoir of each well was filled with 50 µl of test solution. Incubation was at 37°C for 24 hours. The diameter of the growth inhibition zone was measured with a digital caliper (Isnaeni et al., 2017).

Statistical analysis

All results were subjected to analysis of variance (ANOVA) using a completely randomized design with three replications for all treatments. The differences between means were tested at a significance value of $p < 0.05$.

RESULT AND DISCUSSION

Growth curve study of *Lactobacillus plantarum* FNCC 0026

The growth phase of microorganisms consists of four phases: lag phase, exponential growth phase, stationary phase, and decline phase. The OD value approach can be directly correlated with cell concentration. The OD measurements calculate the amount of light lost due to scattering and absorption at a single wavelength (McBirney et al., 2016). The OD value of *Lactobacillus plantarum* FNCC 0026 cell suspension increased hourly until reaching the highest value at 30 hours incubation. The growth curve slopes or showed a steady value until 78 hours, called the stationary phase (Fig. 1). This was consistent with a previous study in which *Lactobacillus plantarum* reached maximum OD values by day 2 (36 hours) (Rahman et al., 2017). Therefore, it could be said that incubating *Lactobacillus plantarum* takes 30 hours to achieve optimal growth.

Wavelength selection is an important consideration for reading OD values. The maximum wavelength is unique to each bacterium due to differences in size and cell characteristic of each bacterium. In this study, there was no significant difference of OD values at different wavelengths during the early stages of growth. At a wavelength of 600 nm, the decline phase occurs after 78 hours, which is different from the other wavelengths. A wavelength of 600 nm is commonly used for bacterial analysis. Previous studies have shown that using a wavelength of 600 nm yields accurate and reproducible results (McBirney et al., 2016).

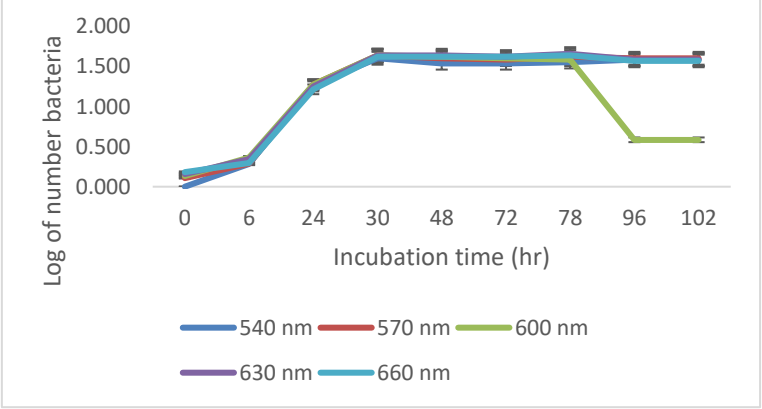

Figure 1. OD reading for *Lactobacillus plantarum* FNCC 0026 at different wavelengths from days 1 to 5. The highest OD value was on 30 hours.

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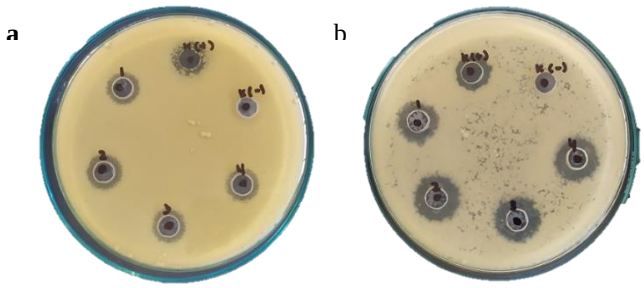

Figure 2. Antibacterial activity of Lactobacillus plantarum FNCC 0026 against a) *Staphylococcus aureus* ATCC 25923 and b) *Escherichia coli* ATCC 8739 was replicated four times. The positive control for SA bacteria ATCC 25293 was vancomycin, and for EC ATCC 8739 was ceftriaxone. The negative control used was MRS broth.

Table 1. Bacterial inhibitory test of each combination against *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 8739

Bacterial test : *Escherichia coli* ATCC 8739

Lactobacillus plantarum replication	Combination 1 (MRS-NA = 25%:75%)		Combination 2 (MRS-NA = 50%:50%)		Combination 3 (MRS-NA = 75%:25%)	
	Growth	Clear zone	Growth	Clear zone	Growth	Clear zone
Replication 1	+	-	+	+	+	+
Replication 2	+	-	+	+	+	+
Replication 3	+	-	+	-	-	-

Bacterial test : *Staphylococcus aureus* ATCC 25923

Lactobacillus plantarum replication	Combination 1 (MRS-NA = 25%:75%)		Combination 2 (MRS-NA = 50%:50%)		Combination 3 (MRS-NA = 75%:25%)	
	Growth	Clear zone	Growth	Clear zone	Growth	Clear zone
Replication 1	+	+	+	+	-	-
Replication 2	+	+	+	+	-	-
Replication 3	+	+	-	-	-	-

Note :
+ : formed
- : not formed

Optimization of bacterial inhibitory test media

Lactobacillus plantarum is a lactic acid bacterium with rich and complex nutritional requirements, so it requires a concentrated growth medium such as MRS medium for optimal growth (De Vos et al., 2009). The decrease in the pH of the medium to 5.7 and the addition of 0.14% sorbic acid to the MRS medium made this medium a suitable medium for probiotics. However, testing antimicrobial activity in a single concentrated medium can yield skewed results because pathogenic bacteria such as SA and E.coli cannot grow on MRS media. Therefore, a media combination between MRS and NA, which is a universal medium, is necessary to meet the needs of probiotics and test bacteria. The concentration in this combination of media differs for each strain according to the nutritional needs of the probiotics and test bacteria. Mixed media were selected based on their ability to grow the test bacteria and form a clear zone around the well. A clear zone indicates that Lactobacillus plantarum can live well so that it can provide inhibitory activity. The choice of combination medium for *Staphylococcus aureus* ATCC 25923 was combination 1 (MRS-NA = 25%:75%) which allowed test organisms to grow and a clear zone was seen around the wells. On the other hand, the selected combination medium for *Escherichia coli* ATCC 8739 was Combination 2 (MRS-NA = 50%:50%) had the most appropriate skills. *E. coli* grew well in combination medium 1 (MRS-NA = 25%:75%), but at this concentration, *Lactobacillus plantarum* could not provide any hindrance. In contrast, combination 3 (MRS-NA = 75%:25%) did not give the desired result for both SA and *E.coli* bacteria (Table 1). The pH of the medium affects the ability of the bacteria to live. MRS media concentrations that are high tend to be acidic, so they are not suitable for SA. while *E.coli* has metabolic flexibility that allows these bacteria to compete for

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carbon and energy sources even though it is in unfavorable conditions (Alteri & Mobley, 2012).

Antibacterial Activity of *Lactobacillus plantarum* FNCC 0026

Antibacterial activity was tested against two pathogenic strains using an adequate diffusion assay (Fig. 2a,b). The difficulty of determining bacterial viability is one of the weaknesses of turbidity measurements. Therefore, activity tests should also be performed to confirm antimicrobial efficacy. Each OD measurement was tested to determine the highest antibacterial activity of *Lactobacillus plantarum* FNCC 0026. See Table 2 and Figure 3. Bacterial suspensions harvested after 30 h had the lowest OD values and the largest clear zones. A small OD value indicates the concentration of the bacterial suspension, it is expected that a high number of bacteria in the suspension. And the clear zone shows the inhibitory capacity of the bacterial suspension. The maximum inhibitory diameters for antibacterial activity against *S. aureus* ATCC 25923 and *E. coli* ATCC 8739 were 17.08 ± 0.51 mm and 16.83 ± 0.54 mm, respectively.

Probiotic growth curves showed that *Lactobacillus plantarum* peaked at 30 hours of the lag phase and tended to decline in exponential antibacterial activity. The results of this study show that *Lactobacillus plantarum* FNCC 0026 can inhibit the growth of both Gram-positive and Gram-negative bacteria. The zone of inhibition diameter for commonly used antibiotics such as vancomycin in SA therapy is 11.97 ± 0.81 mm and ceftriaxone in E. coli therapy is 11.49 ± 0.98 mm. Probiotics have a larger zone of inhibition than antibiotics, indicating that *Lactobacillus plantarum* may replace antibiotics as the treatment of choice. As a functional food, this probiotic can exert advantages as a broad-spectrum antibiotic and acceptable taste than synthetic antibiotics. Therefore, probiotic production could be widely developed as a promising nutritional supplement.

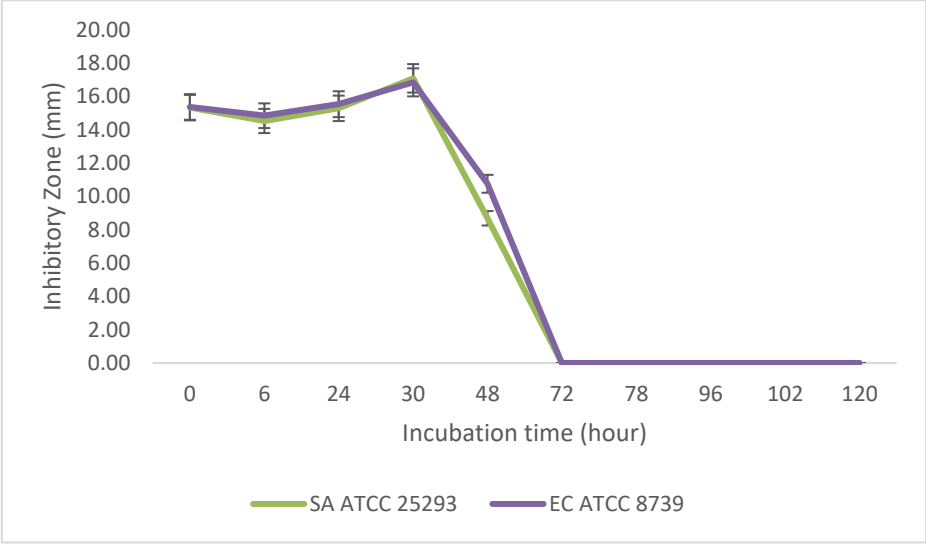

Figure 3. Inhibitory activity curve of Lactobacillus plantarum FNCC 0026 against two pathogenic bacteria (grey line: SA ATCC 25293 and yellow line: EC ATCC 8739) during the continuous incubation time. Each test was carried out three times for replication. And the highest inhibition zone was at 30 hours.

Table 2. Inhibitory activities of *Lactobacillus plantarum* FNCC 0026 against two pathogenic bacteria (mm)

Bacteria	0 h	6 h	24 h	30 h	48 h	72 h	78 h	96 h	102 h	120 h
Staphylococcus aureus ATCC 25923	15.32± 0.66	14.52 ± 0.75	15.28 ± 0.46	17.08 ± 0.51	8.68 ± 0.00	0.00	0.00	0.00	0.00	0.00
Escherichia coli ATCC 8739	15.36 ± 0.22	14.83 ± 1.41	15.52 ± 0.25	16.83 ± 0.54	10.75 ± 0.00	0.00	0.00	0.00	0.00	0.00

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CONCLUSION

Lactobacillus plantarum is a lactic acid bacterium with a broad spectrum of activity and good antibacterial activity. The best performance of these bacteria is in lag phase (30 hours), after which decline phase was obtained significantly. Therefore, the incubation time for these bacteria should be considered to obtain optimal activity.

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