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Research Report

Antibiofilm activity of probiotic bacteria *Lactobacillus plantarum* FNCC 0020 against *Streptococcus mutans* serotype c

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ABSTRACT

Background: *Streptococcus mutans (S. mutans) is a Gram-positive bacterium that plays a role in dental caries. Plaque and biofilm formation can be chemically cleaned using mouthwash, such as chlorhexidine gluconates (CHX) 0.2%, which is the gold standard. Another alternative can be developed to inhibit S. mutans biofilm formation by using Lactobacillus plantarum probiotic, which has antibiofilm potency by producing antimicrobial substances.* ***Purpose:*** *This study tested the biofilm inhibition to determine the Inhibitory Concentration (IC_{50}) that can inhibit 50% of biofilm formation.* ***Methods:*** *This research was conducted as an in vitro experimental laboratory study. Biofilm inhibition testing was performed by using the microdilution method on biofilms formed in 96 well microplates with 0,1% crystal violet staining. Lactobacillus plantarum FNCC 0020 was diluted to several concentrations: 12.5%, 25%, and 50% against Streptococcus mutans biofilm induced by 3% sucrose. The test results in optical density were read using a spectrophotometry with a wavelength of 650 nm. IC_{50} were determined using the GraphPad Prism sigmoidal dose-response method.* ***Results:*** *There is antibiofilm activity of Lactobacillus plantarum FNCC 0020 against Streptococcus mutans, and the inhibitory effect against Streptococcus mutans biofilm showed IC_{50} of 42.43%.* ***Conclusion:*** *Lactobacillus plantarum FNCC 0020 at 50% has biofilm inhibitory activity compared to other concentrations but is less effective than 0.2% CHX.*

Keywords: *Lactobacillus plantarum; antibiofilm; Streptococcus mutans; serotype; probiotic*

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INTRODUCTION

Dental caries is a common dental health problem that can cause damage to dental tissues.[1] The prevalence rate of dental caries increases annually, from children to adults. Riskesdas (2018) stated that dental caries rates reach 88.8% of the population.[2] It is a major and multi-faceted oral health problem which alter activities, characterized by demineralization of tooth hard tissue due to bacterial activity.[3]

Streptococcus mutans (*S. mutans*) is one of the Gram-positive bacteria that plays a role in the occurrence of dental caries. *S. mutans* is classified into serotypes C, E, F, with serotype C being the most common type the oral cavity.[4] *S. mutans* is considered an important pathogen in dental caries, especially in disease initiation. The development of dental caries is associated with biofilm formation.[5] *S. mutans* uses carbohydrates that produce acidic metabolites causing acid damage and dental enamel demineralization, removing mineral materials, and thus causing dental caries.[6]

Biofilm is a collection of bacteria encased in an extracellular polymeric substance (EPS) matrix and attached to solid surfaces.[7] EPS, consisting of polysaccharides, DNA, proteins, and water, is responsible for intercellular interactions and bacterial cell protection. EPS can increase biofilm tolerance to antimicrobials.[8] The biofilm mechanism is a complex process that goes through several stages, including cell attachment, cell-to-cell adhesion, cell proliferation and development, maturation, and release.[9]

Currently, efforts to minimize plaque and biofilm formation can be done chemically and mechanically. Chemical cleaning can use mouthwash; one of the ingredients used for mouthwash is Chlorhexidine gluconate (CHX) 0.2%, which is the gold standard. This material has proven effective in inhibiting plaque formation. However, the use of mouthwash containing CHX can cause side effects such as tooth discoloration and taste sensation changes. [10]

Another alternative material developed to inhibit *S. mutans* biofilm formation is the use of probiotic bacteria.[11] Probiotic bacteria are living bacteria that provide health

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benefits to their host when given at concentrations acceptable to the host. Probiotic bacteria produce antimicrobial agents such as exopolysaccharides that interfere with Quorum Sensing, bacteriocins that damage pathogenic bacterial cell membranes and inhibit biofilm attachment, hydrogen peroxide that damages extracellular enzymes (glucosyltransferase), and organic acids including lactic acid that can lower environmental pH, causing competition for adhesion sites and nutrients.[12,13]

One of the probiotic bacteria commonly used in the oral cavity to inhibit biofilm is the lactic acid bacteria *Lactobacillus*.[12] In research, Lim et al. (2019) proved that *L. plantarum* became the highest biofilm inhibitor compared to *L. Rhamnosus* and *L. Delbrueckii* against *S. mutans*.[14] *L. plantarum* is a type of Lactic Acid Bacteria (LAB), which are bacteria that can form lactic acid as a result of sugar metabolism. LAB are gram-positive bacteria, non-spore-forming, round or rod-shaped, and produce lactic acid as the main metabolic end product during fermentation.[15]

The biofilm inhibition capabilities produced in previous studies proved that *L. plantarum* with different strains at 12.5% concentration could only inhibit (55.6%- 71.8%) biofilm formation, which does not reach the Minimum Biofilm Inhibitory Concentration (MBIC) value stated to be able to inhibit biofilm formation by 90%.[14,16,17] Therefore, in the biofilm inhibition test, it is necessary to determine the Inhibitory Concentration (IC_{50}) value, which is a parameter to determine the concentration that can inhibit 50% of biofilm formation. The determination of IC_{50} value is done by analyzing the non-linear line equation (sigmoidal dose-response with variable slope) to determine the relationship between concentration and percent biofilm inhibition.[18]

The difference in biofilm inhibition produced between strains is possibly due to differences in *L. plantarum* isolation from various fermented foods worldwide that experience differences in climate, weather humidity, and earth surface height. The fermentation process needs to consider extrinsic factors (humidity, temperature, and gas composition), intrinsic factors (nutrients, water activity, and pH), and optimum temperature to improve the quality of antimicrobial compounds, probiotic capabilities, and potential antioxidant activity.[19]

L. plantarum commonly used in Indonesia is obtained from the Food and Nutrition Study Center of Gadjah Mada University, one of which is *L. plantarum* FNCC 0020. Various studies on *L. plantarum* FNCC 0020 have proven to have diverse capabilities ranging from its resistance to acidic conditions and room temperature useful for animal feed, glucose reduction ability in food beneficial for health, and potential to inhibit skin aging process in topical application on rat test animals.[20,21] The characteristics of *L. plantarum* FNCC 0020, which can survive in acidic conditions and room temperature, have the potential to support optimal growth in the oral cavity. Therefore, this study aims to describe the IC_{50} antibiofilm activity of probiotic bacteria *L. plantarum* FNCC 0020 against *Streptococcus mutans* serotype c.

MATERIALS AND METHODS

This research was conducted at the Dental Research Center of Airlangga University and was approved by the ethics committee of the Faculty of Dental Medicine, Airlangga University. This study uses an in vitro experimental laboratory design with a post-test only control group. There were five treatment groups: treatment group 1 (P1), treatment group 2 (P2), treatment group 3 (P3), and positive control (K+), negative control (K-). Based on the number of samples, each treatment was repeated eight times.

The preparation of CFS was carried out by suspending 5 loops of *L. plantarum* bacterial inoculum in 45 mL of MRSB medium and then incubated at 37°C for 48 hours. The suspension was then centrifuged at 5000 rpm for 15 minutes at 4°C. After centrifugation, the formed supernatant was filtered using a 0.22 µm sterile filter paper.[23] The concentration determination was based on the research results of Jang et al. (2024). Based on this research, the Minimum Inhibitory Concentration (MIC) obtained was 12.5%, and in the study by Liang et al. (2023), a concentration of 50% was able to inhibit biofilm formation by 98%.[16,18]

Based on the above description, the concentration variations used in this study included concentrations of 12.5%, 25%, and 50%. The 12.5% concentration was prepared by dissolving 125 µL of CFS formed after centrifugation into BHIB media until reaching a volume of 1 mL. The 25% concentration was prepared by dissolving 250 µL of CFS formed after centrifugation into BHIB media until reaching a volume of 1 mL. The 50% concentration was prepared by dissolving 500 µL of CFS formed after centrifugation into BHIB media until reaching a volume of 1 mL. The suspension was then homogenized using a *vortex*.

The biofilm inhibition test was conducted using the microdilution method. The supernatant of *L. plantarum* FNCC 0020 was serially diluted to concentrations of 12.5%, 25%, and 50% in BHIB media. The negative control was prepared containing BHIB media, 3% sucrose, *S. mutans* serotype c bacteria and distilled water. The positive control contained BHIB media, 3% sucrose, *S. mutans* serotype c bacteria, and chlorhexidine. Treatment group 1 contained BHIB media, 3% sucrose, *S. mutans serotype c* bacteria, and *L. plantarum* FNCC 0020 12,5% concentration. Treatment group 2 contained BHIB media, 3% sucrose, *S. mutans* serotype c bacteria, and *L. plantarum* FNCC 0020 25% concentration. Treatment group 3 contained BHIB media, 3% sucrose, *S. mutans* serotype C bacteria, *L. plantarum* FNCC 0020 50% concentration.

S. mutans bacterial culture stock cultured in Mueller Hinton agar was obtained. Bacterial concentration was adjusted to 1,5 x 10^8 CFU/mL McFarland standard. The bacteria were cultured in BHIB medium containing 3% sucrose and then placed in an anaerobic environment for 24 hours at 37ºC. The biofilm inhibition test was performed by adding 100µl bacterial suspension to each well. It was then incubated for 1 hour at 37°C, after that 100µl *L. plantarum*

FNCC 0020 at concentrations of 12.5%, 25%, and 50% was added to the 96-well microplate and incubated for 24 hours at 37°C. After incubation, planktonic bacteria were removed by washing with phosphate buffered saline (PBS). The remaining biofilm was stained with 100µl 0.1% crystal violet solution, which was incubated for 10 min at room temperature. Afterwards, the crystal violet solution was removed, and the sample was rinsed three times with distilled water to remove residual staining. The adherent stain was then dissolved using 98% ethanol. Biofilm evaluation was performed by measuring the absorbance of the solution using a spectrophotometer at a wavelength of 650 nm. This absorbance value, called Optical Density (OD), describes the amount of biofilm formed. The higher the amount of biofilm, the higher the OD value. The IC_{50} value was calculated after obtaining the average percent inhibition of biofilm formation from each concentration of the treatment group.

With SPSS 26, a statistical analysis was performed on the data derived from the optical density measurement. To determine whether the data is normally distributed, the statistical test starts with a normality test for each group using the Shapiro-Wilk Test (n<50). To determine whether the data was homogeneous, a homogeneity test using the Levene Test was conducted for each group. One Way ANOVA was used in a nonparametric comparative statistical analysis to determine whether treatment groups 1, 2, and 3 differed from the negative control, positive control, or treatment group following treatment. Tukey HSD was used in a post-test (Post Hoc) to determine which groups differed significantly.

RESULTS

Using a spectrophotometer set to 650 nm, the antibiofilm activity of L. plantarum FNCC 0020 against S. mutans serotypes C bacterium was examined and measured.Table 1 displays the findings of the research OD reading.The average *S. mutans* biofilm OD or growth value for each group can be computed using the biofilm OD readings table (Table 2).Treatment groups 1, 2, 3, and positive control (CHX) all had lower biofilm OD values than the negative control (without treatment), according to the results of reading the OD of *S. mutans* biofilm using a spectrophotometer (Figure 1).

Since the negative control group solely included *S. mutans* growing on medium and received no treatment, as seen in Figure 1, there was no suppression in the production of biofilms. The percentage of *S. mutans* biofilm formation in the positive control group was 86.06%. The percentage of *S. mutans* biofilm formation in the treatment group varied between 35.54-73.65%. From this calculation, the results of the inhibition percentage calculation for all treatment groups were obtained. Then, the obtained biofilm formation inhibition percentage data were used to calculate the IC_{50} value using GraphPad Prism (Figure 2).

The analysis showed a dose-dependent pattern, where the increase in *L. plantarum* FNCC 0020 concentration is directly proportional to the increase in biofilm inhibition percentage. The curve shows a minimal response (bottom) of 35.9% at low concentration and a maximal response (top) of 73.7% at the highest concentration, with an IC_{50} value of

Table 1. Optical Density (OD) reading results

Sample	50%	25%	12.5%	K(+)	K(-)
1	0.024	0.08	0.088	0.012	0.156
2	0.023	0.1	0.101	0.019	0.149
3	0.022	0.076	0.095	0.044	0.112
4	0.032	0.118	0.082	0.03	0.184
5	0.054	0.148	0.134	0.01	0.174
6	0.051	0.088	0.101	0.03	0.194
7	0.059	0.112	0.111	0.016	0.134
8	0.064	0.077	0.093	0.013	0.146

Table 2. Result of average Optical Density (OD)

Treatment groups	N	Mean ± SD
50%	8	0.041 ± 0.017
25%	8	0.099 ± 0.025
12.5%	8	0.100 ± 0.016
K(+)	8	0.021 ± 0.011
K(-)	8	0.156 ± 0.027

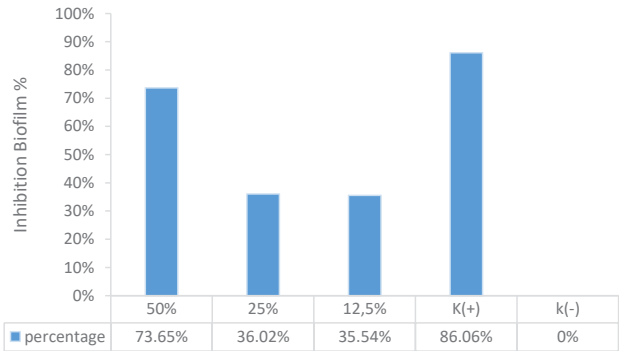

Figure 1. Graph of percentage of inhibition *S. mutans* biofilm formation.

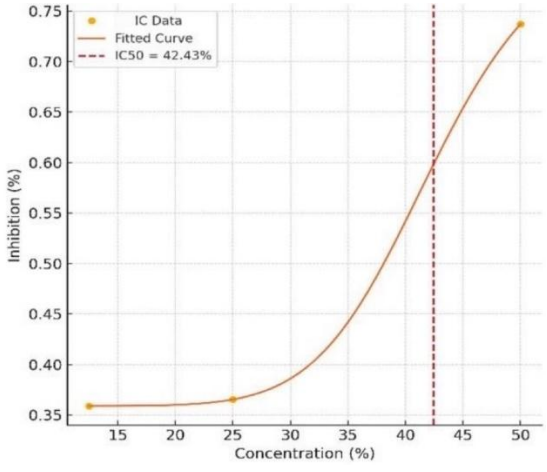

Figure 2. IC_{50} value curve.

42.43%. The slope value of 37.8% indicates good sensitivity of response changes to concentration changes. Therefore, *L. plantarum* FNCC 0020 at a concentration of 42.43% can already inhibit 50% of biofilm formation. Based on these data, *L. plantarum* FNCC 0020 at 50% concentration has greater potential in inhibiting biofilm formation compared to other concentrations (p=0.000).

The study data were found to be normally distributed based on the ShapiroWilk Test results. The research data was homogeneous, according to the Levene Test results. After receiving therapy, at least one group demonstrated a difference, according to the One Way ANOVA results. All groups shown significant differences in suppressing biofilm formation when compared to other groups, according to the Post Hoc Test results table.

DISCUSSION

The results showed the mean Optical Density (OD) concentration values and Figure 1 showing the inhibition percentage of probiotic bacteria *L. plantarum* FNCC 0020 at concentrations of 12.5%, 25%, and 50% demonstrated lower Optical Density (OD) values compared to the negative control group and higher inhibition percentages compared to the negative control group. The Optical Density (OD) readings indicated that the lower the biofilm OD value recorded, the greater the percentage of biofilm reduction.

Probiotic bacteria *L. plantarum* FNCC 0020 at 50% concentration showed inhibitory effects against *S. mutans* biofilm, marked by light purple color in the 96-well microplate, with OD calculations showing *S. mutans* biofilm inhibition of 73.65%, while *L. plantarum* FNCC 0020 at 12.5% and 25% concentrations showed no inhibitory ability against *S. mutans* biofilm. This differs from previous studies, research conducted by Jang et al. (2023), which demonstrated that *L. plantarum* at 12.5% concentration isolated from kimchi could inhibit *S. mutans* biofilm formation by 65.36%.[16] In another study by Lim et al. (2019), *L. plantarum* 20061 isolated from raw crab soaked in fermented tomato kimchi at 12.5% concentration could inhibit *S. mutans* biofilm formation by 71.8%.[17] These differences in results may be due to different isolation sources of *L. plantarum* from various fermented foods worldwide and factors in the fermentation process.[22]

Calculations from Figure 2 showed an IC_{50} value of 42.43%, where *L. plantarum* FNCC 0020 at 42.43% concentration could already inhibit 50% of biofilm formation. Therefore, *L. plantarum* FNCC 0020 at 50% concentration had a strong effect in inhibiting 50% of biofilm formation. This is likely because higher concentrations of *L. plantarum* FNCC 0020 contain more antimicrobial substances, thus increasing effectiveness in inhibiting *S. mutans* biofilm. According to Zaura et al. (2014), the normal oral cavity temperature typically ranges around 37°C, creating a stable habitat for bacteria to live and develop.[23] This aligns with research by Suryani et al. (2023), proving that *L. plantarum* FNCC 0020 growth has resistance in acidic conditions down to pH 2 and survives at 37°C, maintaining viability during 10 days of storage in Ruminant livestock.[20]

In this study, probiotic bacteria *L. plantarum* FNCC 0020 at 50% concentration could grow optimally and produce antimicrobial substances such as lactic acid that can lower environmental pH, exopolysaccharides that disrupt Quorum Sensing communication, bacteriocins that can inhibit bacterial cell membrane integrity, and hydrogen peroxide that inhibits gtfs bonding. These compounds can inhibit the development of pathogenic *S. mutans* bacteria, resulting in minimal biofilm formation. The presence of these compounds is supported by research from Zamani et al. (2017), which demonstrated the antibiofilm power of *L. plantarum spp.* isolated from siahmazgi cheese against pathogenic bacteria *P. aeruginosa*, showing that these compounds (lactic acid, exopolysaccharides, bacteriocins, and hydrogen peroxide) have the ability to inhibit bacterial cell attachment to surfaces, affecting gene expression related to biofilm production and breaking down biofilm in pathogenic bacteria in the digestive system.[24] Based on the research results, the IC_{50} value was found to be 42.43%, so *L. plantarum* FNCC 0020 at 50% concentration could inhibit 50% of biofilm formation, showing an inhibition rate of 73.65%. Meanwhile, the results from administering *L. plantarum* FNCC 0020 at 50% concentration were still lower compared to the positive control, namely the bacterial group treated with 0.2% CHX, which had an inhibition rate of 86.06% against *S. mutans* biofilm. This comparison provides important context for potential clinical applications and suggests areas for future optimization.

In conclusion, *Lactobacillus plantarum* FNCC 0020 shows potential in inhibiting *Streptococcus mutans* biofilm formation. Active compounds in *Lactobacillus plantarum* such as lactic acid, exopolysaccharides, hydrogen peroxide, and bacteriocins are effective in reducing biofilm formation. However, its inhibitory effectiveness depends on the concentration of *Lactobacillus plantarum* FNCC 0020 used, where higher concentrations tend to provide more significant results in inhibiting biofilm growth. Therefore, administration of *Lactobacillus plantarum* FNCC 0020 at a 50% concentration can inhibit *Streptococcus mutans* biofilm with a IC_{50} value of 42.43%.

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