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RESEARCH ARTICLE

The effect of supernatants of *Lactobacillus casei* against *Porphyromonas gingivalis*

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ABSTRACT

Periodontitis is an inflammatory disease of the periodontal tissues primarily caused by *Porphyromonas gingivalis* (*P. gingivalis*). In recent years, probiotics have been proposed as a potential bacteriotherapeutic approach for periodontitis. Probiotic bacteria such as *Lactobacillus casei* (*L. casei*) are known to produce antibacterial compounds, which can be found in their culture supernatants. The ability of *L. casei* to grow and synthesize these substances depends on environmental factors, including pH. Based on preliminary findings, a pH of 6.5 was used in this study. This study aimed to evaluate the antibacterial activity of *L. casei* supernatant against *P. gingivalis* in vitro. The research employed an experimental post-test-only group design. Two control groups were included: a negative control (aquadest) and a positive control (0.2% chlorhexidine). The treatment groups received *L. casei* supernatant at volumes of 20 µl, 50 µl, and 80 µl, with incubation periods of 6, 12, and 24 hours. The results demonstrated that the *L. casei* supernatant exhibited the highest antibacterial activity against *P. gingivalis* at a volume of 80 µl after 12 hours of incubation. The Shapiro–Wilk test indicated that the data were normally distributed, while Levene's test revealed a lack of homogeneity. Post hoc analysis showed significant differences among all treatment groups at the 12-hour incubation point. In conclusion, the supernatant of *L. casei* exhibits in vitro antibacterial activity against *P. gingivalis*.

Keywords: antibacterial; *Lactobacillus casei; Porphyromonas gingivalis;* supernatant

INTRODUCTION

Periodontitis is a disease of the periodontal tissues, with a high prevalence of 74.1% in Indonesia. It is caused by the main subgingival pathogen, *Porphyromonas gingivalis* (*P. gingivalis*).[1] The initial phase of scaling and root planing is necessary to eliminate the etiological agents and predisposing factors of periodontal disease, but the limited effectiveness of these treatments often necessitates systemic antibiotic use, which can lead to bacterial resistance and recolonization.[2]

Several studies have recently suggested the use of probiotics as a form of bacteriotherapy in periodontitis.[3] It has been discovered that *Lactobacillus casei* (*L. casei*), one of the most widely used probiotic bacteria, produces antimicrobial substances such as bacteriocins, organic acids, hydrogen peroxide, and biosurfactants, which are present in its supernatant. These antibacterial substances are known to inhibit the growth of bacteria that cause periodontal disease.[3]

Environmental factors, such as the acidity of the culture medium, greatly affect the growth and ability of *L. casei* to produce antibacterial substances. Therefore, preliminary research was conducted using different pH levels (4.0, 4.5, 5.0, 5.5, 6.0, and 6.5) in the culture medium. The results showed that *L. casei* grows optimally at pH 6.5 after 24 hours of incubation. This study aims to analyze the antibacterial activity of *L. casei* supernatant in inhibiting the growth of *P. gingivalis*. A previous study by Rico et al utilized liquid media treated with varying volumes of cell-free supernatant (CFS): 80, 100, and 120 µL.[4,5]

Based on research by Rico et al,[5] which is supported by the findings of of Kosasi et al (2019)[6] regarding the use of 50 µl and 80 µl supernatant volumes on paper discs, this study used a different method and tested volumes of 20 µL, 50 µL, and 80 µL with the disc diffusion method to examine the antibacterial activity of *L. casei* supernatant cultured in media with an optimal pH of 6.5 in inhibiting the growth of *P. gingivalis* in vitro. In the future, the use of *L. casei* may be developed as a safe and biocompatible clinical treatment for periodontal disease, such as a topical agent applied after periodontal therapy. Therefore, further research is warranted.

To date, there has been no research focusing specifically on the potential of *L. casei* to inhibit the growth of *P. gingivalis*. Yet, *L. casei* is a probiotic bacterium commonly found in fermented foods. Thus, it is not foreign to the human body and holds great promise as a supportive treatment for periodontitis. Furthermore, using bacterial extracts is expected to be faster and easier to cultivate compared to extracts derived from plants or animals.

MATERIALS AND METHODS

This research has been conducted in accordance with the ethical guidelines and regulations of the Faculty of Dentistry, University of Jember, East Java, Indonesia, under approval number No.1588/UN25.8/KEPK/DL/2021. The first stage involved the preparation and optimization of *Lactobacillus casei* growth at different pH levels. *Lactobacillus casei* FNCC 0090 was obtained from PSPG, Universitas Gadjah Mada, and had been cultured on de Man, Rogosa, and Sharpe (MRS) Agar medium and incubated for 48 hours. A starter suspension was prepared by dissolving 1–2 colonies of *L. casei* in 0.85% sterile saline and adjusting it to match McFarland standard 0.5, equivalent to 1.5×10^8 CFU/ml.

Next, *L. casei* was cultivated in MRS Broth (MRSB), adjusted to different pH levels (4.0, 4.5, 5.0, 5.5, 6.0, and 6.5). The broth was placed in Erlenmeyer flasks, and pH was adjusted using CH_3COOH (99%) and 1 N NaOH, with measurements taken using a pH meter and indicator strips. After adjusting the pH, the MRSB was aliquoted into reaction tubes (10 ml per tube) and sealed with cotton plugs. Then, 5 ml of the starter suspension was added to each tube and incubated for 24 hours.

Upon observation, the culture at pH 6.5 exhibited the highest bacterial density. The cell-free supernatant of *L. casei* was then collected by centrifuging the culture grown at pH 6.5 (Oregon centrifuge) at 2325 × g for 10 minutes. The supernatant was then collected using a micropipette and filtered through a sterile membrane.

The next step involved the preparation of *Porphyromonas gingivalis* for antibacterial testing. The strain used was *P. gingivalis* ATCC 33277, obtained from the Bioscience Laboratory, Faculty of Dentistry, Universitas Jember. The bacteria had been revived on blood agar media enriched with 1% glycerol and 25% human blood type O. A bacterial suspension was prepared by picking 1–2 colonies, transferring them into a reaction tube containing 5 ml of 0.9% NaCl, and homogenizing the solution using a vortex. The bacterial density was adjusted to McFarland standard 0.5, equivalent to 1.5×10^8 CFU/ml.

The final laboratory procedure was the inhibition zone test. The *P. gingivalis* suspension was inoculated onto blood agar using the spread plate technique. The test groups were categorized as follows: negative control (aquadest), positive control (0.2% chlorhexidine), and treatment groups receiving *L. casei* supernatant (20 µl, 50 µl, and 80 µl), which was applied to sterile paper discs. After complete absorption, the discs were placed on blood agar plates previously inoculated with *P. gingivalis*. Each group was tested in quadruplicate. The method used was the disc diffusion technique (Kirby–Bauer method), followed by incubation at 37 °C for 6, 12, and 24 hours.

For data analysis, IBM SPSS Statistics 25 was used. All data obtained were analyzed using the Shapiro–Wilk test for normality and Levene's test for homogeneity. Differences between groups were analyzed using Multivariate Analysis of Variance (MANOVA), followed by the Post Hoc Games–Howell test. A significance level of $p < 0.05$ was considered statistically significant.

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RESULTS

The presence of a clear zone around the disc paper indicated that the *L. casei* supernatant at volumes of 20 µl, 50 µl, and 80 µl, as well as the positive control group, exhibited activity in inhibiting the growth of *P. gingivalis*. The diameter of the inhibition zones was measured using a caliper with 0.05 mm precision. The average diameters of the inhibition zones produced by the aquadest (K−), chlorhexidine (K+), and the *L. casei* supernatant treatment groups (20 µl, 50 µl, and 80 µl) are presented in Table 1. It can be observed that after 6, 12, and 24 hours of incubation, the largest inhibition zones were found in the 80 µl *L. casei* supernatant group and the positive control group. All treatment groups, except the negative control, showed their widest inhibition zones at 12 hours of incubation. Each group was tested with four repetitions (Figure 1).

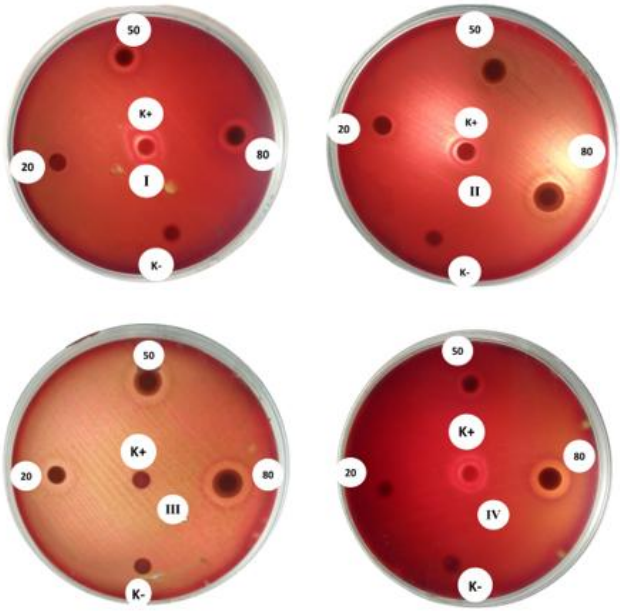

Figure 1. Growth inhibition of *P. gingivalis* treated with aquadest (K−), chlorhexidine (K+), and *L. casei* supernatant (20 µl, 50 µl, 80 µl) in four replicates (I, II, III, IV) at 12 hours

Table 1. The mean diameter of the inhibition zone produced by *L. casei* supernatant on *P. gingivalis* growth

Groups	n	Mean diameter of inhibition zone (mm)[a]			*P*[b] Value
		6 Hours	12 Hours	24 Hours	
Aquadest	4	5.00 ± 0.00	5.00 ± 0.00	5.00 ± 0.00	0.000
Clorhexidin	4	7.98 ± 1.32	12.83 ± 0.29	10.01 ± 2.34	0.006
20 µl	4	6.34 ± 0.22	7.53 ± 0.10	5.96 ± 0.74	0.002
50 µl	4	8.70 ± 1.76	10.63 ± 1.87	6.28 ± 1.28	0.015
80 µl	4	11.51 ± 2.63	13.08 ± 2.28	9.24 ± 1.40	0.091

The data obtained from the experiment were analyzed using SPSS software to assess normality and homogeneity. The results of the Shapiro–Wilk test for normality indicated that the data were normally distributed, with significance values greater than 0.05. In contrast, the Levene's

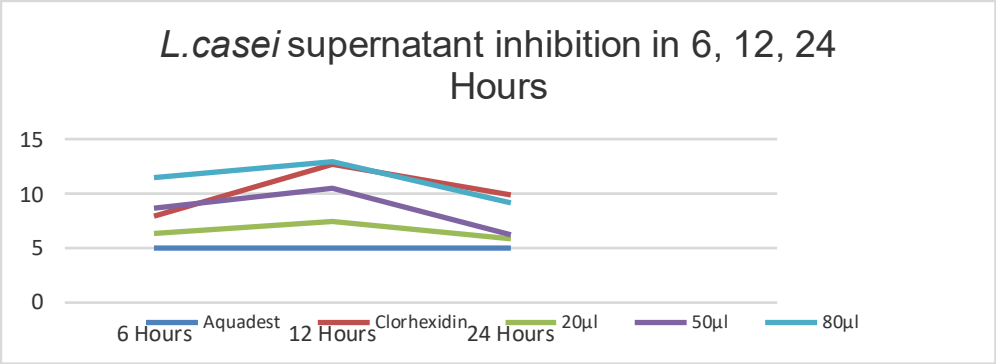

Figure 2. Inhibitory activity of *L. casei* supernatant

Table 2. Post Hoc test

Time	Groups	Aquadest	Clorhexidin	20 µl	50 µl	80 µl
	Aquadest	-	0.076	0.005*	0.091	0.060
	Clorhexidin	-	-	0.295	0.959	0.267
6 Hours	20 µl	-	-	-	0.254	0.107
	50 µl	-	-	-	-	0.469
	80 µl	-	-	-	-	-
	Aquadest	-	0.000*	0.000*	0.036*	0.023*
	Clorhexidin	-	-	0.000*	0.331	0.999
12 Hours	20 µl	-	-	-	0.163	0.063
	50 µl	-	-	-	-	0.517
	80 µl	-	-	-	-	-
	Aquadest	-	0.087	0.269	0.430	0.035*
	Clorhexidin	-	-	0.138	0.175	0.975
24 Hours	20 µl	-	-	-	0.991	0.052
	50 µl	-	-	-	-	0.102
	80 µl	-	-	-	-	-

test for homogeneity showed that the data were not homogeneous, with significance values less than 0.05.

The data were then analyzed using the Multivariate Analysis of Variance (MANOVA), with outputs presented as between-subject effects (Table 1). The positive control group and the *L. casei* supernatant groups (20 µl and 50 µl) showed significance values of 0.006, 0.002, and 0.015 respectively ($p < 0.05$). However, the *L. casei* supernatant at 80 µl showed a significance value of 0.091 ($p > 0.05$), indicating that time had a significant effect on the inhibition zone diameter in the positive control and the 20 µl and 50 µl *L. casei* groups, but not in the 80 µl group.

A follow-up post hoc test was conducted using the Games–Howell test, as the data were not homogeneous. As shown in Figure 2, at 6 hours of incubation, all groups began to exhibit bacterial inhibition activity, although the zones were still not clearly defined. At 12 hours, the inhibitory zones became clearly visible, with the 80 µl *L. casei* supernatant group showing the strongest inhibition. After 24 hours, a decrease in inhibition

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was observed across all groups, and at this point, the positive control (chlorhexidine) demonstrated the highest inhibitory effect.

Table 2 shows that at 6 hours of incubation, a significant difference was observed only between the negative control group and the 20 µl *L. casei* supernatant group. At 12 hours, significant differences were observed among all treatment groups. By 24 hours, significant differences remained only between the negative control group and the 80 µl *L. casei* supernatant group.

DISCUSSION

The results of this study showed that the treatment group receiving *L. casei* supernatant at a volume of 80 µl had the widest inhibition zone diameter compared to the 20 µl and 50 µl supernatant groups. This finding is consistent with the study by Rico et al, which found that higher volumes of supernatant produced stronger antibacterial effects. This may be because a greater supernatant volume contains higher concentrations of accumulated antibacterial substances, thereby enhancing its ability to inhibit *P. gingivalis* growth.[5]

With regard to the different incubation times (6, 12, and 24 hours), the findings showed that the largest inhibition zones across all treatment groups occurred at 12 hours. Observations made after 6 hours indicated an increase in the antibacterial activity of *L. casei* supernatant, peaking at 12 hours, as reflected by the wider inhibition zones, but this effect diminished after 24 hours. This result contrasts with findings from another study, which reported that the largest inhibition zone was observed after only 6 hours of incubation, followed by a gradual decline over time.

The reduction in inhibition zone size after 12 hours in the current study can be explained by the findings of Gefen et al, who showed that after 12 hours, the antibiotic concentration at a certain distance from the disc could fall below the Minimum Inhibitory Concentration (MIC), allowing regrowth of tolerant bacteria. This suggests that the antibacterial activity of the supernatant may be neutralized by metabolic byproducts released by *P. gingivalis* during extended incubation. This explanation is supported by Tavares et al, who reported that *P. gingivalis* reaches its mid-exponential growth phase after 15 hours of incubation, indicating that bacterial proliferation continues as incubation time increases.[7,8]

The presence of inhibition zones resulting from *L. casei* supernatant treatment is likely due to the presence of antibacterial compounds, notably bacteriocins. Bacteriocins are known to disrupt the membrane integrity of *P. gingivalis* by interacting with lipid membranes in a non-specific manner. This interaction is most likely initiated by electrostatic attraction between the cationic bacteriocins and the anionic bacterial membrane, a mechanism similar to that of many other antimicrobial peptides.[9,10]

In addition to bacteriocins, *L. casei* supernatant contains organic acids, such as lactic acid and formic acid. These acids inhibit bacterial growth by acidifying the surrounding environment and lowering the internal pH of target bacteria, thereby disrupting bacterial metabolism. Lower extracellular pH levels cause a decrease in intracellular pH and hinder the active transport of protons, which consumes cellular ATP, ultimately leading to energy depletion in *P. gingivalis*. Lactic acid may also inhibit metabolism by increasing osmotic pressure in the medium. Furthermore, organic acids alter plasma membrane permeability and electrochemical gradients, impairing bacterial growth. In summary, these acids penetrate the bacterial membrane, release hydrogen ions, and lower pH levels, thereby impairing the survival of *P. gingivalis*.[11,12]

The *L. casei* supernatant also contains hydrogen peroxide (H_2O_2), which inactivates essential biomolecules in *P. gingivalis* through superoxide chain reactions and oxidation of thiocyanate, producing toxic oxidation byproducts. H_2O_2's oxidative action can damage bacterial cells by causing DNA degradation and oxidizing proteins and lipids. Additionally, H_2O_2 in the *L. casei* supernatant has been reported to impair *P. gingivalis* hemagglutination and Arg-gingipain enzyme activity.[13]

The potent inhibitory effect of H_2O_2 on *P. gingivalis* may stem from its oxidative properties. At low concentrations (≤ 3 mM), H_2O_2 can induce DNA fragmentation and protein/lipid oxidation in *P. gingivalis*. It is known that *L. johnsonii* NCC 533 produces ≤ 1 mM H_2O_2 under conditions comparable to this study (medium volume, bacterial density, incubation time), making it safe for human cells. However, at higher concentrations (≥ 30 mM), H_2O_2 can disrupt bacterial membranes without causing mutations or damage to host cells.[13,14]

Due to their amphiphilic nature, biosurfactants in the *L. casei* supernatant exhibit emulsifying properties that can interfere with the cell membrane integrity of *P. gingivalis*. Biosurfactants may interact with lipopeptides, leading to membrane disruption. Moreover, recent studies suggest that increased concentrations of biosurfactants can affect eukaryotic cells with low cytotoxicity, indicating their potential safety for topical application. Overall, using the whole supernatant may be more effective than isolating individual antibacterial components, as the various compounds present in *L. casei* supernatant may act synergistically to inhibit the growth of *P. gingivalis*.[15,16]

This study still has limitations, as it did not include testing for the Minimum Inhibitory Concentration (MIC), clinical applications, or safe dosage levels. Therefore, it is necessary to further develop *L. casei* supernatant as a topical therapeutic agent that can serve as an adjunctive treatment to scaling and root planning in the management of periodontitis. Additional research is required to assess the cytotoxicity of *L. casei* supernatant prior to its use in clinical settings, in order to identify any potential side effects.

CONCLUSION

The *L. casei* supernatant in this study demonstrated in vitro antibacterial activity in inhibiting the growth of *P. gingivalis*. Further research is required to determine the efficacy of *L. casei* supernatant in inhibiting *P. gingivalis* and to conduct cytotoxicity testing before it can be applied as a clinical treatment in the oral cavity. Such testing is essential to evaluate any adverse effects. In vivo studies are recommended as a follow-up to this research, to support the potential application of probiotics in periodontal therapy.

CONFLICT OF INTEREST

The authors declare no conflicts of interest related to this study.

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