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Research Paper

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Effectiveness of Probiotic *Lactobacillus reuteri* Lozenges in Reducing Anaerobic Bacterial Population in Gingival Crevicular Fluid After Scaling and Root Planing (Study on Chronic Periodontitis Patients at RSGMP UNSOED)

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Abstract

Background: Chronic periodontitis is a destructive periodontal disease that results periodontal inflammation caused by anaerobic bacteria at subgingival plaque and gingival crevicular fluid. Periodontal treatment is needed to eliminate periodontopathogenic bacteria with non-surgical treatment scaling & root planing and consume antimicrobial medicine as adjuvant therapy. Probiotics such as Lactobacillus reuteri can be used as adjuvant therapy because it can produce bacteriosin as antibacterial substances. **Objectives:** This study aims to determine the effectiveness of *L. reuteri* probiotic lozenges in reducing the population of anaerobic bacteria in the gingival crevicular fluid of chronic periodontitis patients after scaling & root planing. **Methods:** . This study was clinical experimental research with pre-test post-test & control group design. Gingival crevicular fluid sample got from 4 control group patients and 4 treatment group patients. Data formed by Total Plate Count (TPC) of anaerobic bacterial colonies in gingival crevicular fluid which were statistically analyzed using the Paired t-test and Unpaired t-test. Anaerobic bacterial colonies (10^5 CFU/mL) was decreased at 242.25 ± 10.1 in treatment group and 165.75 ± 52.6 in control group. **Key findings:** The result showed that there is a significant difference mean reduction number of anaerobic bacterial colonies between the treatment group and the control group ($p \leq 0.05$). **Conclusions:** Conclusion of this research was that scaling & root planing combine with consume probiotic Lactobacillus reuteri lozenges is effective in reduce the population of anaerobic bacteria that cause chronic periodontitis.

Keywords: Anaerobic bacteria, chronic periodontitis, gingival crevicular fluid, *Lactobacillus reuteri* probiotic, scaling and root planning

Introduction

Chronic periodontitis is an infectious destructive periodontal disease that causes inflammation of the tooth-supporting tissues, with disease progression that tends to be slow and is generally caused by pathogenic bacteria contained in the accumulation of subgingival microbial plaque (biofilm) and calculus. These pathogenic bacteria have mechanisms to induce the body's immune system in responding to inflammation, thereby causing damage to periodontal tissues. The pathogenic bacteria that cause chronic periodontitis are anaerobic bacteria, both gram-negative and gram-positive, which are commonly found in the subgingival plaque and gingival sulcus fluid of patients with periodontitis [2, 5, 15, 26, 58].

Types of anaerobic bacteria frequently found in chronic periodontitis are gram-negative obligate anaerobes, although gram-positive anaerobic bacteria may still be present. The types of gram-negative obligate anaerobic bacteria that may be found include *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia*, *Treponema denticola*, and *Fusobacterium nucleatum*. In addition, types of gram-positive anaerobic bacteria that may be found include *Peptostreptococcus micros* and species of *Eubacterium* [23, 33, 39].

Periodontal therapy in cases of chronic periodontitis needs to be carried out with the aim of eliminating periodontopathogenic bacteria and may also include controlling risk factors that cause chronic periodontitis in the patient. Treatments that may be given to patients with chronic periodontitis include non-surgical treatments that require patient motivation and cooperation throughout treatment. Non-surgical therapy can primarily be performed by providing scaling & root planing (SRP), supported by the consumption of medications that function as antimicrobial agents, such as antibiotics or chlorhexidine digluconate mouthwash. Scaling & root planing (SRP) is a non-surgical periodontal therapy considered the gold standard in the treatment of chronic periodontitis patients with the aim of helping periodontal tissue regeneration and eliminating the etiologic factors of the disease [43]. The consumption of antibiotics is also often used in patients with chronic periodontitis as an adjuvant therapy to non-surgical periodontal treatment; however, in research conducted by Astuti et al. (2017), the high level of antibiotic use for treating oral and dental diseases due to irrational and widespread usage can lead to

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the problem of antibiotic resistance. This may reduce drug effectiveness in treating infections [3].

Antibiotic consumption can also cause several side effects, such as nausea, diarrhea, burning sensations, gastrointestinal disturbances, and others [25, 41]. Therefore, several studies have emerged that consider the presence of alternative therapies in treating oral and dental diseases, including chronic periodontitis. One alternative therapy in treating oral diseases is the use of probiotic preparations, which have been shown to play a role in preventing the growth of microorganisms, thereby improving oral health status in patients [17]. Probiotics may be considered as an adjuvant therapy in periodontal treatment because they are known to have the ability to produce antibacterial substances to fight pathogenic bacteria, have the ability to enhance the protective function of the oral mucosa by acting as host-cell immunomodulators, and are proven safe to consume because they do not cause many specific side effects [41].

Probiotics are living microorganisms commonly referred to as good and healthy bacteria that can be obtained through foods, drinks, or supplements containing probiotics. Probiotics have various benefits for the body, including for oral health through their ability to control chronic oral diseases such as periodontitis, hyposalivation, and dental caries. One type of probiotic bacteria that is widely used as an alternative treatment is *Lactobacillus reuteri*. In a study conducted by Oinike et al. (2018), *Lactobacillus reuteri* probiotics in chewing-gum preparations were effective in increasing salivary pH and flow rate as an effort to prevent dental caries [36]. Research by Haryani et al. (2022) showed that *Lactobacillus reuteri* probiotics in lozenge form were proven effective in increasing salivary secretion as an effort to prevent periodontal disease [17]. Another study conducted by Naureen et al. (2022) in vitro on *Lactobacillus reuteri* showed that these bacteria were effective in inhibiting the activity of periodontopathogenic bacteria such as *P. gingivalis*, *P. intermedia*, and *F. nucleatem* [34].

Based on this discussion, the ability of *Lactobacillus reuteri* to inhibit periodontopathogenic bacterial activity in in-vitro studies, as well as its ability to prevent periodontal disease and dental caries, suggests that it may also have potential as an antimicrobial agent in chronic periodontitis cases. Research on the use of *Lactobacillus reuteri* probiotic lozenges as antimicrobial agents in patients with chronic periodontitis is still rarely found. Currently, *Lactobacillus reuteri* probiotic lozenges are widely consumed and used as additional supplements to maintain digestive tract function and oral health by regulating the balance of normal flora in the oral cavity. Studies that investigate more deeply the role of *Lactobacillus reuteri* probiotic lozenges on the gingival sulcus fluid (GSF) of chronic periodontitis patients as antimicrobial agents against periodontopathogenic bacteria are still limited. Therefore, the author is interested in conducting research on the effectiveness of *Lactobacillus reuteri* probiotic lozenges on reducing the population of anaerobic bacteria causing chronic periodontitis in gingival sulcus fluid (GSF) in patients with chronic periodontitis after scaling & root planing treatment.

Materials and Methods

This type of research is a clinical experimental study. The research was conducted in a planned manner on humans by providing a treatment or intervention to the research subjects, followed by examining and analyzing the effects of the research. The research design used is a pretest–posttest with control group design. This design involves two groups of subjects: the first group received treatment in the form of consuming *L. reuteri* probiotic lozenges after scaling and root planing treatment, while the second group served as the control group and only received scaling and root planing without the consumption of *L. reuteri* probiotic lozenges. The population in this study consisted of patients with chronic periodontitis at RSGMP Unsoed for a period of one month. The sample size was determined using the total sampling technique, meaning the sample size was equal to the population size during one month. The samples in this study were gingival sulcus fluid (GSF) from chronic periodontitis patients at RSGMP Unsoed who met the inclusion criteria. The data sources in this study were primary data on the number of anaerobic bacterial colonies in the gingival sulcus fluid of subjects in the control and treatment groups, obtained directly during the research process.

Tools and Materials

The tools used in this study included a diagnostic set (Schezher, Germany), WHO periodontal probe (Amma, Pakistan), ice box (Lion Star, Indonesia), 5 mL microtubes (OneMed, Indonesia), microtube rack, micropipette, petri dish (OneMed, Indonesia), test tubes (Iwaki Pyrex, Indonesia), incubator (Memmert, Germany), stopwatch, colony counter (Funke Gerber, Germany), vortex (Gemmy, Taiwan). The materials used in this research consisted of probiotic lozenges containing *Lactobacillus reuteri* DSM 17938 and *Lactobacillus reuteri* ATCC PTA 5289 (BioGaia Interlac Pro-D, Sweden), thioglycolate (HiMedia, India), TSA (Tryptone Soya Agar) (HiMedia, India) + 5% sheep blood, 70% alcohol (OneMed, Indonesia), sterile cotton rolls (OneMed, Indonesia), sterile gauze (Dwipa, Indonesia), cotton pellets (OneMed, Indonesia), distilled water, and sterile paper points size 20 (GapaDent, China).

Research Procedure

This study began with submitting an ethical clearance application to the Ethics Committee of the Faculty of Medicine, Jenderal Soedirman University. Patients who met the inclusion criteria and had completed scaling and root planing treatment at RSGMP Unsoed were asked for their willingness to participate in the study. Patients who agreed were provided with a full explanation of the research procedures to be carried out, and then were asked to complete and sign the informed consent form. Allocation of subjects into the treatment and control groups was done randomly using the opaque sealed envelope method for the first research subject, in which the researcher provided a sealed, non-transparent envelope containing the treatment group code (SRP + *L. reuteri* probiotic lozenges) and the control group code (SRP only), then the patient was asked to choose the envelope [21]. The first research subject received an envelope containing the treatment group code; therefore, the second subject was

assigned to the control group, the third subject to the treatment group, and so on for the remaining subjects. All patients were also given education on proper tooth-brushing techniques using the modified Stillman method because it requires simple movements, is efficient, and can reach all tooth surfaces. All patients underwent the first sampling of gingival sulcus fluid after scaling and root planing. The treatment group patients were instructed to consume the probiotic lozenges twice daily after brushing their teeth in the morning and evening for 7 days. All patients underwent the second sampling 7 days after treatment.

The research continued with laboratory procedures, namely bacterial isolation using four-level dilution through the serial dilution method with distilled water, followed by bacterial inoculation on TSA + 5% sheep blood medium using the spread plate method with candle jar incubation. Next, identification of anaerobic bacteria was carried out by macroscopic observation of samples in thioglycolate medium, gram staining, macroscopic observation, and counting the number of anaerobic bacterial colonies. Macroscopic observation of samples in thioglycolate medium is one of the identification tests used to determine the types of bacteria that grow in the medium. Samples in Eppendorf tubes containing thioglycolate medium were incubated for 7 days to allow bacterial growth. Gram staining was conducted as an identification test to determine whether bacteria in the sample were gram-positive or gram-negative. A red color from gram staining indicates gram-negative bacteria, whereas a bluish-purple color indicates gram-positive bacteria. Colony counting was performed using a colony counter with the total plate count method expressed in Colony Forming Units (CFU/mL). Bacteria were inoculated using the spread plate technique. In the pre-research stage, inoculation was carried out using six dilution levels, and only the fourth dilution produced a colony countable according to the required calculation criteria. Therefore, the number of bacterial colonies in this study was obtained from the fourth dilution, and the calculation formula used was:

Number of colonies = (Counted colony number) / (0.1 mL × 10^(-4)) = …… CFU/mL

Data Analysis

Data analysis was performed using SPSS version 22. Univariate analysis was performed descriptively to describe the results of bacterial identification through macroscopic observation of samples on thioglycolate medium, gram staining, and microscopic observation. Bivariate analysis was conducted using the Shapiro–Wilk normality test because the sample size was <50, and the homogeneity test using Levene's test. The paired t-test was used for related samples to observe differences before and after treatment. The unpaired t-test was used to compare the reduction in total anaerobic bacterial colonies between the control and treatment groups.

Results and Discussion

The research subjects were obtained from chronic periodontitis patients at the General Service Unit (UPU) of RSGMP Unsoed who met the subject criteria during a period of 1 month. The ages of the research subjects ranged from 45–61 years, with the mean age of the control group being higher than the mean age of the treatment group. The clinical appearance of all research subjects showed chronic periodontitis conditions in accordance with the inclusion criteria of this study, characterized by alveolar bone resorption on periapical radiographs, the presence of calculus, and gingival recession, as shown in Figures 1 and 2. The isolates of gingival sulcus fluid samples in each Eppendorf tube were incubated for 7 days after bacterial inoculation with the aim of allowing bacteria in the thioglycolate transport medium to grow and to identify the type of bacteria based on the turbidity location within the thioglycolate medium.

The incubation results of all isolates are shown in Figure 3. The isolates of the control and treatment groups on day 0 in Figure 3(A) and 3(C) showed turbidity in the lower 2/3 of the thioglycolate medium, indicating that the bacteria present were obligate anaerobes. The incubation results of samples on day 8 from both groups, shown in Figure 3(B) and 3(D), exhibited turbidity throughout the entire medium and appeared most turbid in the upper 2/3, indicating the presence of facultative anaerobes and obligate anaerobes. Gram staining was carried out on colonies grown on TSA + 5% sheep blood medium to identify whether the bacteria in the samples were gram-negative or gram-positive. The addition of sheep blood to the TSA medium was done to support the growth of anaerobic bacteria by providing good nutrients and to determine hemolytic properties [50]. Gram staining was carried out on the colonies that grew most abundantly on each TSA + 5% sheep blood petri dish. The colonies that grew in all samples were black and gray, so gram staining was performed on colonies of both colors.

Figure 4 shows that the black and gray colonies on TSA + 5% sheep blood exhibited hemolysis. All isolates grown on this medium showed that the color of the blood agar was not as red as before inoculation, indicating that the bacteria exhibited hemolytic activity. All isolates demonstrated total hemolysis or beta-hemolysis, therefore the bacteria present were pathogenic. Bacterial preparations from gram staining were observed microscopically under 100x magnification to examine bacterial morphology. Microscopic images are shown in Figure 5. Preparations containing black-pigmented colonies in Figure 5 showed gram-negative bacteria with a streptococcobacillus shape. Preparations containing gray-pigmented colonies showed gram-negative bacilli.

After 7 days of treatment, both groups experienced a reduction in the number of anaerobic colonies. The paired t-test comparing day 0 and day 8 colony counts showed significant differences in both groups ($p = 0.000$, $p \leq 0.05$). A comparison of the reduction in colony counts between the control and treatment groups was analyzed using the unpaired t-test to determine whether the reduction differed significantly. The results are shown in **Table 1**.

Table 1 shows that the mean reduction in bacterial colonies was greater in the treatment group than in the control group. The unpaired t-test between the reductions in both groups showed a significant difference ($p \leq 0.05$). This indicates that scaling and root planing combined with the consumption of *Lactobacillus reuteri* probiotic lozenges is more effective than scaling and root planing

alone in reducing anaerobic bacterial counts in chronic periodontitis. Isolates on thioglycolate medium incubated for 7 days showed that all tubes contained obligate anaerobes indicated by turbidity at the bottom of the tube. Additionally, 16 sample isolates contained facultative anaerobes due to turbidity in the lower 2/3 of 8 isolates before treatment and turbidity in the upper 2/3 of 8 isolates after treatment. This is consistent with studies identifying bacterial types in chronic periodontitis by Benachinmardi et al. (2015), who reported that 91.74% of bacteria isolated from 121 chronic periodontitis samples were anaerobic [6]. Jindal et al. (2019) also stated that bacteria in chronic periodontitis are dominated by obligate anaerobes followed by facultative anaerobes. The low oxygen level in the gingival sulcus provides a favorable environment for anaerobic bacterial growth [23].

Bacterial growth on TSA + 5% sheep blood appeared as predominantly black and gray colonies. This is consistent with Furoida et al. (2014), who observed that black and gray colonies dominated gingival sulcus samples of chronic periodontitis patients. Hayati et al. (2023) also stated that black-pigmented colonies form when grown on blood agar and that the black pigment derives from hemin, the final product of bacterial metabolism [18]. Tryptone Soy Agar (TSA) contains nutrients such as vitamins, minerals, and nitrogen supporting anaerobic bacterial growth [4]. The addition of 5% sheep blood helps support the growth of specific anaerobes, including periodontopathogens, and allows determination of hemolytic activity. Total hemolysis (beta-hemolysis) indicates pathogenic bacteria. Samples were also incubated using the candle jar method. The candle jar method was used to select for anaerobes. A lit candle inside a sealed jar reduces oxygen and releases carbon dioxide, creating anaerobic conditions that allow only anaerobes to grow [12].

Gram staining involved applying crystal violet, iodine, alcohol, and safranin. Structural differences in bacterial cell walls influence staining results. In this study, black and gray colony preparations stained red, indicating gram-negative bacteria. This occurs because gram-negative cell walls contain thick lipid layers causing crystal violet to wash out with alcohol. In contrast, gram-positive bacteria have thick peptidoglycan layers causing crystal violet retention [32]. Widodo et al. (2014) support this finding, showing that gram-negative bacteria are the most common bacteria isolated from gingival sulcus fluid of chronic periodontitis patients [58]. Newman et al. (2019) note that black-pigmented anaerobic gram-negative colonies are characteristic of red complex bacteria, suggesting possible *Treponema denticola*, *Porphyromonas gingivalis*, or *Tannerella forsythia* [35].

Microscopic images showed that black-pigmented colonies resembled gram-negative coccobacilli, consistent with Widodo et al. (2014), who reported that 58.82% of black-pigmented colonies were coccobacilli [58]. According to Reddy (2018), these may include *P. gingivalis*, *P. intermedia*, and *A. actinomycetemcomitans* [42]. Gray-pigmented colonies appeared as gram-negative bacilli, consistent with Widodo et al. (2014) and Lindawati et al. (2018), who noted that gray-pigmented bacilli may include *Fusobacterium nucleatum* and *Capnocytophaga* species [30, 58].

In the control group, scaling and root planing alone significantly reduced anaerobic bacterial colony counts. Scaling and root planing is the gold standard for chronic periodontitis, aiming to remove etiologic factors and thus reducing pathogenic bacteria [40]. Isaac et al. (2015) and Tekce et al. (2015) similarly observed significant reductions in bacterial loads following SRP [22, 52]. Reductions in anaerobic bacteria after SRP are associated with improved clinical parameters such as PD, BOP, PI, and GI [52]. Periodontopathogens in chronic periodontitis trigger inflammation, increasing sulcus fluid volume, bleeding, and inflammatory mediator release. SRP eliminates these pathogens, allowing tissue recovery and normalization of sulcus fluid volume [42].

Healthy gingival epithelial cells after SRP produce antimicrobial peptides (HBDs) active against key pathogens such as *A. actinomycetemcomitans*, *F. nucleatum*, *P. gingivalis*, *P. micros*, *S. mutans*, *S. sanguis*, and *Candida spp.* [16, 60]. Brushing technique also influences bacterial load. All subjects were educated to brush twice daily with the Stillman technique. Rahmawati et al. (2019) showed that toothbrushing with toothpaste reduces *P. gingivalis* levels. Stillman brushing effectively removes plaque across all tooth surfaces [31, 40].

The treatment group, receiving SRP + *L. reuteri* lozenges for 7 days, showed significantly greater reductions than the control group. This indicates that *L. reuteri* lozenges are an effective adjuvant therapy. Hadzic et al. (2021) showed similar findings, with significant reductions in *A. actinomycetemcomitans* and *P. gingivalis* after 40 days of *L. reuteri* lozenges [15]. Widyarman et al. (2018) also found significant reductions in *P. gingivalis*, *A. actinomycetemcomitans*, and *S. mutans* after 2 weeks [59]. *L. reuteri* produces antimicrobial organic acids, ethanol, and bacteriocins such as reuterin and reutericyclin, which inhibit pathogenic bacteria. *L. reuteri* also adheres strongly to host tissues, preventing pathogenic attachment [58]. Additionally, *L. reuteri* has strong immunomodulatory and anti-inflammatory properties, enhancing the host immune response and reducing pro-inflammatory cytokines, promoting periodontal tissue regeneration [44]. The limitation of this study was the small number of subjects because not all UPU RSGMP Unsoed patients met the inclusion criteria.

Conclusion

Based on this study, it can be concluded that *Lactobacillus reuteri* probiotic lozenges are effective in reducing anaerobic bacterial populations causing chronic periodontitis in gingival sulcus fluid (GSF) of chronic periodontitis patients after scaling and root planing. In addition, there was a significantly greater reduction in the mean anaerobic bacterial colony count in patients who consumed *Lactobacillus reuteri* lozenges after SRP (242.2 ± 10.1) compared with the control group (165.7 ± 52.6).

Supplementary Material

None

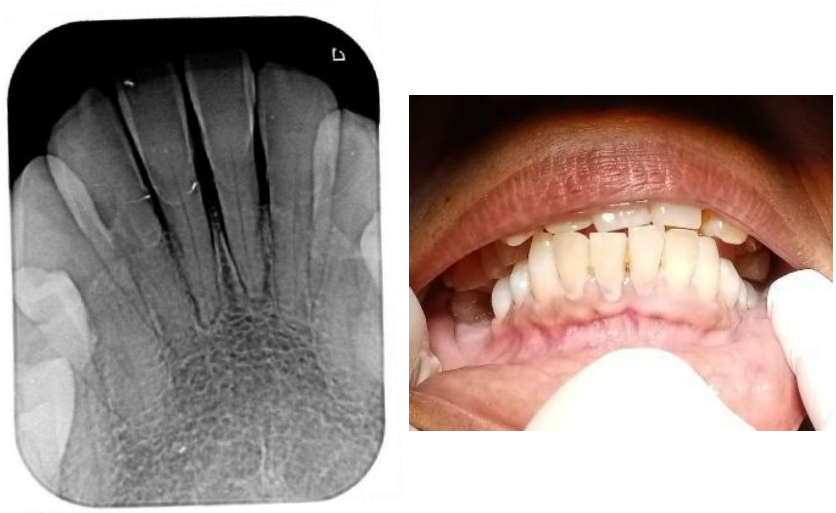

Figure 1. Clinical appearance of control group patients before SRP. (A) Periapical radiograph showing alveolar bone resorption; (B) Intraoral image showing calculus and gingival recession.

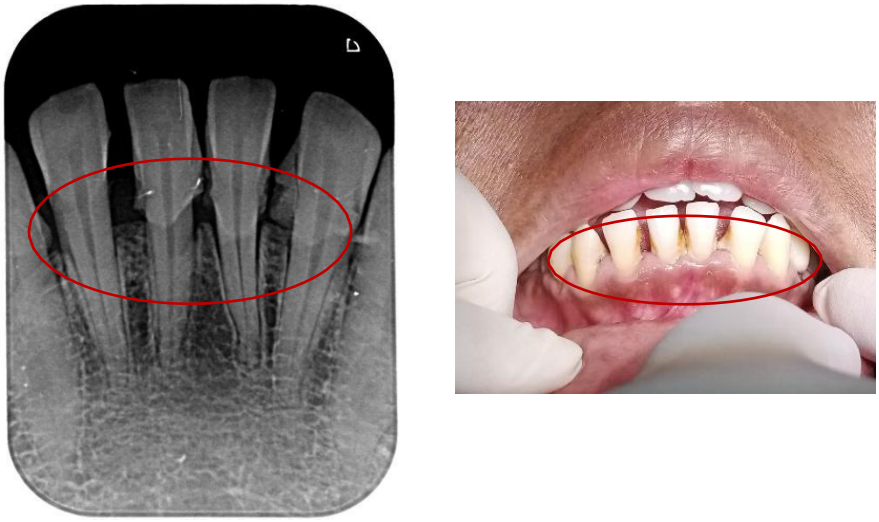

Figure 2. Clinical appearance of treatment group patients before SRP. (A) Periapical radiograph showing alveolar bone resorption; (B) Intraoral image showing calculus and gingival recession.

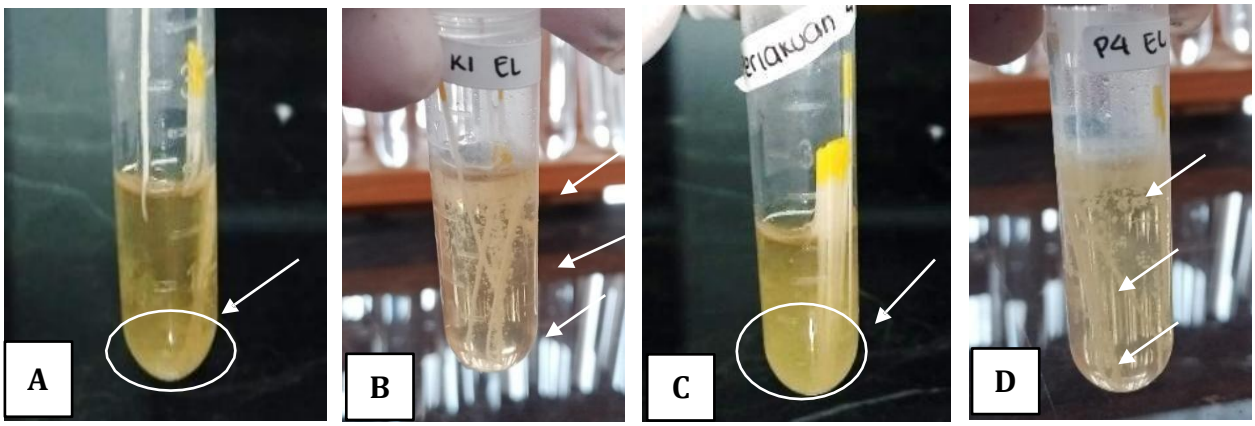

Figure 3. Isolates on thioglycolate medium. (A) Control group isolate on day 0; (B) Control group isolate on day 8; (C) Treatment group isolate on day 0; (D) Treatment group isolate on day 8.

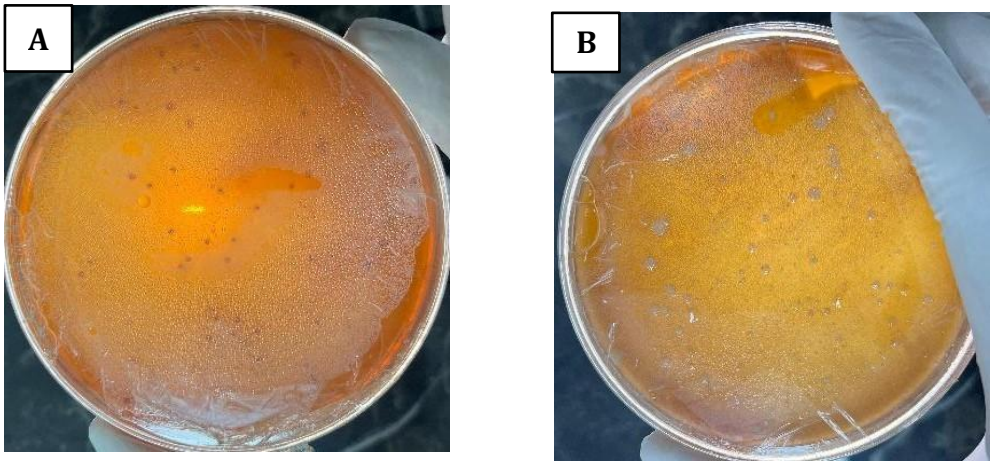

Figure 4. Morphology of colonies on TSA + 5% sheep blood. (A) Black colonies with hemolysis; (B) Gray colonies with hemolysis.

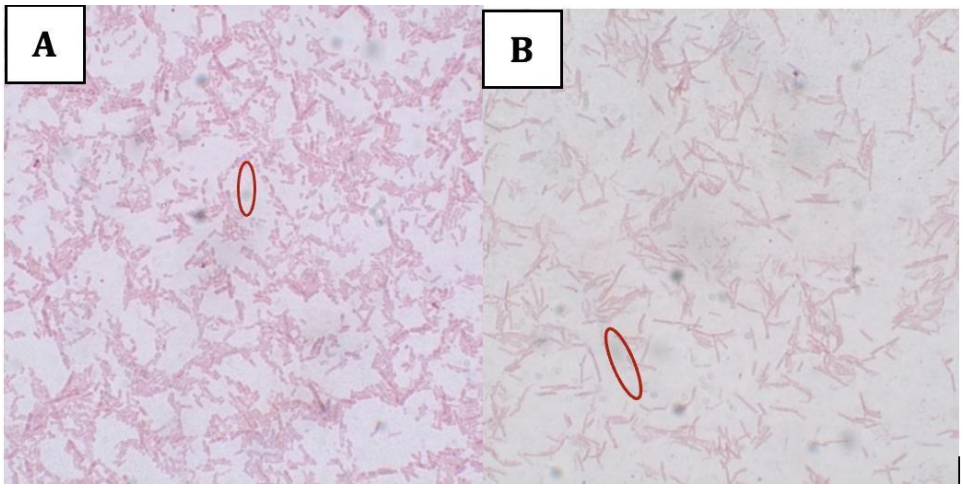

Figure 5. Cell morphology in Gram staining (100x magnification). (A) Black colony: gram-negative streptococcobacillus; (B) Gray colony: gram-negative bacillus.

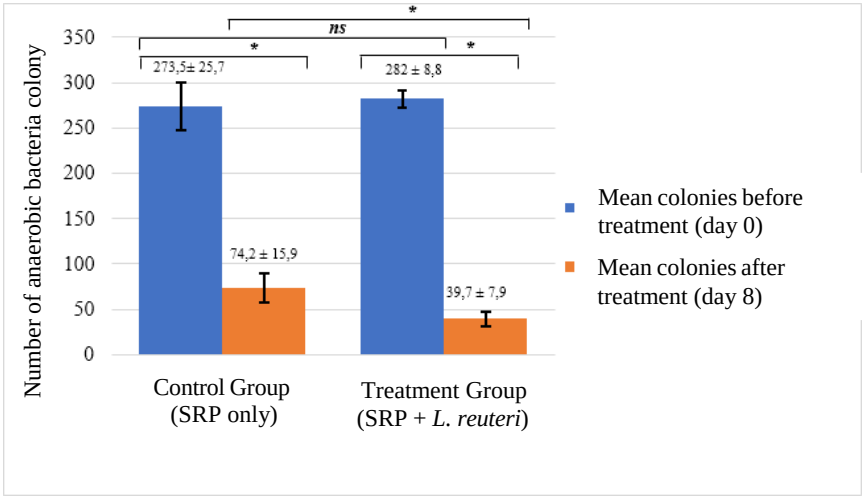

Figure 6 shows that the mean number of anaerobic bacterial colonies in both groups before treatment was higher than after treatment. The results of the unpaired t-test on day 0 colony counts between the control and treatment groups showed no significant difference ($p = 0.566$, $p > 0.05$).

Tabel 1 The comparison of reduction in bacterial colonies

Groups	n	Mean number of colonies (CFU/mL x 10^5) ± SD		Mean reduction bacterial colonies (CFU/mL x 10^5) ± SD	Unpaired t-test ($p \leq 0,05$)
		Day 0	Day 8		
Control	4	273,7 ± 25,7	74,2 ± 15,9	165,7 ± 52,6	0,029
Treatment	4	282,0 ± 8,8	39,7 ± 7,9	242,2 ± 10,1	

Author Contributions

FTMS : Conceptualization, Methodology, Writing-Original Draft. **TSS** : Data Curation, Formal Analysis, Visualization. **IC** : Supervision, Funding Acquisition, Writing- Review & Editing.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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