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The Effect Of Hydrogen Peroxide (H_2O_2) in Yoghurt (*Lactobacillus bulgaricus* and *Streptococcus thermophilus*) on Inhibiting the Growth of *Streptococcus pyogenes*

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ABSTRACT

Yoghurt is a food product made from milk fermented with lactic acid bacteria (LAB), which provides various health benefits, including enhancing immunity through the production of lactic acid, hydrogen peroxide, diacetyl, and bacteriocins. Several studies have reported that LAB-fermented yoghurt can inhibit the growth of pathogenic bacteria. Streptococcus pyogenes is a gram-positive bacterium that forms chain-like colonies, is small, round in shape, has a smooth surface, and produces grayish-white pigmentation on blood agar. S. pyogenes can cause respiratory infections such as pharyngitis, and skin infections such as impetigo, cellulitis, and erysipelas. This study aimed to investigate the role of hydrogen peroxide in yoghurt (L. bulgaricus and S. thermophilus) in inhibiting the growth of S. pyogenes. This was an experimental study using a post-test-only control group design. Bacterial colony counts were performed using a colony counter. A total of 60 samples were used, with three repetitions, two dilutions, and ten concentration groups (0%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%), with 0% serving as the control. Data were analyzed using the Kruskal-Wallis test, followed by the Spearman Rho test. The results showed that yoghurt inhibited the growth of S. pyogenes, and the 30% yoghurt concentration, containing 0.32 mmol/L hydrogen peroxide, was the minimum inhibitory concentration. Kruskal-Wallis analysis showed significant differences among groups (p<0.05), and the Spearman Rho test indicated a significant correlation (p<0.05) between the variables.

1. INTRODUCTION

Yoghurt is a food product made from milk fermented with the bacteria *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, which provides various health benefits, such as boosting immunity, improving digestion, reducing cholesterol levels, and promoting skin health (Handayani et al., 2017). The fermentation process results in yoghurt possessing antimicrobial properties such as lactic acid, diacetyl, bacteriocins, and hydrogen peroxide (Wigati et al., 2019).

One of the antimicrobial substances produced during yoghurt fermentation is hydrogen peroxide. Hydrogen peroxide has the ability to kill bacteria by damaging functional biomolecular groups in cells, inhibiting bacterial nucleic acid synthesis, and denaturing proteins. In a study by Huda (2013), which examined the role of hydrogen peroxide in inhibiting the growth of *Staphylococcus aureus and Escherichia coli* in honey, it was shown that honey concentrations of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100% could inhibit bacterial growth, as observed by the size of the inhibition zone.

Streptococcus pyogenes is a Group A *Streptococcus* bacterium that causes various diseases such as skin infections, pharyngitis, rheumatic fever, acute glomerulonephritis, and others (Brown & Smith, 2015). *S. pyogenes* is a gram-positive, cocci-shaped bacterium that forms chains. It is a facultative anaerobe and grows optimally at 37°C. Group A *Streptococcus* bacteria form beta-hemolytic zones and have capsules composed of hyaluronic acid. These capsules inhibit phagocytosis and the deposition of C3b, making it necessary to use medications to dissolve the bacterial capsule so that phagocytic cells can function and fight the bacteria (Brigitta et al., 2021).

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S. pyogenes produces toxins such as *streptokinase, deoxyribonucleases, hyaluronidase, pyrogenic exotoxins*, and *hemolysins*, which can attack proteins that bind to cells (Mahmudah & Hamzah, 2014).

The current standard treatment for infectious diseases is the use of antibiotics. However, eradication remains a challenge, especially due to the emergence of resistance. One cause of resistance is inadequate or improper therapy (Wikananda et al., 2019). Therefore, it is essential to seek alternative preventive strategies to avoid complications that could threaten patients' lives. Additionally, antibiotic resistance can lead to increased healthcare costs due to prolonged hospital stays (Desrini, 2015). Natural substances with minimal side effects and a lower risk of resistance are considered safer alternatives (Setiawati, 2015). The effect of hydrogen peroxide generated by lactic acid bacteria in yogurt has been the subject of limited research. This has prompted the authors to further explore the role of hydrogen peroxide in yoghurt in inhibiting the growth of *S. pyogenes*.

2. METHOD

This study falls under the category of True Experimental Design using the posttest-only with control group design. This method is used to observe the inhibitory effect of lactic acid bacteria in yoghurt on *Streptococcus pyogenes* and to compare its effects across different yoghurt concentrations. To compare the effectiveness of various yoghurt concentrations in inhibiting the growth of *Streptococcus pyogenes*, the following concentrations were tested: 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, along with a negative control (0% concentration), which only contained distilled water.

The bacterial sensitivity test against antibacterial agents was conducted in vitro using the liquid dilution method. This method involves serial dilutions to reduce the number of bacteria suspended in the liquid. Its purpose is to determine how much antibacterial agent is required to inhibit the tested bacteria (Brooks et al., 2012). Milk was mixed with two starter cultures of *Lactic Acid Bacteria* and incubated at 37°C for 24 hours to produce yoghurt.

The data analysis in this study was conducted using univariate and bivariate analyses. Univariate analysis was used to identify and describe the characteristics of the dependent variable, namely the growth of *Streptococcus pyogenes*, across yoghurt concentrations of 0%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, and 45%, with the assistance of IBM SPSS Statistics version 22. Bivariate analysis was used to assess data normality using the *Shapiro-Wilk test*, due to the sample size being fewer than 50. Homogeneity of variance was evaluated using *Levene's Test*. The data obtained were analyzed using the non-parametric Kruskal–Wallis test to determine which groups showed significant differences in the variable. This was followed by the *Spearman Rho test* to determine whether there was a correlation between the variables.

3. RESULT AND DISCUSSION

This study measured the inhibitory effect of yoghurt on the growth of *S. pyogenes* after treatment. The inhibition percentages at concentrations of 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, and 45% were recorded in Table 1 as follows: 95.07%, 96.22%, 96.79%, 97.99%, 98.04%, 99.71%, 99.72%, 99.93%, and 99.96%, respectively. These results indicate that higher concentrations of yogurt correspond to greater inhibition of *S. pyogenes*. This suggests that, in addition to lactic acid, other components present in yogurt contribute significantly to the inhibition of *S. pyogenes* growth.

The average number of *S. pyogenes* colonies in the treatment group with 0% yoghurt concentration was 531.6 x 10^3 CFU/mL. This average was higher compared to the colony counts at 5% (26.2 x 10^3 CFU/mL), 10% (20.1 x 10^3 CFU/mL), 15% (17 x 10^3 CFU/mL), 20% (10.7 x 10^3 CFU/mL), 25% (10.4 x 10^3 CFU/mL), 30% (1.5 x 10^3 CFU/mL), 35% (1.4 x 10^3 CFU/mL), 40% (0.3 x 10^3 CFU/mL), and 45% (0.2 x 10^3 CFU/mL).

Table 1. Percentage of Yoghurt Inhibition against *S. pyogenes*

Group	∑ Sample	Average (CFU/ml)	% Inhibition
K1 (0%)	3	531,6 x 10^3	0
K2 (5%)	3	26,2 x 10^3	95,07
K3 (10%)	3	20,1 x 10^3	96,22
K4 (15%)	3	17 x 10^3	96,79
K5 (20%)	3	10,7 x 10^3	97,99
K6 (25%)	3	10,4 x 10^3	98,04
K7 (30%)	3	1,5 x 10^3	99,71
K8 (35%)	3	1,4 x 10^3	99,72
K9 (40%)	3	0,3 x 10^3	99,93
K10 (45%)	3	0,2 x 10^3	99,96

The inhibition data in Table 1. shows that increasing yoghurt concentration correlates with a decrease in the average growth of *S. pyogenes*. The results of the permanganometric titration are presented in Table 2. Hydrogen peroxide levels in yoghurt were measured using the permanganometric titration method by mixing $KMnO_4$ reagent into the yoghurt. The measured concentrations of hydrogen peroxide were as follows: K1 0% (0.15 mmol/L), K2 5% (0.17 mmol/L), K3 10% (0.22 mmol/L), K4 15% (0.22 mmol/L), K5 20% (0.27 mmol/L), K6 25% (0.32 mmol/L), K7 30% (0.32 mmol/L), K8 35% (0.33 mmol/L), K9 40% (0.35 mmol/L), and K10 45% (0.35 mmol/L). These findings indicate that yoghurt contains measurable levels of hydrogen peroxide, with the highest concentrations found at 40% and 45% yoghurt.

The hydrogen peroxide levels assessed through permanganometric testing in this study demonstrate that as yogurt concentration increases, H_2O_2 levels also rise. This phenomenon can be attributed to *L. bulgaricus* being a microaerophilic bacterium, which thrives in the presence of oxygen but not in excessive quantities. Excess oxygen is converted into hydrogen peroxide through oxidation-reduction reactions facilitated by enzymes such as pyridine nucleotide oxidase, pyruvate oxidase, and α-glycerophosphate oxidase (Oktavia *et al.*, 2015).

Table 2. Results of Permanganometric Titration Test

Yoghurt Concentration	**Hydrogen Peroxide**
0%	0 mmol/L
5%	0,17 mmol/L
10%	0,22 mmol/L
15%	0,22 mmol/L
20%	0,27 mmol/L
25%	0,32 mmol/L
30%	0,32 mmol/L
35%	0,33 mmol/L
40%	0,35 mmol/L
45%	0,35 mmol/L

The diagram illustrating the percentage inhibition of *S. pyogenes* and the hydrogen peroxide levels at various yoghurt concentrations can be seen in Figure 1.

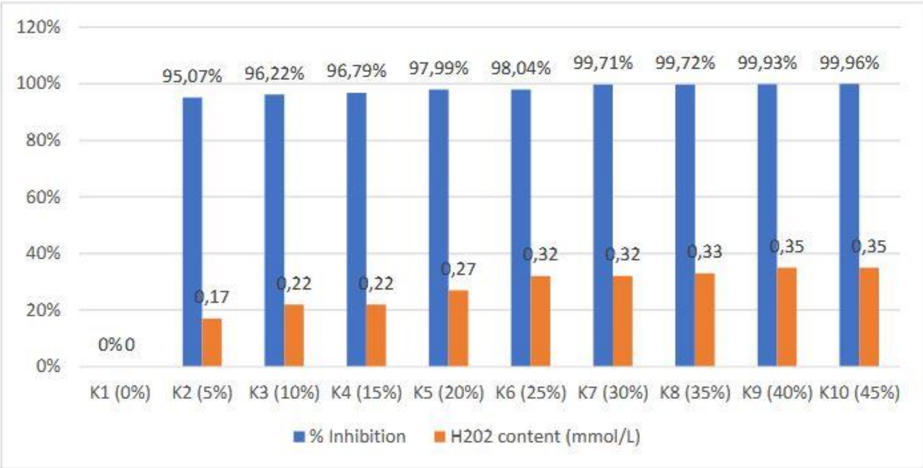

Figure 1. Diagram of the Percentage Inhibition of *S. pyogenes* and Hydrogen Peroxide Levels at Each Yoghurt Concentration

The pH measurement results using a pH meter showed that the pH value of 0% yoghurt concentration was 6.8, followed by 5% (5.7), 10% (5.7), 15% (5.6), 20% (5.6), 25% (5.7), 30% (5.6), 35% (5.7), 40% (5.7), and 45% (5.7). The pH meter test results are presented in Table 3.

Table 3. pH Values of Yoghurt at Each Concentration

Yoghurt Concentration	**pH Value**
0%	6,8
5%	5,7
10%	5,7
15%	5,6
20%	5,6
25%	5,7
30%	5,6
35%	5,7
40%	5,7
45%	5,7

Lactic acid bacteria (LAB) like *L. bulgaricus* convert lactose into lactic acid and are classified as homofermentative, producing both lactic acid and ethanol, typically maintaining a pH around 5.5. The production of lactic acid contributes to increased acidity and a corresponding decrease in pH (Hidayat et al., 2013). The bacteria utilized in yogurt production include lactic acid-producing strains such as *Lactobacilli* and *S. thermophilus*. The combination of *L. bulgaricus* and *S. thermophilus* (double strain) is commonly used in yogurt manufacturing, promoting symbiotic growth that results in higher acid production compared to using a single strain (Rahman et al., 2019). This underscores the role of lactic acid as a metabolic byproduct of LAB, contributing to acidification and lowering pH levels.

The collected data were then analyzed using SPSS. The bacterial inhibition data were described and tested for normality using the *Shapiro-Wilk* method and for homogeneity using *Levene's Test.* The results showed that the data were not normally distributed ($p<0.05$) and not homogeneous ($p<0.05$). Therefore, a non-parametric *Kruskal-Wallis test* was performed to determine significant differences between groups, with significance considered at $p<0.05$.

The *Spearman Rho test* showed significant differences between groups when $p<0.05$, and each variable was tested accordingly. From the *Spearman Rho test* results comparing the control group K1 (0%) to other groups, p-values were as follows: $p=0.04$ for K7 (30%) and K8 (35%), and $p=0.01$ for K9 (40%) and K10 (45%). These results indicate that K7 (30%), K8 (35%), K9 (40%), and K10 (45%) had significant differences compared to the control group K1 (0%), suggesting that these concentrations are ideal for inhibiting *S. pyogenes*.

The *Spearman Rho test* results, shown in Figure 2, were used to assess the presence of significant correlations ($p<0.05$) between variables. The analysis revealed a significant relationship between hydrogen peroxide (H_2O_2) levels and both bacterial inhibition and colony counts. Inhibition was significantly correlated with both H_2O_2 levels and colony counts. Colony counts were also significantly correlated with H_2O_2 levels and inhibition. However, pH was not found to be significantly related to H_2O_2 levels, inhibition, or colony counts.

Table 4. Correlation Between *S. pyogenes* Growth Inhibition, Hydrogen Peroxide Levels, and pH

Correlations

			h2o2	Hambatan	Koloni	pH
Spearman's rho	h2o2	Correlation Coefficient	1.000	.891**	-.882**	-.126
		Sig. (2-tailed)	.	.000	.000	.507
		N	30	30	30	30
	Hambatan	Correlation Coefficient	.891**	1.000	-.996**	-.214
		Sig. (2-tailed)	.000	.	.000	.256
		N	30	30	30	30
	Koloni	Correlation Coefficient	-.882**	-.996**	1.000	.191
		Sig. (2-tailed)	.000	.000	.	.312
		N	30	30	30	30
	pH	Correlation Coefficient	-.126	-.214	.191	1.000
		Sig. (2-tailed)	.507	.256	.312	.
		N	30	30	30	30

**. Correlation is significant at the 0.01 level (2-tailed).

The hydrogen peroxide measured and produced by *Lactic Acid Bacteria* (*L. bulgaricus* and *S. thermophilus*) in this study is a strong oxidizing agent that can kill or inhibit bacterial growth and is often used as a disinfectant. Hydrogen peroxide is characterized by a higher viscosity compared to water, is colorless, and in concentrated form has an acidic odor. It works by denaturing proteins and inhibiting protein synthesis or interfering with the function of bacterial nucleic acids, leading to cell wall damage (Huda, 2013). Another effect of hydrogen peroxide is oxidative damage to bacterial cells, particularly the sulfhydryl groups of cellular proteins, which denature several enzymes and lead to lipid peroxidation and increased membrane permeability (Surono, 2016).

Hydrogen peroxide can also generate free radicals such as hydroxyl and superoxide, which can attack vital cellular components including lipids, proteins, and even bacterial DNA. Furthermore, it can decompose into oxygen, which itself contributes to bacterial cell death. Hydrogen peroxide and other antimicrobial compounds are capable of penetrating into the cytoplasm (Utami et al., 2020). Based on the above discussion, it is evident that hydrogen peroxide plays a role in inhibiting the growth of *S. pyogenes*.

One limitation of this study is that lactic acid levels were only inferred through pH reduction, and therefore the exact concentration of lactic acid could not be determined, as it may have been mixed with other organic acids formed during fermentation. Another factor that could contribute to bacterial inhibition, aside from lactic acid and hydrogen peroxide (H2O2), is the presence of bacteriocins produced during the yoghurt fermentation process. Thus, further research is needed to explore the effects of bacteriocins on the growth of *S. pyogenes*.

4. CONCLUSION

Lactic Acid Bacteria (L. bulgaricus and S. thermophilus) in yoghurt at concentrations of 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, and 45% can inhibit the growth of *S. pyogenes*. The minimum concentration of yoghurt required to inhibit the growth of *S. pyogenes* is 30%, as this concentration showed a significant reduction in *S. pyogenes* growth by 99.71%. The higher the yoghurt concentration, the higher the level of H_2O_2. As H_2O_2 levels increase, the inhibitory effect on *S. pyogenes* becomes greater. Further research was conducted on other compounds such as *Bacteriocins* in yogurt for their role in inhibiting bacterial growth

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