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The Role of Hydrogen Peroxide (H_2O_2) in Yogurt (*Lactobacillus bulgaricus* and *Streptococcus thermophilus*) on the Growth of *Staphylococcus aureus* ATCC 29213

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ABSTRAK

Yoghurt merupakan produk pangan susu fermentasi dengan bantuan bakteri asam laktat (BAL). BAL merupakan bakteri yang dapat menghasilkan metabolit asam laktat, bakteriosin dan hidrogen peroksida. Beberapa penelitian menyampaikan BAL dalam yoghurt memiliki kemampuan menghambat pertumbuhan bakteri patogen. *S. aureus ATCC 29213* adalah Gram positif, *coccus* yang tersusun tidak beraturan berkelompok seperti buah anggur. *S. aureus ATCC 29213* adalah bakteri patogen utama manusia. Penelitian ini bertujuan untuk mengetahui peranan hidrogen peroksida pada yoghurt (*L. bulgaricus* dan *S. thermophilus*) terhadap pertumbuhan *S. aureus ATCC 29213*. Penelitian ini merupakan penelitian eksperimental dengan *post-test only with control group design.* Jumlah sampel yang digunakan sebanyak 60 sampel dengan dua kali pengulangan dan terbagi dalam 10 kelompok konsentrasi (0%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%) dengan 0% sebagai kontrol negative. Analisis penelitian ini menggunakan *Kruskal Wallis* (p=0,003). Hasil ini menunjukkan bahwa terdapat perbedaan signifikan antar kelompok. Kemudian dilanjutkan uji *Mann Whitney* untuk mengetahui perbandingan masing-masing kelompok. Penelitian ini dapat disimpulkan terdapat penghambatan pertumbuhan *S. aureus ATCC 29213* oleh yoghurt dengan konsentrasi 5%, kandungan hidrogen peroksida 0,17 mmol/L, pH 5,7 sebagai konsentrasi yoghurt terendah.

ABSTRACT

Yogurt is a fermented milk product created through the action of lactic acid bacteria (LAB). These bacteria can produce various metabolites, including lactic acid, bacteriocins, and hydrogen peroxide. Research has demonstrated that LAB in yogurt can effectively inhibit the growth of pathogenic bacteria. Staphylococcus aureus ATCC 29213, a Gram-positive bacterium, is known for forming irregular clusters resembling grapes and is a significant human pathogen. This study aims to investigate the impact the hydrogen peroxide produced in yogurt (specifically from L. bulgaricus and S. thermophilus) on the growth of S. aureus ATCC 29213. The research employed an experimental design involving a post-test with a control group. a total of 60 samples were utilized, divided into 10 concentration groups (0%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%), with 0 % serving as the negative control. Statistical analysis was performed using the Kruskal Wallis test (p=0.003), which indicated significant differences among the groups. Subsequent Mann-Whitney tests were conducted to compare the individual groups. The findings suggest that yogurt with a concentration of 5% hydrogen peroxide (0.17 mmol/L, pH 5.7) effectively prohibits the growth of S. aureus ATCC 29213 at the lowest concentration tested.

1. INTRODUCTION

Yogurt is a fermented dairy product made with the help of lactic acid bacteria (LAB) (Hidayat, 2013). These bacteria play a vital role in the production of probiotic beverages,

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significantly influencing their quality (Agustine, 2018). LAB can generate various metabolites, including lactic acid, bacteriocins, and hydrogen peroxide (H_2O_2) (Hamidah *et al.*, 2019). Research conducted by Purbaningrum (2020) has demonstrated that LAB derived from *L. bulgaricus* and *S. thermophilus* can inhibit the growth of S. aureus. The activity of LAB in yogurt effectively suppresses *S. aureus* growth, with pH levels serving as a relevant measurement.

The antimicrobial properties of hydrogen peroxide stem from its oxidizing capabilities and the production of more toxic hydroxyl radicals, which contribute to the damage of bacterial cells. Lactic acid bacteria produce several antibacterial compounds, including hydrogen peroxide, bacteriocins, CO_2, acetoin, ethanol, ammonia, diacetyl, and various organic acids (such as lactate, citrate, acetate, fumarate, and malate). Hydrogen peroxide is particularly known for its ability to damage proteins, lipids, and nucleic acids due to its bactericidal nature (Murphy & Friedman, 2019).

S. aureus ATCC 29213 is a Gram-positive bacterium distinguished by its spherical shape and irregular clusters resembling grapes under microscopic examination. It grows easily in a variety of media, possesses an active metabolism, and produces pigments that can range from white to deep yellow (Jawetz & Adelberg's, 2019). *S. aureus* is capable of causing infections that range from mild skin infections to severe systemic diseases, often beginning in the skin or throat and leading to abscesses and other complications (Yuliati, 2017).

Current treatment strategies for *S. aureus* infections primarily involve antibiotics such as cloxacillin, dicloxacillin, and erythromycin. However, eliminating *S. aureus* presents challenges, especially due to the rise of antibiotic resistance, which often results from insufficient treatment (Wikananda et al., 2019). Therefore, it is essential to investigate alternative approaches to prevent resistance, thus avoiding further complications that could threaten patient lives. Additionally, the increasing costs of healthcare related to prolonged hospital stays further compound this issue (Desrini, 2015). Preventive measures could include the use of natural substances that have fewer side effects and lower potential for resistance, making them safer options (Setiawati, 2015). One promising alternative for combating S. aureus infections is the use of lactic acid bacteria inoculated in yogurt (Hamidah *et al.*, 2019).

Despite the potential benefits, research on the role of hydrogen peroxide produced by lactic acid bacteria in yogurt remains limited. This knowledge gap has motivated the current investigation into how hydrogen peroxide in yogurt can inhibit the growth of *S. aureus ATCC 29213*.

2. METHOD

This study employed a post-test only control group design to determine the optimal yogurt concentration for inhibiting the growth of *S. aureus ATCC 29213*. By comparing various concentrations against a control group, the research aimed to identify the most effective level of inhibition. Preliminary findings indicated optimal concentrations of 0%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, and 45%, establishing a framework for evaluating the effectiveness of H_2O_2 in yogurt against *S. aureus ATCC 29213*.

The research utilized an in vitro liquid dilution method. Yogurt containing varying concentrations of H_2O_2 was tested against the pathogenic bacteria *S. aureus ATCC 29213*, and turbidity levels were measured to evaluate antibacterial activity. Turbidity was standardized to a McFarland value of 0.5. The pathogenic bacteria and the yogurt were incubated together for 24 hours at 37°C. Each concentration of the fermented milk and the control was diluted five times. Subsequently, samples were plated on Mannitol Salt Agar, employing a duplicate technique to ensure accuracy, and were incubated at 37°C for another 24 hours (Dahlan, 2014).

Data analysis involved both univariate and bivariate methods. Univariate analysis described the dependent variable, specifically the growth of *S. aureus ATCC 29213* across all concentrations. Bivariate analysis assessed the inhibitory effects of lactic acid bacteria in yogurt on the growth of *S. aureus ATCC 29213*. Data were analyzed using the non-parametric Kruskal-Wallis test, followed by the Mann-Whitney test to identify significant differences among groups. SPSS version 25 was used for statistical analysis, with a confidence level set at 95% (Dahlan, 2014).

3. RESULT AND DISCUSSION

The inhibitory effect of yogurt was quantified by comparing the colony counts in the control group to those in the treatment groups. In group 1 (K1, 0%), the inhibition value was recorded at 0%. The inhibition values for groups K2 (5%), K3 (10%), K4 (15%), K5 (20%), K6 (25%), K7 (30%), K8 (35%), K9 (40%), and K10 (45%) were 94.16%, 94.85%, 95.52%, 96.46%, 96.69%, 97.18%, 97.92%, 98.32%, and 98.43%, respectively. A summary of the growth of *S. aureus ATCC 29213* after exposure to yogurt is presented in Table I and Figure 1. The results indicated a positive correlation between yogurt concentration and the inhibitory effect; higher concentrations resulted in greater inhibition. This finding is consistent with research by Purbaningrum (2020), which showed that increased yogurt concentrations led to enhanced inhibition of *S. aureus ATCC 29213* growth.

Table 1. Percentage Inhibition of Yogurt Against *S. aureus ATCC 29213*

Group	Sample Size	Average (CFU/ml)	Inhibition
0%	6	1055×10^3	0%
5%	6	$61{,}6 \times 10^3$	94,16%
10%	6	$54{,}3 \times 10^3$	94,85%
15%	6	$47{,}2 \times 10^3$	95,52%
20%	6	$37{,}3 \times 10^3$	96,46%
25%	6	$34{,}9 \times 10^3$	96,69%
30%	6	$29{,}7 \times 10^3$	97,18%
35%	6	$21{,}9 \times 10^3$	97,92%
40%	6	$17{,}7 \times 10^3$	98,32%
45%	6	$16{,}5 \times 10^3$	98,43%

Based on Table I, it is clear that increasing the concentration of yogurt enhances the inhibition percentage of *S. aureus ATCC 29213* growth, as reflected by the decrease in average bacterial growth following treatment. The data indicate that yogurt with a concentration of 0% does not inhibit *S. aureus ATCC 29213* (P = 0%), while yogurt at a 5% concentration effectively inhibits the bacteria by 94.16%. This suggests that the minimum yogurt concentration required to inhibit the growth of *S. aureus ATCC 29213* is 5% (K2, group 2). These findings are consistent with the research conducted by Purbaningrum (2020), which also determined that 5% is the minimum concentration effective against *S. aureus ATCC 29213*.

The lactic acid content in the yogurt was measured using a pH meter, while H_2O_2 levels were evaluated through permanganometric testing. As shown in Figures 1 and 2, the pH of the 0% yogurt concentration was recorded at 6.8, which is close to normal pH levels. In contrast, pH levels at concentrations ranging from 5% to 45% decreased to more acidic values, ranging from 5.6 to 5.7. The H_2O_2 concentration for the 0% yogurt was measured at 0 mmol/L, while the 45% yogurt concentration produced 0.35 mmol/L of H_2O_2. This indicates that higher yogurt concentrations are associated with increased H_2O_2 levels, resulting in a lower pH (greater acidity).

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.

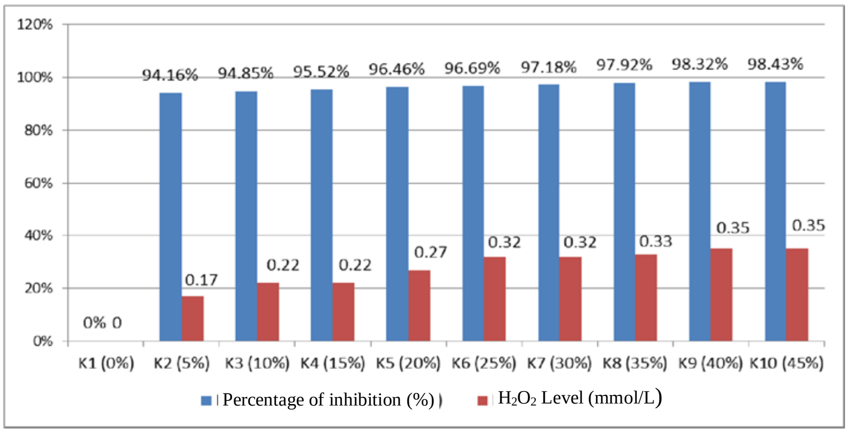

Figure 1. Graph of Average Growth and Hydrogen Peroxide (H_2O_2) Levels

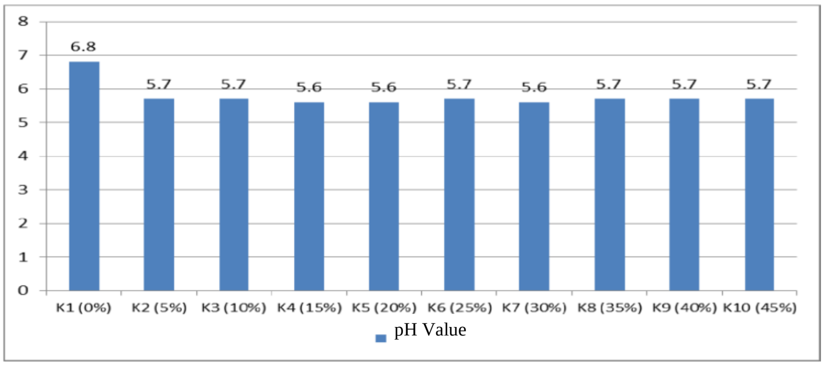

Figure 2. Graph of Average pH of Yogurt for Each Treatment

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 the *Kruskal-Wallis Test*

Treatment Group	N	p-value
K1	6	
K2	6	
K3	6	
K4	6	
K5	6	
K6	6	0,003
K7	6	
K8	6	
K9	6	
K10	6	

The statistical analysis from the non-parametric Kruskal-Wallis test, as shown in Table II, reveals a p-value of less than 0.05. This indicates a significant difference among the yogurt treatment groups regarding the growth of *S. aureus ATCC 29213*. These findings suggest that yogurt effectively inhibits the growth of *S. aureus ATCC 29213*. This outcome is consistent with

Naurah's research (2021), which noted the formation of clear zones around *S. aureus ATCC 29213* colonies when treated with yogurt, indicating successful bacterial growth inhibition.

Table 3. Mann-Whitney Test Results

V	K1	K2	K3	K4	K5	K6	K7	K8	K9	K10
K1		0,004	0,004	0,004	0,004	0,004	0,004	0,004	0,004	0,004
K2	0,004		0,521	0,873	0,423	0,522	0,262	0,261	0,150	0,109
K3	0,004	0,521		0,520	0,199	0,199	0,199	0,107	0,054	0,037
K4	0,004	0,873	0,520		0,337	0,337	0,150	0,295	0,078	0,109
K5	0,004	0,423	0,199	0,337		0,688	0,575	0,630	0,297	0,262
K6	0,004	0,522	0,199	0,337	0,688		0,423	0,520	0,199	0,377
K7	0,004	0,262	0,199	0,150	0,575	0,423		1,000	0,337	0,337
K8	0,004	0,261	0,107	0,295	0,630	0,520	1,000		0,261	0,148
K9	0,004	0,150	0,054	0,078	0,297	0,199	0,337	0,261		1,000
K10	0,004	0,109	0,037	0,109	0,262	0,377	0,337	0,148	1,000	

The results of the Mann-Whitney test shown in Table III indicate p-values below 0.05 when comparing group K1 to groups K2, K3, K4, K5, K6, K7, K8, K9, and K10, as well as between groups K3 and K10. The Mann-Whitney test criteria state that if the p-value ≤ α, then the null hypothesis (H0) is rejected; conversely, if the p-value ≥ α, H0 is accepted (Utomo, 2021). Thus, the Mann-Whitney test results lead to the rejection of H0, confirming significant differences in colony counts between group K1 and groups K2 through K10, as well as between group K3 and K10. This variation can be attributed to differences in the mean colony counts across these groups.

The lactic acid bacteria *L. bulgaricus* and *S. thermophilus* in yogurt operate synergistically to inhibit pathogenic bacteria through their metabolic byproducts. As noted by Brooks *et al.* (2013), several mechanisms contribute to bacterial growth inhibition, including the interference with cell wall and nucleic acid synthesis. The inhibition of *S. aureus ATCC 29213* by lactic acid bacteria in yogurt is primarily due to antimicrobial substances such as hydrogen peroxide, lactic acid, bacteriocins, and diacetyl (Fitriarnani, 2014). Lactic acid is a significant antimicrobial agent with high efficacy and a broad spectrum of inhibition, leading to an imbalance in the internal and external pH of bacterial cells (Rahman *et al.*, 2019).

Acidic environments can hinder the formation of bacterial cell walls and increase the permeability of cell membranes. Consequently, H+ ions from the external environment diffuse into the cell, leading to enzyme denaturation and subsequent damage to the membrane. This process allows other antimicrobial agents, such as hydrogen peroxide (H_2O_2), to penetrate the cytoplasm. Hydrogen peroxide inhibits glucose transport, nucleic acid synthesis, hexokinase activity, and glyceraldehyde-3-phosphate dehydrogenase activity by oxidizing the sulfhydryl groups in these enzymes. Additionally, hydrogen peroxide can disrupt lipid membrane synthesis, accelerating cell lysis and ultimately resulting in bacterial cell death (Herlambang *et al.*, 2018; Surono, 2016). As an oxidative biocide, hydrogen peroxide generates free hydroxyl radicals that cause oxidative damage to DNA, proteins, and lipid membranes, leading to bacterial lysis and death.

Yogurt contains antibacterial compounds such as lactic acid and hydrogen peroxide (H_2O_2), which can lower pH and inhibit the growth of *S. aureus ATCC 29213*. At a concentration of 45%, yogurt can inhibit bacterial growth by as much as 98.43%, and even at a 5% concentration, significant inhibition of 94.16% is observed. The pH values across all treatment groups remained fairly consistent, ranging from 5.5 to 5.7. This consistency suggests that other factors, such as bacteriocins, may also play a role in bacterial inhibition (Murphy & Friedman, 2019). *S. aureus ATCC 29213*, a Gram-positive bacterium, can interact with bacteriocins via teichoic acid receptors. The complex formed by the bacteriocin and teichoic acid destabilizes the cell membrane, creating pores in the cytoplasmic membrane, which alters the membrane potential gradient and allows extracellular substances to enter (Nurhajati, 2019). The formation of these pores disrupts the proton motive force (PMF), essential for energy production and utilized in all cellular activities, including bacterial metabolism. When the PMF fails, all energy-dependent reactions cease, leading to cell death (Suwayvia, 2017).

One limitation of this study is that lactic acid levels were inferred based solely on pH reduction, making it impossible to accurately measure the concentration of lactic acid produced, as it may combine with other organic acids formed during fermentation. Additionally, other compounds contributing to growth inhibition, apart from lactic acid and hydrogen peroxide (H_2O_2), include bacteriocins produced during yogurt fermentation. Thus, further research is warranted to explore the effects of bacteriocins on the growth of *S. aureus ATCC 29213*.

4. CONCLUSION

1) Yoghurt (*L. bulgaricus* and *S. thermophilus*) concentrations of 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, and 45% effectively inhibit the growth of *S. aureus ATCC 29213*, as indicated by a decrease in average bacterial colony growth.
2) Higher concentrations of yogurt correspond to increased levels of H_2O_2. As H_2O_2 levels rise, the inhibition of *S. aureus ATCC 29213* growth also intensifies.
3) The minimum yogurt concentration that can inhibit *S. aureus ATCC 29213* growth is 5%, which contains 0.17 mmol/L of hydrogen peroxide.

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