Research Article

Lactic Acid Bacteria (Lactobacillus bulgaricus and Streptococcus thermophilus) in Yogurt Inhibit the Growth of Escherichia coli, Salmonella typhimurium, and Shigella sp. In Vitro

Uji Aktivitas Bakteri Asam Laktat (*Lactobacillus bulgaricus* dan *Streptococcus thermophilus*) pada Yogurt dalam Menghambat Pertumbuhan Bakteri *Escherichia coli*, *Salmonella typhimurium* dan *Shigella sp.* Secara In Vitro

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ABSTRACT

Diarrhea is defecation with a frequency more often than usual (three times or more) a day (10 mL/kg/day) with a soft or liquid consistency, even in the form of water alone. Pathogenic bacteria, such as *Escherichia coli*, *Salmonella typhimurium*, and *Shigella sp.*, play a role in many cases, to which antibiotics are prescribed as the first-line therapy. However, since antibiotic resistance cases are often found, preventive therapies are needed, such as consuming yogurt, which is produced through a fermentation process by lactic acid bacteria (LAB). This research aimed to determine the activity of lactic acid bacteria (*Lactobacillus bulgaricus* and *Streptococcus thermophilus*) in yogurt in inhibiting the growth of the pathogenic bacteria *E. coli*, *S. typhimurium*, and *Shigella sp.* The research applied in vitro with the liquid dilution test method and the true experimental design research method with post-test-only and control group design. The design was used to see the inhibitory effect of yogurt LAB on the growth of *E. coli*, *S. typhimurium*, and *Shigella sp.* to compare the effect of several different yogurt concentrations, namely 20%, 40%, 60%, and 80%. The results of the Least Significance Different analysis showed that there was a significant difference between yogurt with a concentration of 0% and that with various concentrations in inhibiting the growth of *E. coli*, *S. typhimurium*, and *Shigella sp.* with a p-value of <0.05. Whereas, there was no significant difference in the various concentrations of yogurt in inhibiting the growth of the three kinds of bacteria with a p-value of >0.05.

Keywords: Diarrhea, *Escherichia coli*, *Salmonella typhimurium*, *Shigella sp.*, yogurt Lactic Acid Bacteria (LAB)

ABSTRAK

Escherichia coli, *Salmonella typhimurium* dan *Shigella sp.* merupakan bakteri patogen penyebab diare. Diare adalah buang air besar dengan konsistensi lembek atau cair, bahkan dapat berupa air saja dengan frekuensi lebih sering dari biasanya (tiga kali atau lebih) dalam satu hari (10 ml/KgBB/hari). Antibiotik merupakan terapi lini pertama penyakit ini, namun sekarang sudah sering dijumpai kasus resistensi antibiotik sehingga perlu adanya terapi pencegahan. Terapi pencegahan bisa dilakukan dengan mengonsumsi yogurt. Yogurt merupakan salah satu produk minuman yang dihasilkan melalui proses fermentasi oleh bakteri asam laktat (BAL). Tujuan penelitian adalah untuk mengetahui aktivitas bakteri asam laktat (*Lactobacillus bulgaricus* dan *Streptococcus thermophilus*) pada yogurt dalam menghambat pertumbuhan *E. coli*, *S. typhimurium* dan *Shigella sp.* penyebab diare. Penelitian dilakukan secara *in vitro* dengan metode uji dilusi cair dan metode penelitian *true experimental design* dengan *post test-only* dan *control group design*. Desain yang digunakan untuk melihat efek penghambatan pertumbuhan *E. coli*, *S. typhimurium* dan *Shigella sp.* oleh BAL yogurt dan membandingkan efeknya pada beberapa konsentrasi yogurt yang berbeda, yaitu 20%, 40%, 60% dan 80%. Hasil analisis Least Significance Different menunjukkan bahwa terdapat perbedaan bermakna antara konsentrasi 0% dengan berbagai konsentrasi yogurt dalam menghambat pertumbuhan *E. coli*, *S. typhimurium* dan *Shigella sp.* dengan nilai $p < 0,05$, tetapi tidak terdapat perbedaan yang bermakna antar konsentrasi yogurt dalam penghambatan pertumbuhan *E. coli*, *S. typhimurium* dan *Shigella sp.* dengan nilai $p > 0,05$.

Kata Kunci: Bakteri Asam Laktat (BAL) yogurt, diare, *Escherichia coli*, *Salmonella typhimurium*, *Shigella sp.*

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INTRODUCTION

Diarrhea is defecation with a soft or liquid consistency, even in the form of water, with a frequency more often than usual (three times or more) in one day (10 mL/kg/day) (1). It can be caused by abnormal transport of water and electrolytes in the intestines. There are approximately 500 million children worldwide who suffer from diarrhea each year, and 20% of all deaths in children living in developing countries are related to diarrhea and dehydration—(2).

Diarrhea is a symptom of gastrointestinal infection or gastroenteritis. This disease can be caused by infection with parasites, viruses, or bacteria. Bacterial infections usually cause more severe diarrhea and are usually the result of consuming contaminated food or drink. The groups of bacteria that cause diarrhea are *E. coli*, *Salmonella*, and *Shigella*.

Most *E. coli* bacteria are harmless and often live normally in the human digestive tract. However, some of these bacteria can release toxins that cause diarrhea and cause acute infections, urinary tract infections, severe anemia, kidney failure, and even death. *E. coli* bacterial infection generally occurs due to direct contact with either animals' or humans' feces, primarily through food and drinks contaminated with the feces. These bacteria are often found in raw meat or dairy products and raw fruit and vegetables. This diarrhea-causing bacteria can also be spread between humans from shaking hands and the equipment used together.

There are two types of *Salmonella enterica*, namely *S. typhi* serotype *typhimurium* and *S. typhi* serotype *enteritidis*. Children and people with immune system disorders are particularly susceptible to infection with *S. typhimurium* bacteria, which can be found in raw meat or milk-based products, as well as in the feces of pets, especially animals suffering from diarrhea. Reptiles, chicks, ducks, and small rodents such as hamsters are very likely to carry *S. typhimurium*. Contact with animals or food and drinks that have been contaminated with these bacteria can also trigger diarrhea. *Salmonella typhimurium* can develop rapidly and the symptoms can appear within 12 hours to three days and can last up to seven days. *Salmonella typhimurium* is a reasonably weak bacteria, which can die at high temperatures. So, to kill bacteria can be done by cooking food until thoroughly cooked.

Shigella sp. that causes diarrhea spreads through water and food contaminated with feces. It can release toxins that can irritate the intestines. This type of bacteria often causes diarrhea containing blood. An environment with poor sanitation and a lifestyle that is less hygienic become a vital factor in the spreading of these bacteria. Washing hands regularly and adequately can prevent infection with these bacteria. Medical drugs, such as antibiotics can help kill the bacteria that cause diarrhea.

The primary treatment to overcome diarrhea is to replace lost body fluids, such as oralit or *Oral Rehydration Solution* (ORS) administration. Patients who experience bacteria-caused diarrhea need antibiotics to break the colonization of the pathogenic bacteria. However, recently, there have been many incidents of antibiotic resistance that have led to ineffective treatment using single antibiotics. Several studies showed that *E. coli*, *S. typhimurium*, and *Shigella*

sp. are starting to become resistant to streptomycin, chloramphenicol, and tetracycline (3). Therefore, to control the number of antibiotic resistance, it is better to prevent the colonization of the bacteria that cause diarrhea, namely by consuming probiotics (4). Probiotic bacteria can prevent the colonization of pathogenic bacteria in the intestinal epithelium and compete to occupy the epithelial site of the pathogenic bacteria (5). There are many kinds of probiotic-containing drinks on the market, such as yogurt.

Yogurt is a fermented milk product with some benefits for your health. It is made from pasteurized milk, fermented using certain bacteria to produce a distinctive smell, acidity, and taste, with or without other ingredients. As a functional drink, it has beneficial properties for health due to lactic acid bacteria (LAB) (6). Consuming it regularly can reduce the risk of gastrointestinal diseases and increase immunity(7).

According to Rahmiati and Mumpuni, LAB is useful in maintaining the balance of normal intestinal flora and inhibiting pathogenic bacteria's growth (8). It produces bacteriocins and has the ability to create an acidic atmosphere in the digestive tract. Bacteriocin is bacteriostatic, thus inhibiting other bacteria. An in vitro study conducted by (9), proved that LAB inhibited colonization of intestinal pathogenic bacteria by forming a significant inhibition zone.

Based on the description above, an in vitro study was carried out to test the ability of yogurt LAB to inhibit the growth of *E. coli*, *S. typhimurium*, and *Shigella* sp., which cause diarrhea.

METHOD

Research Design

The research was conducted *in vitro* at the Microbiology Laboratory of the Faculty of Medicine, Universitas Jenderal Soedirman, Purwokerto, using three test bacteria, namely *Escherichia coli*, *Salmonella typhimurium*, and *Shigella* sp., which was obtained from the collection of the laboratory. The test was carried out using the liquid dilution test method and the true experimental design research method with post-test-only and control group design. The design used was to see the yogurt LAB's inhibitory effect on the growth of *E. coli*, *S. typhimurium* and *Shigella* sp. and to compare the effect of several different yogurt concentrations. The study was carried out in six repetitions, by cultivating the bacteria in duplo at the last two dilutions. The independent variable in this study was the activity of yogurt, which come from the fermentation of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* in various concentrations, namely 20%, 40%, 60% and 80%. The dependent variables were the growth of *E. coli*, *S. typhimurium*, and *Shigella* sp. colonies on petri dishes and the level of acidity (pH).

Preparation of Yogurt that Contains *Lactobacillus Bulgaricus* and *Streptococcus Thermophilus*

Yogurt was made in the Microbiology Laboratory by means of 500 ml of fresh cow's milk and 25 grams of skim milk powder. After pasteurization, 2% lactic acid bacteria were inoculated with a ratio of *Lactobacillus bulgaricus* to *Streptococcus thermophilus* of 1:1 and then incubated at 37°C for 1 x 24 hours(10).

Yogurt was prepared in several concentrations using distilled water based on the following dilution formula: (11)

$$C1.V1 = C2.V2$$

Note C1: Cow milk yogurt stock concentration; V1: Cow milk yogurt stock volume; C2: Cow milk yogurt concentration; V2: Final volume of cow milk yogurt made

The level of acidity of yogurt was measured after the yogurt was made in various concentrations using a calibrated pH meter. A total of 1 mL of yogurt from each concentration was taken using a micropipette and then transferred to a beaker glass and measured until it showed a stable number (12).

Measurement the Growth of Escherichia Coli, Salmonella Typhimurium, and Shigella Sp

The test bacteria (*E. coli*, *S. typhimurium*, and *Shigella* sp.) were standardized at 0.5 Mac Farland (10^8 CFU/ml), by adding the test bacteria colonies in physiological NaCl solution to a turbidity level of 0.5 Mac Farland. In 10 mL of yogurt from each concentration, 1 mL of test bacteria was added, then incubated at 37°C for 1 x 24 hours (13).

The test bacteria, after the 24-hour incubation, were then diluted using the serial delution method to 10^{-4} using physiological NaCl. In the results of the 10^{-2} , 10^{-3} , and 10^{-4} dilutions, cultivation was carried out in Petri dishes containing Eosin Methylene Blue Agar (EMBA) for *E. coli* and *Salmonella Shigella* Agar (SSA) for *S. typhimurium* and *Shigella* sp., to be incubated at 37°C for 1 x 24 hours (14).

The data obtained were quantitative data with a ratio numeric scale. The data were obtained from the calculation of the number of bacterial colonies of *E. coli*, *S. typhimurium*, and *Shigella* sp. that grew after coming into contact with yogurt. The results obtained were then recorded and converted into percentages with the following formula: (15)

$$(1) \text{ actual number of colonies} = \text{number of colonies on the dish} \times \frac{1}{10^{-x}}$$

$$(2) P = \frac{K_0 - K_1}{K_0} \times 100\%$$

Note

X : the number of stratified dilutions performed

P : inhibition of yogurt LAB against bacteria (%)

K0 : the average number of colonies in the control group (CFU/mL)

K1 : the average number of colonies in the treatment group (CFU/mL)

Data Analysis

The data was analyzed by using analysis of varians (ANOVA) and correlation analysis, and if the result of ANOVA showed significant different, continued by least significant different test (LSD) at 5%.

RESULTS

The results showed that the concentration of *L. bulgaricus* and *S. thermophilus* in yogurt fermentation was able to suppress the population growth of *E. coli*, *S. typhimurium*, and *Shigella* sp. Populations of *E. coli*, *S. typhimurium*, and *Shigella* sp. at various concentrations of *L. bulgaricus* and *S. thermophilus* in yogurt, it looks lower than the concentration in the control treatment, where the population of *E. coli*, *S. typhimurium*, and *Shigella* sp. respectively 9.95 x 10⁶ CFU/mL, 4.41 x 10⁷ CFU/mL, and 2.25 x 10⁷ CFU/mL (Table 1). However, there was no difference in the effect between concentrations of *L. bulgaricus* and *S. thermophilus* on the inhibition of growth of *E. coli*, *S. typhimurium*, and *Shigella* sp. The percentage of inhibition of various concentrations showed a value greater than 99%.

Table 2. Results of measurement of yogurt LAB pH at various concentrations

Treatments	<i>E. coli</i>	<i>S. typhi</i>	<i>Shigella</i> sp.
K1 (0%)	5.50 a	5.50 a	5.50 a
K2 (20%)	5.30 b	5.30 b	5.30 b
K3 (40%)	5.10 c	5.10 c	5.10 c
K4 (60%)	4.80 d	4.80 d	4.80 d
K5 (80%)	4.40 e	4.40 e	4.40 e

Note: The number followed by same letter at the same colour is not significant different according Least Significance Difference (LSD) 5%

Increasing concentrations of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* in yogurt fermentation showed an effect on the pH of yogurt inoculated with *E. coli*, *S. typhimurium*, and *Shigella* sp. The results showed that the lowest yogurt pH was achieved at a concentration of 80% of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* in yogurt fermentation, both inoculated with *E. coli*, *S. typhimurium*, and *Shigella* sp. each showed a pH value of 4.40 (Table 2). Treatment with 0% concentration of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* in yogurt fermentation showed the highest pH value of 5.50, respectively. Based on the results of correlation analysis showed that the

Table 1. Yields of yogurt LAB against *E. coli*, *S. typhimurium*, and *Shigella* sp.

Treatments	<i>E. coli</i>		<i>S. typhimurium</i>		<i>Shigella</i> sp.	
	Average (CFU/ml)*	% of inhibition	Average (CFU/ml)*	% of inhibition	Average (CFU/ml)*	% of inhibition
K1 (0%)	9.95 x 10 ⁶ a	0	4.41 x 10 ⁷ a	0	2.25 x 10 ⁷ a	0
K2 (20%)	6 x 10 ⁴ b	99.39	2.98 x 10 ⁴ b	99.89	1.11 x 10 ⁵ b	99.56
K3 (40%)	1 x 10 ⁴ b	99.89	4.20 x 10 ⁴ b	99.90	8.30 x 10 ⁴ b	99.67
K4 (60%)	1 x 10 ³ b	99.89	3.10 x 10 ⁴ b	99.92	5.85 x 10 ⁴ b	99.77
K5 (80%)	5 x 10 ³ b	99.95	2.25 x 10 ⁴ b	99.94	4.85 x 10 ⁴ b	99.80

Note: the numbers followed by the same letter in the same column were not significantly different according to the 5% Least Significance Difference (LSD). * data in Log(x) transformation

Table 3. Correlation among yogurt pH and Inhibition against growth of *E. coli*, *S. typhimurium*, and *Shigella* sp.

	pH <i>E. coli</i>	pH <i>S. typhimurium</i>	pH <i>Shigella</i> sp	<i>Shigella</i> sp	<i>E. coli</i>	<i>S. typhimurium</i>
pH <i>E. coli</i>	1.00000					
pH <i>S. typhimurium</i>	0.98419*	1.00000				
pH <i>Shigella</i> sp	0.98898*	0.99071*	1.00000			
<i>Shigella</i> sp	0.55515*	0.53847*	0.53570* 0.66065*	1.00000		
<i>E. coli</i>	0.67643*	0.65704*	0.52374*	0.96676*	1.00000	
<i>S. typhimurium</i>	0.52293*	0.54252*		0.85769*	0.84980*	1.00000

Note:* close correlation

population growth of *E. coli*, *S. typhimurium*, and *Shigella* sp. has a close correlation with the pH value of yogurt.

DISCUSSION

Table 1. shows the potential for yogurt LAB to inhibit the growth of *E. coli*, *S. typhimurium*, and *Shigella* sp. can be seen from the decrease in the number of colonies of the three types of bacteria after being given yogurt with various concentrations. A treatment is said to inhibit if the number of treated bacteria, which grow, is less than that in the control group. The results of the calculation of the inhibition against the growth of *E. coli*, *S. typhimurium*, and *Shigella* sp. can be used to determine the minimum inhibitory concentration. The minimum inhibitory concentration in this study was the LAB concentration, which showed a large inhibition but at low antibacterial levels (16). Based on the research, the concentration of yogurt LAB K2 (20%) was able to inhibit the growth, reaching over 50%, namely *E. coli* (99.39%), *S. typhimurium* (99.89%), and *Shigella* sp. (99.56%).

The results of the analysis in Table 2. show a significant difference between the effect of K1 and the various concentrations (K2, K3, K4, and K5) on the growth of the test bacteria (*E. coli*, *S. typhimurium*, and *Shigella* sp.) with a *p*-value of < 0.05 (indicated by letter a). This means that the various concentrations can inhibit the growth of the test bacteria. When the treatment groups (K2, K3, K4, and K5) were compared to each other, no significant difference was found with a *p*-value of >0.05 (indicated by letter b).

The inhibition against the growth of *E. coli*, *S. typhimurium*, and *Shigella* sp. in this study was caused by the activity of yogurt lactic acid bacteria (LAB) in yogurt, namely *L. bulgaricus* and *S. thermophilus*. These two bacteria synergized to inhibit pathogenic bacteria by producing antimicrobial substances. The mechanism of the antimicrobial substances produced by these bacteria consists of four mechanisms, namely inhibition of cell wall synthesis, disruption of cell membrane function, inhibition of protein synthesis processes, and inhibition of lactic acid synthesis — (17). According to (18), the antibacterial substances produced by the lactic acid bacteria in yogurt are lactic acid, hydrogen peroxide (H_2O_2), diacetyl, and bacteriocin.

Lactic acid in yogurt has several stages in suppressing bacterial growth, digesting the cell membrane disrupting membrane permeability and changing intracellular pH of gram-negative bacteria, resulting in inhibition of protein synthesis. Disrupted membrane permeability will result in damage to the mitochondrial, plasma, and lysosomal

membrane of the cell. Damage to cell membranes due to increased membrane permeability will result in decreased ATP production so that cells undergo lysis(18).

Hydrogen peroxide in yogurt is converted into hypochlorite components that are highly reactive and become hydroxyl free radicals. These free radical components oxidize membrane lipids and form cross-reactions on proteins mediated by sulfhydryl groups so that degradation is increased or enzyme activity is lost. Hydrogen peroxide can also cause DNA damage due to the free radical process(18).

Diacetyl will deactivate the enzyme so that the catalyst side of the enzyme changes. Besides, no sources discuss diacetyl antimicrobial compounds(18).

In addition to the mechanism of action of the antimicrobial compounds possessed by yogurt LAB, some factors influence bacterial growth, like temperature, humidity, osmotic value, nutrition, and pH. The factors including temperature, humidity, osmotic value, and nutrition have been adjusted to the optimum value of the tested bacteria so that pH affects the occurrence of inhibition against the growth of *E. coli*, *S. typhimurium*, and *Shigella* sp.—(17). Based on Table III, all concentrations of yogurt had different pH values. The control treatment (K1) had a relatively higher pH than the K2, K3, K4, and K5 treatments did. All control groups and the treatment of the tested bacteria in this study were grown on plates with selective media. Each group had the same temperature and incubation period, there was 37°C for 24 hours. The difference in environmental factors in this study lies in the intervention in each treatment group, with different yogurt concentrations, which provide a pH value below the optimum pH for the tested bacteria's growth.

Based on Table 3. the level of acidity (pH) produced by all treatment concentrations of yogurt (K1, K2, K3, K4, and K5) influences the inhibitory activity against the growth of the test bacteria. The level of acidity (pH) of yogurt is correlated with the inhibition against the growth *E. coli* with a value of $r < .0001$, while the inhibition against the growth of *S. typhimurium* and *Shigella* sp. is significantly correlated with yogurt pH values with $r < .0020$ and $r < .0023$, respectively. A pH value below the optimum pH value indicates an increasingly acidic environment for bacterial growth. When the total acid increases, the growth of *E. coli*, *S. typhimurium*, and *Shigella* sp. are further inhibited(19).

The acidity of yogurt is influenced by glucose fermented into lactic acid by LAB. So, the higher the glucose level, the more acidic the resulting taste or the lower the acidity. A

low pH will cause fat to dissolve and diffuse through the cell membrane, allowing the cell to die.(20)

Based on this research, there are several limitations as follows: the parameter measured in the inhibition against the growth of *E. coli*, *S. typhimurium*, and *Shigella* sp. was only the level of acidity or the pH of the yogurt concentration, thus only representing the acidity of each research sample; this study could not determine the most influential substance produced by yogurt's LAB in inhibiting the growth of bacteria *E. coli*, *S. typhimurium*, and *Shigella* sp., and; this study only examined the inhibitory effect of yogurt's LAB in vitro, thus requiring further research in vivo.

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