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Andyanita Hanif Hermawati 🔤; Eka Puspitasari; Chalies Diah Pratiwi

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Combination of Forest Honey with Probiotic Milk as an Antibacterial Against *Escherichia coli* and *Staphylococcus aureus* in vitro

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Abstract. Antibacterial activity of the combination of forest honey with probiotic milk produced by *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Lactobacillus casei*, and *Lactobacillus rhamnosus* against *Escherichia coli* ATCC 8739 and *Staphylococcus aureus* ATCC 6538 has been studied. The fermented milk was prepared by inoculating each probiotic in milk at 43°C and then incubated at room temperature for 24 hours. The diffusion agar technique by using nutrient agar media was used for antibacterial activity with three replication. Fermented milk is made by inoculating Lactobacillus acidophilus, Lactobacillus bulgaricus, Lactobacillus casei, and Lactobacillus rhamnosus against *Escherichia coli* ATCC 8739 with a ratio of 2:8 had inhibition zone diameters of 16.38mm, 11.47mm, 14.73mm, and 16.52mm. The combination of forest honey with probiotic milk *Lactobacillus casei*, and *Lactobacillus bulgaricus*, *Lactobacillus acidophilus*, *Lactobacillus against Staphylococcus aureus* ATCC 6538 in a ratio of 2:8 has inhibition zone diameters of 15.65mm, 12.02mm, 15.45mm, and 15.18mm so that it has potential as an antibacterial. The result was analyzed using MANOVA and showed that all the treatments had a significant effect p-value < α (<0.05). The combination of forest honey with probiotic milk has antibacterial activity.

INTRODUCTION

Alternative medicine using natural ingredients continues to be developed, one of which is honey. Honey is a sweet liquid derived from plant nectar that is processed by bees and stored in beehive cells. Honey can inhibit the growth of bacteria such as *Escherichia coli* and *Staphylococcus aureus* based on the inhibition zone produced in the media that has been planted with these bacteria [1]. This study used poly flora honey. Polyflora honey comes from the nectar of several types of flower plants, such as forest honey. Forest honey is produced by wild bees. This honey comes from a wild bee named *Apis dorsata*. The source of food for these bees is medicinal plants that grow in tropical rainforests in Indonesia. Indonesians have long-known milk. Fermented milk products that are well-known in Indonesia include yogurt, sour milk, kefir, and flavored fermented milk drinks. Since 2003, several competitors have emerged in the fermented milk beverage industry with added probiotics in it. Probiotics are living organisms that can provide beneficial effects on the health of the host when consumed in sufficient quantities by improving the balance of intestinal microflora when entering the digestive tract [2]. Several microorganisms are currently used as probiotics. The most commonly used probiotics are bacteria belonging to the genus Lactobacillus, the first and largest group of microorganisms considered probiotics, and Bifidobacterium [3].

There are three ways probiotics work, namely suppressing the growth of pathogenic microorganisms in the digestive tract through the antimicrobial substances they produce; competition for food and competing for receptors in the intestinal epithelium; altering microbial metabolism by increasing the activity of beneficial enzymes [1]. Lactic acid bacteria (LAB) are efficacious in preserving food and improving the nutritional quality of various fermented food products. In particular, LAB exerts an antimicrobial effect by producing lactic acid, which increases the acidity of the environment, resulting in the loss of viability of pathogenic bacteria [4]. Low molecular weight compounds, such as hydrogen peroxide, carbon dioxide, diacetyl (2,3-butanedione), and bacteriocins also contribute

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to the antimicrobial effect of LAB because they substantially inhibit the growth of pathogenic bacteria in food systems [5].

Honey that contains flavonoids can damage bacterial cell membranes. To date, flavonoids, especially catechins, have been extensively studied for their antimicrobial properties in Gram-positive and Gram-negative bacteria. The interaction of flavonoids with the lipid bilayer involves two mechanisms. The first is associated with the partition of more non-polar compounds in the hydrophobic interior of the membrane, while the second includes the formation of hydrogen bonds between the polar head groups of lipids and more hydrophilic flavonoids at the membrane interface [6]. So there is a decrease in the defense function of the bacterial membrane so that the bacterial colonies formed on the surface will also decrease.

Probiotic milk and honey each have antibacterial properties with different mechanisms. The combination of probiotic milk and honey was made to increase antibacterial activity compared to probiotic milk and honey. One mechanism of the combination of probiotic milk and honey is to produce antimicrobial compounds. In this study, preparations will be made from probiotic milk containing *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Lactobacillus casei*, and *Lactobacillus rhamnosus* and forest honey solution. The test bacteria used were *Escherichia coli* and Staphylococcus aureus. The purpose of this study was focused on testing the combination of forest honey with probiotic milk as an in vitro antibacterial against the growth of *Escherichia coli* and *Staphylococcus aureus*. Based on the content of compounds in forest honey solution and microbes from probiotic milk, it is expected to have antibacterial power from the combination of forest honey and probiotic milk.

MATERIALS AND METHODS

Materials

The material used in this research is forest honey obtained from honey farmers in the mountainous area of Pasuruan, East Java. The probiotics used were *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Lactobacillus casei*, and *Lactobacillus rhamnosus* cultured in deMann Rogosa and Sharpe (MRS) broth media. *Escherichia coli* ATCC 8739 and *Staphylococcus aureus* ATCC 6538 which were used as the test bacteria in this study were cultivated in Nutrient Agar media.

Making probiotic milk

Using the inoculum oseed from the bacteria *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Lactobacillus casei*, and *Lactobacillus rhamnosus* to tilt into the MRS Broth medium then shake it at 150 rpm for 48 hours. A total of 50 mL of fresh cow's milk was then added with 2ml of inoculum from the bacteria *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Lactobacillus casei*, and *Lactobacillus rhamnosus* which had been shaken for 48 hours.

Making a Combination of Forest Honey with Probiotic Milk

The combination of forest honey and probiotic milk used is 2: 8. A total of 10 ml of forest honey added with 40 ml of probiotic milk *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Lactobacillus casei*, and *Lactobacillus rhamnosus* which had been shaken for 48 hours. Then, close tightly the milk that has been added inoculum and store it at room temperature for 24 hours.

Inhibition Activity Test

Conducted using diffusion method on double-layered Nutrient Agar Merck (base layer and seed layer) to measure the growth inhibition zone around the plate. Pour 30 ml of nutrient media into the Petri, let it cool, and solidify as a base layer

Conducted on Nutrient agar with a base layer of 30 mL and a seed layer of 20 mL using *Escherichia coli* ATCC 8739 and *Staphylococcus aureus* ATCC 6538 test bacteria made inoculum with 25% transmittance at a wavelength of 580 nm. A total of 10 μ L of tested bacterial inoculum, was added to the seed layer, shaken with vortex, poured on the surface of the base media (base layer) in a petri dish, and allowed to stand until solidified. A hole was made, filled with 150 μ L of the sample solution, 150 μ L of sterile water as a negative control, and 50 μ L of standard Streptomycin 100ppm as a positive control then incubated at 32.5°C for 24 hours. The diameter of the zone formed around the rig is measured (mm).

Methods

This research path is like the research conducted by Hermawati et al (2015) with the title inhibition of mango (Mangifera indica)-milk honey probiotic combination on the growth of *Escherichia coli* and *Staphylococcus aureus* [1]. The combination of honey and probiotic milk used is 2:8.

RESULTS AND DISCUSSION

Forest honey obtained from Lumbang District, Pasuruan Regency, East Java is poly flora honey because honey bees eat nectar (a sweet liquid found in flowers) from various types of plants. Characterization of forest honey is needed to ensure reproducibility (Figure 1).



FIGURE 1. Forest Honey

From these data, it can be seen that the characterization of forest honey includes dark chocolate, sweet taste, distinctive smell, and thick form. The darker the honey color, the higher the mineral content in the honey. The color of honey is believed to be based on its mineral content, source of nectar, processing methods such as honey extraction, and its management [1].

Lactobacillus sp. produces several metabolites, including hydrogen peroxide and bacteriocins, which can inhibit growth and/or kill pathogenic bacteria. Other mechanisms that cause probiotics to fight pathogenic microbes are competitive antagonists through adhesion competition on epithelial cells, competition for nutrient use, and enhancement of the host immune system [1].

The study was conducted by characterizing the probiotic milk of *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Lactobacillus casei*, and *Lactobacillus rhamnosus* (Figure 2). The probiotic starter *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Lactobacillus casei*, and *Lactobacillus casei*, and *Lactobacillus rhamnosus* were added to fresh cow's milk and incubated at 37°C for 24 hours into probiotic milk.



FIGURE 2. Probiotic milk



From these data, it can be seen that the characterization of organoleptic-based probiotic milk can be seen that probiotic milk is a thick liquid with a white solid, has a distinctive odor like milk, and has a sour taste.

There are several methods for testing antibacterial activity, namely the dilution and diffusion methods. The agar diffusion method is the formation of an inhibition zone around the reservoir on nutrient agar media that has been inoculated with the *Escherichia coli* ATCC 8739 and *Staphylococcus aureus* ATCC 6538 test bacteria. The highest zone of inhibition was produced by the combination of forest honey with probiotic milk *Lactobacillus rhamnosus* against *Escherichia coli* ATCC 8739 of 16.52mm (Figure 3) and the combination of forest honey with probiotic milk *Lactobacillus acidophilus* against *Staphylococcus aureus* ATCC 6538 with a ratio of 2:8 of 15.65mm (Figure 4).

Combination of forest honey with probiotic milk to determine the antibacterial activity against *Escherichia coli* and Staphylococcus aureus. An antibacterial activity test was carried out on *Escherichia coli* and *Staphylococcus aureus* with three replications. The inhibition zone diameter obtained was then analyzed to determine the highest antibacterial activity. The inhibition zone produced by the combination of forest honey with probiotic milk of *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Lactobacillus casei*, and *Lactobacillus rhamnosus* against *Escherichia coli* and *Staphylococcus aureus* was 16.38 mm, 11.47 mm, 14.73 mm, 16.52 mm, and 15.65mm, 12.02mm, 15.45mm, and 15.18mm. The research data were analyzed using Manova and the result are shown in Table 1.

Honey and probiotic milk each have antibacterial properties with different mechanisms [7]. Honey can inhibit the growth of pathogenic bacteria such as *Escherichia coli*, Listeria monocytogenes, and Staphylococcus aureus. This can be seen from the inhibition zone caused by honey given to the media that has been inoculated with the bacteria [8]. Based on the results of the Kaligis (2020) examination, the content of forest honey appears to have a higher water content, a more acidic pH level, higher protein content, lower levels of hydrogen peroxide, higher levels of flavonoids, and lower sugar content than black honey [9]. This content is the content of active compounds in forest honey as antibacterial power.





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FIGURE 4. Zone of Inhibition by the Combination of Forest Honey with Probiotic Milk Against Staphylococcus Aureus

TADLE 1 Multivariate Test

TADLE 1. Wullivallate Test												
							Noncent.	Observed				
Effect		Value	F	Hypothesi	is dfError df	Sig.	Parameter	Power ^d				
Combinatio	o Pillai's Trace	1.300	4.458	10.000	24.000	.001	44.575	.987				
n Hone	yWilks' Lambda	.007	24.174 ^b	10.000	22.000	.000	241.739	1.000				
with	Hotelling's Trace	98.595	98.595	10.000	20.000	.000	985.952	1.000				
Probiotic	Roy's Larges	t98.146	235.550°	5.000	12.000	.000	1177.748	1.000				
Milk	Root											

The results of Manova show that a significant effect is p $(0.00) < \alpha$ (0.05). Overall, the independent variables have a significant effect on the dependent variable group of 95%. The results of the antibacterial activity test of the combination of forest honey and probiotic *Lactobacillus rhamnosus* milk against *Escherichia coli* and the combination of forest honey with *Lactobacillus acidophilus* probiotic milk against *Staphylococcus aureus* obtained data on the largest diameter of the inhibition zone.

The ability of lactic acid bacteria to combat infectious diseases can be caused by the metabolic products produced, including lactic acid, hydrogen peroxide (H2O2) and other organic acids as well as antimicrobial peptide compounds called bacteriocins. This metabolite plays the most important role in fighting infection. The advantages of bacteriocins compared to other antimicrobial compounds are that they work selectively, are safe and can overcome resistance. Acidocin A and Lactacin are examples of bacteriocins produced by *Lactobacillus acidophilus* (L.a), while caseicin is a bacteriocin produced by *Lactobacillus casei* (L.c). Bacteriocin is an antibacterial peptide that is synthesized ribosomally, is produced by several bacteria and has a bactericidal or bacteriostatic effect on bacteria that have a close relationship with the producing bacteria [10].

Lactobacillus rhamnosus GG (LGG), was originally discovered by Sherwood Gorbach and Barry Goldwin, who conducted research by isolating bacterial samples from healthy adult humans. These good bacteria are natural grampositive bacteria that are identified as a type of probiotic because these bacteria can withstand stomach acid and bile, have a good growth rate, and can adhere (adhesion) to the epithelial lining of the intestine. Since the discovery of this bacteria, it has been widely researched and can easily be found in various commercially available probiotic products [11]. Yoghurt is a product that is generally used as a carrier for various probiotic agents. It is known from research conducted by Nuraida (2014) that *Lactobacillus rhamnosus* is one of the bacteria that can be combined with yoghurt [12]. The results of this study showed that yoghurt can not only carry one probiotic agent but can also carry two probiotics with different functional properties. Yoghurt that has been fermented by these bacteria is known to last for 32 days in cold storage and still maintains the levels of the probiotic.

Each type of probiotic bacteria has various compounds including different bacteriocins. The presence of bacteriocins produced by more than one type can help protect the producing bacteria from the effects of antimicrobial compounds produced by forest honey and stronger resistance to pathogenic bacteria.

CONCLUSION

Each combination of forest honey solution with probiotic milk showed antibacterial activity against *Escherichia coli* ATCC 8739 and *Staphylococcus aureus* ATCC 6538. The combination of forest honey with probiotic milk resulted in antibacterial activity at a ratio of 2:8. The inhibition zone of combining forest honey with probiotic milk is *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Lactobacillus casei*, and *Lactobacillus rhamnosus* had antibacterial activity against *Escherichia coli* ATCC 8739 and *Staphylococcus aureus* ATCC 6538 with an average inhibition zone of 16.38 mm, 11.47 mm, 14.73mm, 16.52mm. The best combination is forest honey with probiotic milk Lactobacillus rhamnosus.

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