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Research Article

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Comparison of the inhibitory effects of butterfly pea flower *(Clitoria ternatea L.)* extract and fermented milk probiotics on the growth of *Salmonella* sp.

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ABSTRACT

The increasing resistance of pathogenic bacteria such as *Salmonella* sp. to antibiotics has prompted the need for alternative antibacterial agents derived from natural sources. This study aimed to compare the inhibitory effects of prebiotics and probiotics against *Salmonella* sp. in vitro. The research was a laboratory experimental study using a post-test-only control group design. The prebiotic used was derived from butterfly pea flower (*Clitoria ternatea* L.) extract, while the probiotic was obtained from cow's milk fermented with Lactobacillus casei. The test organism was *Salmonella* sp., obtained from a pure culture. The well diffusion method was used to evaluate antibacterial activity by measuring the diameter of the inhibition zone using a vernier caliper. Five concentrations (0.65%, 1.25%, 2.5%, 5%, and 10%) were tested in triplicate. The data were analyzed using descriptive statistics to calculate the mean inhibition zone diameter. At a concentration of 1.25%, the average inhibition zone of the probiotic was 8.08 mm, while the prebiotic produced a zone of 7.4 mm. Both agents showed larger inhibition zones compared to the negative control. The findings indicate that both prebiotics and probiotics exhibit antibacterial activity against *Salmonella* sp., with probiotics showing slightly stronger effects. These substances have the potential to be developed as natural antibacterial agents.

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INTRODUCTION

The five primary causes of child morbidity and mortality in developing nations are malnutrition, measles, diarrhea, acute respiratory infections (ARI), and malaria. The leading cause of death for newborns and young children is diarrhea, an infectious disease. According to its etymology, diarrhea can be caused by immunological inadequacies, allergies, chemical or dietary toxicity, malabsorption, and pathogenic attacks. *Enterotoxigenic E. Coli, Enteropathogenic E. Coli, Enteroaggregative E. Coli, Enteroinvasive E. Coli, Enterohemorrhagic E. Coli, Shigella* spp., *Campylobacter jejuni/Helicobacter*

jejuni, Vibrio cholerae 01 and V. cholerae 0139, and Salmonella nontyphoidal are among the pathogenic bacteria that cause diarrhea. *Salmonella typhi, Shigella species,* and *Escherichia coli* are among the bacteria that cause diarrhea in children (Rusdianto et al., 2022).

Salmonella sp. is one of many commonly found bacteria that causes food contamination and can cause damage and loss of nutrients (Baskara et al., 2014). Typhoid fever can be brought on by *Salmonella* sp. contamination in food items. The symptoms include a high temperature, constipation, abdominal pain, lightheadedness, skin itching, the development of red patches, and even unconsciousness. Raw food items like eggs and raw chicken flesh can harbor salmonella, which can grow if improper food preparation is carried out (Suhandayati et al., 2024).

Salmonella sp. bacteria have the ability to reproduce quickly because a warm and humid environment can stimulate their growth (Ihsan et al., 2018). Then the diseases generally caused are those of the digestive organs (Amiruddin et al., 2017). *Salmonella* sp. bacteria belong to the group of Gram-negative bacteria with shorter stem cell shapes. Gram-negative bacteria generally have a higher percentage of lipids and fats compared to gram-positive bacteria (Asih et al., 2023).

The conventional treatment for diarrhea is oral antibiotics. Giving antibiotics allows bacteria to adapt to antibiotics by forming new strains, causing resistance to antibiotics. This condition makes research begin to be directed toward finding effective alternative treatments (Hermawati & Islamy, 2023). Nowadays, the rate of use of antibiotics or antibacterials is rising rapidly. To suppress the use of over-fabricated antibiotics, it's necessary to do a study to make an antibacterial with natural ingredients. Antibacterial manufacturing with natural ingredients is expected to be able to reduce the harmful side effects of growing. Besides, the materials that are present in the surrounding environment or nature should be exploited as best as possible.

The most important side effect of overusing antibiotics is the development of antibiotic resistance. No less important than the effect is the increasing side effects on health, so the cost of treatment will rise (Fatmah et al., 2019). The widespread misuse of antibiotics leads to antibiotic resistance in bacteria. Antibiotic resistance in bacteria can have fatal consequences, as infectious diseases caused by bacteria resistant to treatment increase the morbidity and hospitalization of individuals. When treatment becomes slow or even fails, patients can become carriers of bacteria. This allows antibiotic resistance to spread to more people (Hermawati et al., 2024).

One of the ingredients in functional foods that are always changing in the modern world is prebiotics. Because these prebiotics can increase the growth and activity of probiotic bacteria, they are beneficial, particularly for humans (Setiarto et al., 2015). One of the functional food ingredients being explored at the moment is prebiotics. Because they can promote the growth and activity of probiotic bacteria in the colon, these prebiotics are beneficial, particularly for humans (Yuliastuti et al., 2024). Due to their low production costs and lack of toxicity, natural substances are in high demand for treatment. The utilization of telang flowers is one among them. The telang flower, also called butterfly pea (*Clitoria ternatea L.*), has recently gained popularity among Indonesians as a flower with numerous health advantages (Hermawati & Islamy, 2023).

Butterfly pea flower (*Clitoria ternatea L*) belongs to the Leguminoceae plant family, which has pharmacological benefits. In Indonesia, this plant is found in Java, Sumatra, Maluku, and Sulawesi (Hawari et al., 2022). The study used calf as a prebiotic because it contained chemical components such as anthocyanins, flobatanins, saponins, tannins, proteins, carbohydrates, phenols, flavonoids, triterpenoids, antrakuinon, volatile oils, steroids, and flavol glycosides. One of the phytochemicals that

can prevent microbial cells from synthesizing nucleic acids, interfere with the metabolism of microorganisms, and alter cell membrane activity is flavonoid (Yurisna et al., 2022).

Probiotics are dietary supplements containing live microorganisms that can help the hosts they are consumed by. Probiotics are highly promising as anti-inflammatory drugs (Aviany & Pujiyanto, 2020). Probiotics are single or mixed cultures of living microorganisms that are administered to humans or animals as fermentation products or dried cells. They can help the host by boosting the amount of normal body flora. Lactic acid bacteria (LAB) are the probiotics that have the greatest beneficial effects on the human digestive system. These are Gram-positive bacteria that can survive in both aerobic and non-aerobic environments (Hermawati et al., 2016).

One outcome of biotechnological applications including a number of lactic acid bacteria exploitation techniques is the production of probiotic goods. In the production of probiotic goods, Lactobacillus species—particularly Laktobacilli casei—are frequently utilized. Bacteria must be safe for human consumption, have good stability and viability, and grow well in vitro in order to be employed as probiotic microbes (Sunaryo et al., 2014). The production of bacteriocins with bactericidal qualities by lactic acid bacteria has been crucial in improving the safety of fermented food products. Nisin, a bacteriocin produced by lactic acid bacteria, is the only one that has been identified and used in food products to date. Acidophilucin A is a bacteriocin that Lactobacillus acidophilus can produce. Bacteriocins and organic acids may be responsible for Lactobacillus acidophilus's antibacterial qualities. Its antibacterial qualities may also be influenced by the presence of bacteriocins, other organic acids, and microbial competition with other bacteria. Probiotics have a number of positive effects, including as reducing cholesterol, preventing colon cancer, and treating dermatitis (Yuliastuti et al., 2024).

Using the characteristics of Lactic Acid Bacteria (LAB) strains, Lactobacillus-fortified milk is used as a probiotic in this study. These strains are promising because they generate bacteriocins, which are bactericidal bioactive peptides and enzymes that efficiently inhibit the growth of infections and the formation of biofilms. Some LAB strains have demonstrated remarkable effectiveness against Staphylococcus aureus that produces biofilms (Hermawati et al., 2020).

Although *Salmonella* sp.-caused diarrhea has a major impact in Indonesia, especially in urban and rural areas, there is a dearth of targeted research addressing the unique problems and solutions associated with this problem. By examining how well natural probiotics and prebiotics work to prevent *Salmonella* sp. infections, this study seeks to close this gap. While many elements of treating diarrhea have been studied in the past, few studies have specifically evaluated the effectiveness of probiotics and prebiotics made from natural sources in the area against *Salmonella* sp. For example, Yuliastuti et al. (2024) concentrated on the health advantages of probiotics, while Hermawati et al. (2023) demonstrated the potential of natural components in lowering antibiotic resistance. Comprehensive investigations that explicitly examine the inhibitory effects of these two strategies on *Salmonella* sp. are still lacking, nevertheless. By comparing the inhibitory effects of probiotics and prebiotics on *Salmonella* sp., our study will add to the body of literature already in existence and make the novelty of our research more clear.

The primary objective of this research is to assess and contrast the ways in which probiotics and prebiotics suppress the growth of *Salmonella* sp. bacteria in vitro using an experimental laboratory designed with the Post-evaluate-Only Control Group. We hope to get knowledge on the efficacy of these natural substitutes in order to offer suggestions for better ways to treat pediatric diarrhea, which would ultimately improve health outcomes in developing nations.

RESEARCH METHODS

This study employed a post-test-only control group design and a laboratory experimental methodology. Comparing the antibacterial activity of probiotics and prebiotics against Salmonella sp. in vitro was the aim. The research was carried out at the Test Services Unit of the Faculty of Pharmacy, Universitas Airlangga, Surabaya, from July to August 2022. Salmonella sp., a pure culture supplied by the Test Services Unit of the Faculty of Pharmacy, Universitas Airlangga, served as the biological sample for this investigation. Among the treatment samples were: 96% ethanol was used to extract the prebiotic extract from dried butterfly pea flower *(Clitoria ternatea L.).* Lactobacillus casei-inoculated fresh cow's milk is fermented to create a probiotic solution. Both prebiotic and probiotic samples were tested at five concentrations: 0.65%, 1.25%, 2.5%, 5%, and 10%.

Treatment type (probiotic vs. prebiotic) and concentration (five levels) are independent factors. The diameter of the inhibition zone that developed around the wells (in millimeters) is the dependent variable. Control variables include the test organism *(Salmonella* sp.*)*, temperature (36°C), and incubation duration (24 hours). Vernier caliper (for determining the diameter of the inhibitory zone), analytical balance (for sample weighing), micropipette (to apply solutions), Incubator (for the development of germs), To standardize bacterial turbidity at 25% transmittance at 580 nm, use a spectrophotometer. Sterile cork borers and Petri dishes (to create wells), shaker, vortex, and autoclave. The butterfly pea flower *(Clitoria ternatea L.)* is the model for this prebiotic. Butterfly pea flower *(Clitoria ternatea L)* is extracted, and this extract is used as a prebiotic in this study.

A total of 500 grams of dried butterfly pea flower *(Clitoria ternatea L.)* powder was weighed and placed in a beaker glass. The powder was then macerated using 1000 mL of 96% ethanol (solvent ratio 1:2) at room temperature for 24 hours. After the first extraction period, the mixture was filtered to separate the filtrate and residue. The residue was remacerated with the same volume of solvent under the same conditions for another 24 hours. This process was repeated two more times. All filtrates obtained from each extraction cycle were combined and concentrated using a rotary evaporator at an appropriate temperature. The remaining extract was further dried in an oven until a thick extract was obtained.

For this milk-based probiotic, 100 milliliters of fresh cow's milk, heating it to 70°C, letting it cool, adding Lactobacillus casei bacteria, sealing the container, and letting it sit at room temperature for a whole day.The antibacterial activity of both probiotics and prebiotics was tested using the well diffusion method. The test bacteria *(Salmonella* sp.*)* were adjusted to 25% transmittance at 580 nm and added to the seed layer (20 mL), followed by the foundation layer (30 mL) in each petri dish. After solidification, wells were made in the agar. Each well was filled with 100 µL of either the probiotic or prebiotic sample at concentrations of 10%, 5%, 2.5%, and 1.25%. Sterile distilled water was used as the negative control for probiotics, pure ethanol was used as the negative control for prebiotics, and kanamycin served as the positive control for both. The plates were then incubated at 36°C for 24 hours. The diameters of the inhibition zones were measured using a vernier caliper and recorded in millimeters.

The primary data in this study were obtained from the results of an in vitro antibacterial assay using the well diffusion method. Data collection was carried out by measuring the diameter of the inhibition zones formed around the wells after treatment with prebiotic and probiotic samples at various concentrations (10%, 5%, 2.5%, and 1.25%). Each treatment was replicated three times. After 24 hours of incubation at 36°C, the clear zones around each well were measured using a vernier caliper in millimeters. The average inhibition zone diameter for each treatment group was recorded and used as the primary research data.The inhibition zone diameters obtained from each treatment group were

measured three times and analyzed using both descriptive and inferential statistics. Descriptive analysis was used to calculate the mean and standard deviation of the inhibition zone diameters at each concentration level. To determine whether there were statistically significant differences in antibacterial activity between concentrations and between treatment types (prebiotic vs. probiotic), one-way ANOVA was employed. An independent samples t-test was also conducted to compare the effects of prebiotics and probiotics at equivalent concentrations. Statistical significance was set at $p < 0.05$.

FINDING AND DISCUSSION

Based on research conducted using diffusion to form an inhibition zone around the well, The prebiotic and probiotic concentrations used in this barrier force test used five different concentrations, with each concentration replicated three times. The diameter formed around the inhibition zone is measured using the length of the spindle. Here's a picture (Figure 1) of the inhibition zone formed against prebiotics:

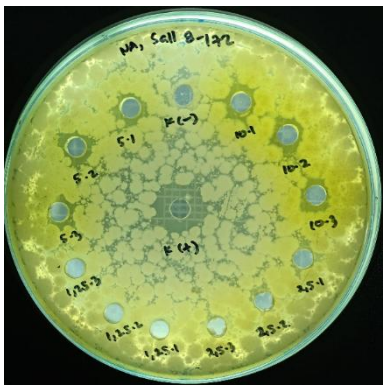

Figure 1. Prebiotic Resistance Test Results Against *Salmonella* sp. Bacteria

In addition to the use of prebiotics, the inhibitory test was performed using probiotics. The probiotic is the result of the processing of fresh cow's milk that was added with the bacteria Lactobacillus casei. Here's a picture (Figure 2) of the inhibition zone formed against probiotics: The prebiotic inhibitory test produced an inhibition zone at 7.4 mm and a minimum barrier at 1.25% concentration. The results of the probiotic inhibitory test showed an inhibition zone of 8.08 mm and a minimum barrier at a dosage of 1.25%. The average inhibitory zone created by prebiotic and probiotic use is shown in the following Table 1.

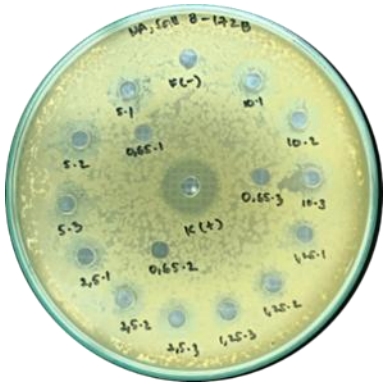

Figure 2. Results Of A Probiotic Resistance Test Against *Salmonella* sp. Bacteria

Table 1. Results Of Prebiotic And Probiotic Resistance Test Against *Salmonella* sp. Bacteria

Concentration (%)	Prebiotic (mm)	Probiotic (mm)
10	11,85	10,92
5	10,15	9,47
2.5	8,72	8,93
1.25	7,40	8,08
Control (+)	19.05	20,35
Control (-)	7,40	7,25

The Independent Samples Test results show that p > 0.05, so there is no significant difference between prebiotics and probiotics at the same concentration, as shown in the following Table 2. The results of one-way ANOVA showed that p < 0.05, so there is a significant difference between concentrations within one treatment type (prebiotic or probiotic), as shown in the following Table 3.

Table 2. Independent Samples Test (Levene's Test for Equality of Variances 95% Confidence Interval of the Difference)

Inhibitory zone	F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Equal variances assumed	1.257	.305	.160	6	.878	.18000	1.12651	-2.57648	2.93648
Equal variances not assumed			.160	5.029	.879	.18000	1.12651	-2.71077	3.07077

Table 3. One-way ANOVA

Inhibitory zone	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	205.327	3	68.442	48.608	.000
Within Groups	33.793	24	1.408		
Total	239.120	27			

To compare the antibacterial efficacy of probiotics and prebiotics against *Salmonella* sp., statistical analysis was performed. When prebiotics and probiotics were compared at the same concentration, the independent samples t-test revealed p-values greater than 0.05, suggesting that there was no statistically significant difference in the mean inhibitory zone widths between the two treatment types. At the 95% confidence level, the difference between probiotics and prebiotics was not significant, despite the fact that probiotics often created slightly wider inhibitory zones.

In contrast, p-values less than 0.05 were found in both the probiotic and prebiotic treatment groups by the one-way ANOVA, indicating that concentration levels significantly impacted the inhibitory zones' diameter. This indicates that the dosage used affected both drugs' antibacterial effectiveness, with higher concentrations typically resulting in bigger inhibition zones. According to these results, probiotics and prebiotics have comparable levels of antibacterial activity at the same concentration, but their inhibitory effects are much increased at higher concentrations. Therefore, in order to maximize antibacterial effectiveness, the concentration level must be optimized.

The ability of a chemical or substances to stop the development of bacteria is tested using an inhibitory test. The development of an inhibition zone indicates a substance's capacity to inhibit microorganisms. This barrier area is a transparent region that surrounds a disc or well (Lestari et al., 2020). The methods that can be used in this test are divided into two: the diffusion method and the dilution method. However, the most commonly used method is the diffusion method with wells (Retnaningsih et al., 2019). similar to the one employed in this research. Due to the method's benefit, measuring the inhibition zone's diameter is made simpler.

This study was done using three repetitions of the examination at each concentration. It was done to increase the number of repetitions, which increases the severity of the results (Putri et al., 2023). The results of the minimum inhibitory concentration test for prebiotics against *Salmonella* sp. bacteria showed that the average inhibition zone diameter was 7.4 mm at a prebiotic concentration of 1.25%. The positive control's inhibition zone had a diameter of 19.05 mm, while the negative control's was 7.4 mm. Absolute alcohol served as the negative control, and the antibiotic kanamycin served as the positive control. Both gram-positive and gram-negative bacteria can be effectively combatted by the broad-spectrum antibiotic kanamycin. The only characteristic that sets this antibiotic apart as a constitutional isomer is the quantity of amino and hydroxyl groups it possesses (Fanayoni et al., 2019).

The average inhibition zone diameter was 8.08 mm at a probiotic dosage of 1.25%, based on the results of the probiotics' minimum inhibitory concentration test against *Salmonella* sp. bacteria. An inhibitory zone measuring 20.35 mm in diameter was generated by the positive control, and one measuring 7.25 mm by the negative control. The antibiotic kanamycin served as the positive control in the probiotic and prebiotic inhibitory studies. However, sterile water was employed as the negative control in the probiotic inhibition test. Based on the results above, it shows that prebiotics and probiotics have the ability to act as antibacterials. Using low quantities of probiotics and prebiotics, the inhibitory zone's average diameter, it was able to form a large inhibition zone. It's probable that the diameter of the inhibitory zone that forms will increase with larger prebiotic and probiotic concentrations.

Butterfly pea flower *(Clitoria ternatea)* prebiotics have antibacterial qualities, especially against dangerous bacteria like Streptococcus mutans, a major cause of dental cavities. Alkaloids, flavonoids, and tannins are among the phytochemical components found in the flowers that are thought to have antibacterial properties. These substances are more effective as natural antibacterial agents since they not only prevent bacterial growth but also interfere with the production of biofilms. The processes and efficacy of butterfly pea extracts are explained in more detail in the sections that follow.

The butterfly pea flower *(Clitoria ternatea L)* leaf extract provided the prebiotics used in this investigation. Flavonoid phytochemical substances are present in the extract of butterfly pea flowers *(Clitoria ternatea L)*. Water-soluble polyphenols and their secondary metabolites are called flavonoids. These flavonoids generally have biological advantages as anti-inflammatory and anti-virus substances (Abdillah et al., 2022). Furthermore, triterpenoid phytochemical substances possess antimicrobial properties. The secret is to harm the membrane of the bacterial cell. If the antibacterial active component dissolves lipids and their permeability system or reacts with the active side of the bacterial membrane, cell membrane damage results. Antibacterial substances can infiltrate bacterial cells by increased permeability, lyse their membranes, or coagulate their cytoplasm (Widhowati et al., 2022). Consequently, it is consistent with studies to ascertain if prebiotics derived from butterfly pea flower *(Clitoria ternatea L)* extract had antibacterial properties.

Butterfly pea flowers' complex phytochemical makeup is largely responsible for their antibacterial properties. These substances, which have been demonstrated to function as natural antibacterial agents, include alkaloids, flavonoids, tannins, saponins, and ternatins (Salmiah & Nainggolan, 2024). Bioactive substances with antibacterial qualities can be found in butterfly pea flowers. It is well known that alkaloids and flavonoids cause bacterial cell membranes to rupture, which results in cell lysis. Particularly, flavonoids are well known for their capacity to damage bacterial cell membranes and obstruct vital enzymatic processes, which in turn prevents the growth of the bacteria (Adhiningtyas et al., 2023).

Lactic acid, hydrogen peroxide (H_2O_2), and other organic acids, as well as antimicrobial peptide compounds known as bacteriocins, are among the metabolic products that lactic acid bacteria make and may contribute to their capacity to fight infectious illnesses. In the battle against infection, this metabolite is the most crucial. When compared to other antimicrobial substances, bacteriocins have the advantages of being safe, selective, and capable of conquering resistance (Hermawati et al., 2024). It can even stop the growth of strains of extended-spectrum beta-lactamase (ESBL) and methicillin-resistant Staphylococcus aureus (MRSA) (Purwijantiningsih, 2014).

A crucial component of probiotic supplements is their survivability and functional activity. Probiotics can stop harmful bacteria from growing and acting, especially those that are already within the body (Purwijantiningsih, 2014). Probiotics are used to keep harmful bacteria from growing in humans and to balance the number of good microorganisms. This strategy can lessen the need for antibiotics, which can lead to the development of resistance in dangerous bacteria. Additionally, a rise in bioactive chemicals during the fermentation process activates the mechanism (Turista et al., 2023). This hypothesis is consistent with data showing that *Salmonella* sp. bacteria are harmful bacteria and that probiotics are among the things that can stop harmful bacteria from growing.

According to the study's findings, probiotics made from fermented cow's milk and prebiotics made from butterfly pea flower *(Clitoria ternatea L.)* extract both showed antibacterial activity against *Salmonella* sp. The probiotic generated an average inhibitory zone of 8.08 mm at a concentration of 1.25%, whereas the prebiotic created a zone of 7.4 mm. While the effects of both treatments were similar, the probiotic's antibacterial capacity was somewhat higher. The presence of antimicrobial metabolites in both agents—such as bacteriocins and organic acids in the probiotic and flavonoids and triterpenoids in the prebiotic—supports these conclusions.This conclusion provides an answer to the study's goal, which was to compare the in vitro inhibitory effects of probiotics and prebiotics against *Salmonella* sp. The findings reveal that both substances work well, with probiotics exhibiting a larger inhibitory zone at the same dose.The results of this investigation can be used as a guide to help create natural antibacterial products that contain probiotic and prebiotic substances. It is advised that these medicines be tested in vivo and at larger concentrations in future studies, particularly for therapeutic or food safety applications. These findings could also help the public by reducing foodborne bacterial illnesses without the need for synthetic antibiotics.

CONCLUSION

This study aimed to compare the inhibitory effects of prebiotics and probiotics against *Salmonella sp.* in vitro. The results showed that at a concentration of 1.25%, the probiotic formed a larger inhibition zone (8.08 mm) than the prebiotic (7.40 mm), indicating that both agents have antibacterial activity, with probiotics exhibiting slightly stronger effects. These findings suggest that both prebiotics and probiotics can serve as natural antibacterial agents and have potential to be developed further as alternatives to conventional antibiotics. Further research is recommended to examine higher concentrations and in vivo applications of both agents. Communities and industries may also consider utilizing local plant-based prebiotics and dairy-based probiotics as affordable and natural approaches to support food safety and public health.

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