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The Effectiveness of Chocolate in Reducing the Number of Methicillin-Resistant *Staphylococcus aureus* Colonies in *Rattus norvegicus*

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Abstract
Chocolate has long been understood to provide positive emotions and a good mood if consumed in moderation. Chocolate contains prebiotics naturally from its constituent ingredients produced during the production process. Prebiotics, frequently oligosaccharides, are substances which cannot be metabolized by the human digestive system but can be employed by a group of bacteria in the gut, understood as probiotics. The positive relationship among them provides benefits for the host in eliminating pathogens. One of the well-known pathogens which frequently cause infection either in the community or in hospitals is methicillin-resistant *Staphylococcus aureus* (MRSA). Since it was first identified in 1960, MRSA has caused health problems until today. Research conducted on two groups of *Rattus norvegicus* infected with MRSA and then fed chocolate revealed a decrease in the average number of bacterial colonies on the skin compared to the control group. In the group fed chocolate at a dose of 50 mg/day, the bacterial colonies decreased to 1.28 x 10^8 CFU/cm^2 in 7 days, lower than in the control group (1.46 x 10^8 CFU/cm^2) at the same time. While those fed 75 mg/day chocolate decreased to 2.70 x 10^7 CFU/cm^2 and the three groups were significantly different (0.000<0.05). Prebiotics fermented by probiotics release short-chain fatty acids (SCFA), which compete with the pathogens for attaching to the epithelial wall so that pathogens lose space and nutrients to survive. However, the adverse effect of chocolate may occur because it contains sugar which is a nutrient for bacteria, but if the balance of normal flora and adequate intake of prebiotics are administered, the pathogen could be eliminated.

Keywords: Chocolate, Prebiotics, Probiotics, Methicillin-resistant Staphylococcus *aureus*.

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1. INTRODUCTION

Chocolate is a popular food consumed worldwide and able to produce the effect of increasing pleasure sensory and positive emotions (Konar, et al., 2016). Chocolate can be employed as an excellent means of generating prebiotics into the body. Prebiotics are nutrients for beneficial bacteria (probiotics) in the human digestive tract (Ejtahed, et al., 2011; Rad, et al., 2016; Rad, et al., 2012; Rad, et al., 2012; Homayouni, et al., 2012; Homayouni, et al., 2012; Rad, et al., 2013; Rad, et al., 2013). Prebiotics such as inulin, fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS) have been assigned as the subject of research for a long time (Gonzalez, et al., 2011; Morais, et al., 2014). Prebiotics have also been revealed contributing to the treatment of diseases encompassing allergic contact dermatitis (ACD), acne and aging primarily by increasing the probiotics growth (Lolou, & Panayiotidis, 2019). With these advantages, chocolate is considered as a healthy food (Rad, et al., 2018; Scheid, et al., 2013). This feature makes chocolate admitted as an attractive non-fermented product which functions as a protector for the probiotic bacteria survival (Granato, et al., 2010; Lahtinen, et al., 2007).

The World Health Organization (WHO) defines probiotics as living organisms providing benefits to their host if they occur in sufficient quantities (Lin, et al., 2014). Probiotics are able to formulate vitamins, antioxidants, short chain fatty acids (SCFA) and compete with pathogens by various mechanisms, for instance through protein defensins (Roberfroid, et al., 2010; Wallace, et al., 2011). *Escherichia coli* strain Nissle, lactic acid producing Lactobacillus and a group of Bifidobacteria are primary probiotic organisms among the many microbes which meet the WHO criteria (Lin, et al., 2014).

One of the well-known pathogens infecting wounds is methicillin-resistant *Staphylococcus aureus* (MRSA) (Sikorska, & Smoragiewicz, 2013). *Staphylococcus aureus* is a spherical, Gram-positive, nonmotile, coagulase-positive bacterium, and a member of the phylum Firmicutes. *Staphylococcus aureus* is a commensal microbe of the nasal mucosa in 20-40% of the total population (Lee, et al., 2018). A year after methicillin was employed in the clinic, MRSA was identified from a hospital patient in 1960 (Turner, et al., 2019).

It has been proven that oligosaccharides, polysaccharides and lactose play a role as a prebiotic and is able to decrease the number of Salmonella in the digestive tract (El-Hack, et al., 2021), and probiotics such as *Lactobacillus acidophilus* and *Lactobacillus casei* which possesses the ability as an antibacterial agent against MRSA (Karska-Wysocki, et al., 2010). However, the reason why chocolate can be a source of prebiotics in reducing the number of bacteria is still not extensively identified.

2. RESEARCH METHOD

The research was conducted from October to November 2020 at the Laboratory of Microbiology, Department of Medical Laboratory Technology of Politeknik Kesehatan Kemenkes Pontianak. The objective of this study is to determine whether chocolate as a source of prebiotics is able to reduce MRSA infections. This study administered an in vivo quasi-experimental approach to healthy male *Rattus norvegicus*, aged 8-10 weeks and weighing 150-250 grams, and received a letter of approval from the Health Research Ethics Commission of Politeknik Kesehatan Kemenkes Pontianak No. 311/KEPK-PK.PKP/X/2020 on October 14, 2020.

The preparation of MRSA suspension: 1 colony was obtained from the MRSA culture stock and mixed into 0.9% sodium chloride solution. The turbidity established was compared to a standard McFarland turbidity 0.5 (0.05 mL of 1.175% barium chloride dihydrate ($BaCl_2.2H_2O$), with 9.95 mL of 1% sulfuric acid (H_2SO_4)). A suspension which turbidity is equivalent to this standard owns a density of 1.5 x 10^8 colony forming units (CFU)/mL (Aryal, 2020).

The preparation of Plate Count Agar: Into an Erlenmeyer containing 1 liter of distilled water, 17.5 grams of PCA were added and dissolved while heated, then sterilized by autoclaving at 121°C for 20 minutes. This medium was stored for up to 7 days at 2-8°C until utilized (Thermo Fisher Scientific, 2001).

A total of 45 rats undergone acclimatization were infected with MRSA on 1 square centimeter of skin. After the infection of wound occurred, each 15 rats not provided any treatment (control, group A), were fed chocolate (Bella, PT Dolphin, Indonesia) at a dose of 50 mg chocolate/day orally (group B) and were fed 75 mg chocolate/day orally (group C) (Eor, et al., 2019). The treatment had been performed for 7 consecutive days.

After the treatment period, the number of bacteria was assessed by the Total Plate Count (TPC) method as follows: swabs were conducted on the injured (and healing) rat skin by employing a sterile and moist cotton swab, placed into a tube containing 9 mL of 0.9% NaCl, then it was diluted in a dilution series of 1/10 to 1/1,000,000. Every 1 mL of the dilution tube was transferred to a sterile petri dish then poured with sterile, 45-55°C PCA. It was then mixed and let solidified at room temperature, then it was incubated at 37°Celsius for 24 hours. Colonies growing after the incubation were counted and considered as CFU/cm^2.

The number of bacterial colonies from the three groups was statistically processed by implementing the Kruskal Wallis and Mann-Whitney U test to determine if there were significant differences between groups. The test was conducted with the SPSS application.

3. RESULTS AND DISCUSSION

The bacteria growing on the skin surface of *Rattus norvegicus* are derived from MRSA suspension with a concentration of 1.5 x 10^8 CFU/mL, and normal skin flora coexisting. After measuring the bacteria by administering the TPC method, various data were obtained from the three treatment groups. Although there were outliers and extreme values (Figure 1), the three groups presented a linear decrease in the average number of bacterial colonies from the original number (Table 1). There was a difference in the number of colonies of 1.81 x 10^7 CFU/cm^2 between the control group and the group fed 50 mg chocolate/day and 1.19 x 10^8 CFU/cm^2 and fed 75 mg chocolate/day.

Table 1. Description of Bacteria Number (CFU/cm^2).

Group	Mean	Median	Standard Deviation
A	1.46 x10^8	1.53 x10^8	3.86 x 10^7
B	1.28 x 10^8	9.82 x 10^7	8.20 x 10^7
C	2.70 x 10^7	2.28 x 10^7	2.50 x 10^7

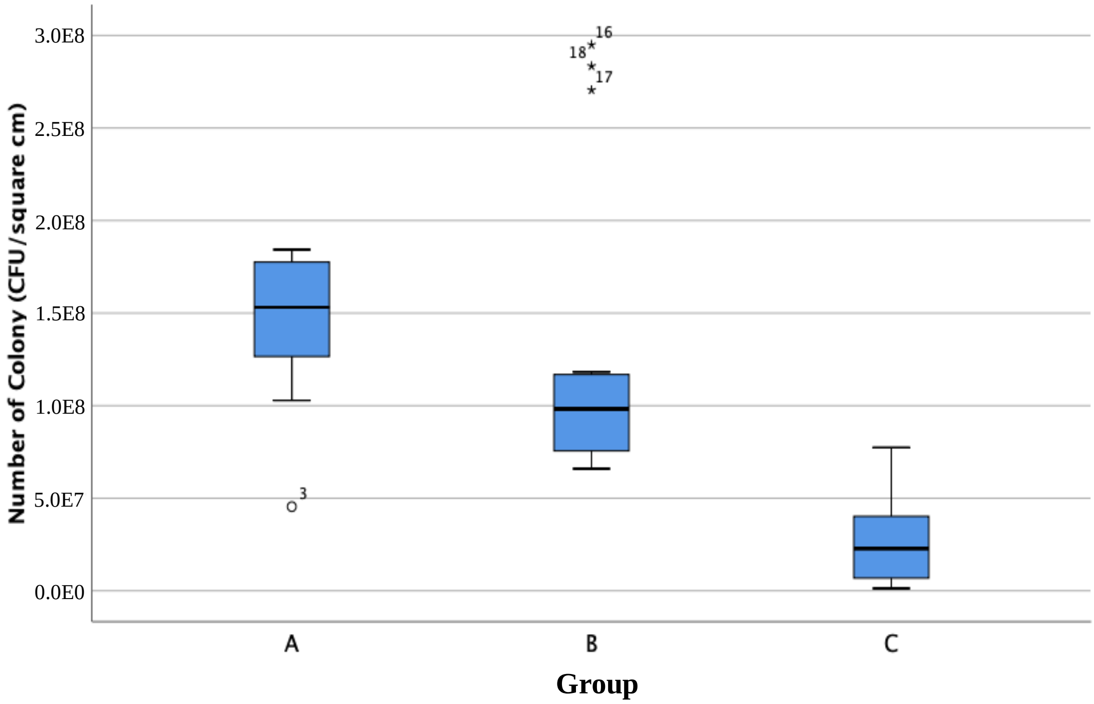

Figure 1. Distribution of research data.

The results of the Shapiro-Wilk test for the three groups presented a significant value of 0.034, 0.000, and 0.046 (< 0.05), respectively, indicating that the data is not normally distributed. After conducting a Levene's test, it was obtained a significant value of 0.004 which means that the data variance is not homogeneous. Hence, the results of these two tests became the assumptions for non-parametric tests. Kruskal Wallis test produced a significant value of 0.000, followed by the Mann-Whitney U test with the results as demonstrated in table 2. The last two tests displayed a significant difference ($\alpha < 0.05$) both between the three groups and between each group.

Table 2. The different of mean rank of test groups.

Group	**A**		**B**		**C**	
	Mann-Whitney U	**Sig.**	**Mann-Whitney U**	**Sig.**	**Mann-Whitney U**	**Sig.**
A			62.000	0.036	3.000	0.000
B	62.000	0.036			5.000	0.000
C	3.000	0.000	5.000	0.000		

Prebiotics in chocolate originate from natural ingredients, such as milk, cocoa and nuts or from synthetic ingredients administered directly to chocolate. Oligosaccharides such as inulin, FOS, GOS, soybean-oligosaccharide (SOS), lactulose, lactosucrose, xylo-oligosaccharides (XOS), isomalto-oligosaccharides (IOS) and resistant starch (Vinayak, et al., 2021) possess low molecular weight and cannot be digested by humans due to the absence of enzymes catalyzing them, but can be utilized by healthy microbes (probiotics) in the digestive tract (Al-Sheraji, et al., 2013; Khangwal & Shukla, 2019; Quigley, 2019; Vinayak, et al., 2021).

Dominant probiotics such as Lactobacillus and Bifidobacteria are able to ferment prebiotics and generate SCFA such as butyric acid, acetic acid, lactic acid and propionic acid (Ahmad & Khalid, 2018; Vinayak, et al., 2021) associated with the increased function of mineral absorption, digestion, glucose regulation, and lipid metabolism (Sarao, & Arora, 2017). If probiotics thrive in the digestive tract, the growth of pathogenic bacteria is inhibited.

The significant decrease in the average number of bacterial colonies revealed in this study indicates the suppression of MRSA growth which might be caused by the prebiotic performance in chocolate. The higher the dose of chocolate provided, the lower the number of bacterial colonies. There are two mechanisms of prebiotics in corroborating the host deal with pathogenic bacteria. The first mechanism is to inhibit pathogens directly through the formation of antimicrobial compounds and compete in attaching to the epithelial wall, for instance the SCFA formation, carbohydrate modulation and lipid metabolism. In the second mechanism, prebiotics help enrich the probiotics growth by increasing the absorption of essential nutrients and minerals such as calcium and magnesium (Slavin, 2013).

As MRSA is infected in the skin, the role of prebiotics which is more frequently to occur in study subjects is the first mechanism. It is because probiotics stimulated by prebiotics (second mechanism) reside in the gastrointestinal tract and reluctantly migrate to the skin and compete directly with MRSA, particularly if the host is in good physiological condition. Meanwhile, with the first mechanism, the compounds of SCFA are able to circulate with the bloodstream and modulate carbohydrate and lipid metabolism thereby inhibiting bacteria from attaching to the epithelium (as described above) and ultimately unable to survive.

However, some research subjects displayed an increase in the number of bacterial colonies. It should be concerned that in addition to containing prebiotics, chocolate also encompasses sugar which possesses nutritional value for bacteria in general. This sugar may originate from milk or administered directly to chocolate. Sugars discovered in chocolate encompass sucrose (the highest, 90% of all sugars), glucose, fructose, mannitol, galactose, sorbose, arabinose, xylose, and inositol (Barišić, et al., 2019). *Staphylococcus* is glucose, lactose, sucrose, and mannitol fermenter (El-Hadedy & Abu El-Nour, 2012; Ribeiro de Souza da Cunha, 2018). Fermentation is one of the metabolic pathways performed by microbes in producing energy. The ability of *Staphylococcus aureus*, including MRSA, in fermenting sugars contained in chocolate provides an advantage in maintaining life. It occurs in the host with unfavorable physiological conditions, such as stress or lack of nutrients so that the balance of normal flora in the digestive tract is disturbed. Instead of decreasing the number of pathogens, this condition produces nutrients and benefits for MRSA so that their numbers increase.

The dynamic conditions between the host and the pathogen cause variations in the response established. In reducing the number of pathogens and the possible risks, the host should be in ideal conditions or homeostasis, hence, it remains in a superior position and controls the pathogen. This condition can be obtained by maintaining the number of probiotics in the digestive tract and assisting it by providing prebiotics. Therefore, it becomes a worthy competitor for pathogens.

4. CONCLUSION

This study has revealed that consuming chocolate can help reduce the number of MRSA colonies on *Rattus norvegicus* skin through the SCFA formation mechanism which is effective in preventing bacterial attachment to epithelial cells and modulating carbohydrate and lipid metabolism. Furthermore, it should be considered that there may be an adverse effect because chocolate encompasses sugar which is a nutrient for bacteria. However, it can be enhanced if the host possesses a good balance of normal flora, adequate nutrition and is not stressed. Further research is required to conduct on what is the ideal dose of consuming chocolate so that the good effects can be obtained and the adverse effects can be prevented.

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