

## Antibacterial potential of red dragon fruit peel yogurt (*Hylocereus* spp.) against *Bacillus subtilis* bacteria in hypercholesterolemic wistar rats

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### ABSTRACT

**Background:** Fruit peel is a part of red dragon fruit that weighed 30-35% of the fruit weight and has not been used optimally. Red dragon fruit peel contains fiber, vitamin, flavonoid, tannin, alkaloids and has the potential as an antibacterial. Red dragon fruit peel can be processed into yogurt.

**Objectives:** This research examined the antibacterial potential of red dragon fruit peel yogurt against *Bacillus subtilis* in hypercholesterolemic Wistar rats.

**Materials and Methods:** Materials tested in this study were negative control, positive control, and caecum of hypercholesterolemic Wistar. This study used chloramphenicol as the positive control (K+) and DMSO 10% as the negative control (K-). The K1; K2; K3 were orally administered with 1.8 mL; 2.7 mL; 3.6 mL of red dragon fruit peel yogurt, respectively. Red dragon fruit peel yogurt was administered daily for 28 days. Caecum was collected and tested for antibacterial activity using disk diffusion (Kirby Bauer). The *Bacillus subtilis* was obtained from the Microbiology Laboratory of Center for Food and Nutrition Studies Universitas Gadjah Mada.

**Results:** The average inhibition zone in K-; K+; K1; K2; K3 were  $0.00 \pm 0.00$  mm;  $11.5 \pm 1.41$  mm;  $11.5 \pm 0.96$  mm;  $10.13 \pm 0.66$  mm;  $10.38 \pm 1.12$  mm, respectively. The experimental animal groups, which received 2.7 mL and 1.8 mL of red dragon fruit peel yogurts, showed a significant difference compared to the positive control group ( $p = 0.026$  and  $p = 0.021$ , respectively). When the dose was increased to 3.6 mL, it showed no statistical difference in results ( $p = 1.000$ ).

**Conclusions:** Red dragon fruit peel yogurt has an antibacterial potential against *Bacillus subtilis*.

**Keywords:** Antibacterial; *Bacillus subtilis*; Caecum; Red dragon fruit peel; Yogurt.

### BACKGROUND

Antibacterial is a substance produced by microbes or other sources that can inhibit growth and even eradicate pathogenic bacteria that are harmful to humans. Bacterial infection is one of the health problems in Indonesia. Gastrointestinal disorders are complaints caused by bacterial infection.<sup>1</sup> Pharmacological therapy using antibiotics is often chosen to treat bacterial infection problems. Bacterial infection therapy using antibiotics can cause problems. Antibiotics are components produced by microbes that can kill or inhibit the growth of other microbes. Antibiotics are also defined as a natural molecular inhibitor.<sup>2</sup> The problem that arises from antibiotic therapy is the occurrence of resistance. This resistance event is triggered by the use of antibiotics that are not following the proper dosage, thus causing pathogenic microorganisms to become resistant and infection therapy to be ineffective. *Bacillus subtilis* can survive in various environmental conditions on land, water, and even in anaerobic conditions inside

the digestive tract. These bacterial spores can even be blown by the wind to allow long-distance migration.<sup>3</sup> *Bacillus subtilis* can activate its host defense system so that the host becomes resistant to pathogens. *Bacillus subtilis* is a gram-positive bacterium commonly found in rotten bread.<sup>4</sup>

The discovery of new antibacterial compounds derived from natural ingredients can be an alternative solution to overcome the problem of antibiotic resistance. The sensitivity of each microorganism to a drug determines the lowest drug levels that can inhibit the growth of microorganisms in vitro. These antibacterial compounds can be obtained from plants. Plants contain compounds that have antibacterial potential with a new mechanism of action and have not yet experienced resistance. One of the plants that can function as an antibacterial is red dragon fruit.

Red dragon fruit is one type of dragon fruit species widely found and consumed in Indonesia. Dragon fruit is commonly known as pitaya, comes from the *Cactaceae* family<sup>5</sup> and is a tropical fruit.

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Pitaya has a CAM (photosynthetic crassulacean acid metabolism pathway) which allows efficient use of water.<sup>6</sup> Dragon fruit peel is a part of dragon fruit that weighed 30-35% of the fruit weight and has not been used optimally.<sup>7</sup> Red dragon fruit peel contains polyphenols and other active compounds. Active compounds other than polyphenols that can be found on red dragon fruit peel are alkaloids, terpenoids, flavonoids, saponins, tannins, carotenes, and phytoalbumins. Polyphenols such as flavonoids are more commonly found in the peel of red dragon fruit than in the flesh. Polyphenols as phenol components have many antioxidant activities. Betalain is a component associated with anthocyanins and is a reddish pigment found in red dragon fruit peel.

Processing of products from red dragon fruit peel which are considered as waste has already begun. To date, people know that red dragon fruit peel has many properties such as antioxidants, anti-hypercholesterolemia, and anti-diabetics. In research on antibacterial activity of n-hexane fraction from red dragon fruit peel against *Staphylococcus aureus* ATCC 25923, it was known that the inhibition zone diameter of the n-hexane fraction at concentrations of 20 and 40 mg/mL are  $11.17 \pm 1.69$  mm and  $12.80 \pm 1.11$  mm respectively.<sup>8</sup> Another research using ethanol extract of red dragon fruit peel (*Hylocereus polyrhizus*) on *Staphylococcus aureus* ATCC 25923 in vitro showed that the minimum inhibitory concentration was obtained at a concentration of 25% and the best inhibition zone was produced by an extract with a concentration of 100%.<sup>9</sup> Other research on red dragon fruit peels also stated that red dragon fruit peel extract can inhibit the growth of *Salmonella pullorum* with an average inhibition zone diameter at concentrations of 60 mg/mL, 40 mg/mL, and 20 mg/mL are 9.6 mm, 9.4 mm and 9.3 mm, respectively.<sup>10</sup> Research on the potential of red dragon fruit peel has not been done much, and it can be a great opportunity to explore red dragon fruit peel. Septiana's pre-clinical research shows that steeping dragon fruit peel can reduce plasma MDA levels in Sprague Dawley dyslipidemic rats. MDA is one of the parameters for free radical compounds. An increase in MDA value indicates an increase in oxidative stress in body cells.<sup>11</sup> Mardiana's research on red dragon fruit peel shows that red dragon fruit peel can be processed into yogurt and has the potential to be a functional food.<sup>12</sup> Yogurt can support human health and survival and improve dysbiosis due to aging.<sup>13</sup> Red dragon fruit added to yogurt with concentrations of 3%, 5%, and 7% showed antimicrobial activity with moderate to high inhibition zones because the growth of *E. coli* reached the range of 6.06 - 15.29

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mm.<sup>9</sup> Research on the antibacterial activity of red dragon fruit peel is only limited to the extraction process and there has been no further research related to processed dragon fruit peel products. This study aims to determine the antibacterial properties of red dragon fruit skin yogurt against *Bacillus subtilis* in hypercholesterolemic rats.

## **MATERIALS AND METHODS**

### **Materials and Tools**

This research uses experimental animal handling and facilities of CNFS laboratory, Universitas Gadjah Mada, Yogyakarta based on the Guidelines for Care and Use of Laboratory Animals. The ethical letter was obtained from the Committee on Ethics of Health, Universitas Negeri Semarang number 140/KEPK/EC/2019. House of Experimental Rats CNFS, Universitas Gadjah Mada, Yogyakarta Indonesia is the provider of Wistar rats used in this study. The inclusion criteria were male Wistar rats, active, aged 8-12 weeks, weighed 150-240 grams with fasting blood glucose level  $<110$  mg/dL, while the exclusion criteria were Wistar rats with anatomical abnormalities and had a weight loss of more than 10% during the study. During the study, no Wistar rats were dropped out because all animals were able to follow the intervention until the end.

The laboratory environment is conditioned to have a dark/light cycle with a ratio of 12 hours:12 hours with a room temperature of  $25 \pm 1$  °C to maintain room humidity. The cages were always kept clean by removing feces every day, to minimize the stress of Wistar rats during the study. Wistar rats were placed in individual cages made of stainless steel and received standard feed with ad libitum water access. Wistar rats had an adaptation period of seven days before the study was carried out.

### **Hypercholesterolemia Induction**

Hypercholesterolemic conditions in Wistar rats were obtained through single intraperitoneal injection of feed powder made of 1% cholesterol and 0.5% cholic acid given for 14 days.<sup>14</sup> Cholesterol and cholic acid powder were obtained from Sigma Aldrich, Japan.

### **Experimental Design**

Materials used in this study were caecum from each group of hypercholesterolemic Wistar rats. The study used were K-: DMSO 10% as negative control; K+: chloramphenicol as positive control; K1: caecum of hypercholesterolemic Wistar rats received red dragon fruit peel yogurt as much as 1.8 mL/kg b.wt/day; K2: caecum of hypercholesterolemic

Wistar rats received red dragon fruit peel yogurt as much as 2.7 mL/kg b.wt/day and K3: caecum of hypercholesterolemic Wistar rats received red dragon fruit peel yogurt as much as 3.6 mL/kg b.wt/day. Other materials required in this study were aqua dest, nutrient agar, chloramphenicol, and Dimethylsulfoxide (DMSO).

The tools used were separating funnel, rotary evaporator, ose needle, petri dish, vennai caliper, incubator, and autoclave. Tools such as separating funnels, ose needles, petri dishes, and vernier caliper were sterilized in an autoclave for 15 minutes at 121°C by regulating a pressure of 1.5 atm having previously been washed clean, dried, and wrapped in paper.

### **Procedures for Producing Red Dragon Fruit Peel Yogurt**

Red dragon fruit peel yogurt was produced based on research by Mardiana<sup>13</sup> and Putriningtyas.<sup>15</sup> Production of red dragon fruit peel yogurt begins with making a starter. Culture rejuvenation or yogurt starter making was carried out using *Lactobacillus bulgaricus* or *Streptococcus thermophilus*, each of which was inoculated in full cream pasteurized milk for 18 hours at 42°C. The next step is selecting or sorting the red dragon fruit peel. Fresh red dragon fruit peel is separated from the pulp and cleaned using running water. Red dragon fruit peel is steamed for 15 minutes to remove the distinctive aroma of the peel and reduce the pectin content in the red dragon fruit peel. Red dragon fruit peel is blended with full cream milk in a ratio of 1:4. Suspension of steamed red dragon fruit peel with full cream milk is then added with 10% sucrose, and covered with aluminum foil for the next pasteurization process at a temperature of 75-85°C for 15 minutes. The result of the pasteurized mixture of steamed red dragon fruit peel, full cream milk, and sucrose is cooled to a temperature of 35-37°C then added with 5% *Lactobacillus bulgaricus* and 5% *Streptococcus thermophilus* yogurt starter. The last step in making red dragon fruit peel yogurt is incubation at 42°C for seven hours.

### **Testing the Antibacterial Activity of Red Dragon Fruit Peel Yogurt**

The antibacterial activity test of the sample was carried out on *Bacillus subtilis* obtained from the Microbiology Laboratory of the Center for Food and Nutrition Studies, UGM. *Bacillus subtilis* was rejuvenated by inoculating pure culture into nutrient agar and incubated at 37°C for 24 hours. Antibacterial activity was measured using the Disc diffusion method (Kirby Bauer).<sup>16</sup> Bacteria from the rejuvenation media were spread on nutrient agar.

Empty test discs that have been soaked for 15 minutes in each caecum group of hypercholesterolemic Wistar rats were hygienically placed on the surface of the nutrient agar. The empty discs were immersed in 10 mL of caecum from each group of hypercholesterolemic rats. Media containing test discs were incubated at 37°C for 24 hours and then the diameter of the clear zone was measured using a vernier caliper. The negative control used was DMSO 10%, which was dripped as much as 20 µL on a paper disc. The positive control used in this study was chloramphenicol 0.5 mg. Chloramphenicol is a broad-spectrum antibiotic so it is appropriate to inhibit the growth of gram-positive and negative bacteria. All numerical data were expressed as mean±standard deviation from twice measurements. One-way analysis of variance (ANOVA) with Tukey's test was used to determine significant differences ( $p < 0.05$ ) between means.

### **RESULTS**

Determination of the antibacterial activity of red dragon fruit peel yogurt was carried out by the Kirby-Bauer disc diffusion method, which is the determination of the sensitivity of bacteria to certain substances that may have antibacterial activity, using paper discs. The results of the antibacterial activity of red dragon fruit peel yogurt against *Bacillus subtilis* in each group can be seen in table 1.

The average inhibition zone of K+, K-, K1; K2; K3 are each 11.50±1.41 mm; 0.00±0.00 mm; 11.50±0.96 mm; 10.13±0.66 mm; 10.38±1.12 mm. DMSO 10% as negative control has no inhibition zone. The categories of antibacterial inhibitory strength are inhibition zone diameter of 5 mm or less is categorized as weak, inhibition zone diameter of 5-10 mm is categorized as a medium, inhibition zone diameter of 10-20 mm is categorized as strong while the diameter of more than 20 mm is categorized as very strong.<sup>10</sup> The positive control using chloramphenicol (K1), which is the caecum of the experimental animal group which received 1.8 mL of red dragon fruit peel yogurt for 28 days, shows the widest inhibition zone.

The DMSO negative control group shows significant differences in the inhibition zone of each treatment group ( $p < 0.05$ ). Based on these results, it can be concluded that the red dragon fruit peel yogurt has antibacterial activity against *Bacillus subtilis*. The difference in the results of the antibacterial activity test in each group can be seen in table 2. Table 2 shows that there is no difference between the positive control group and the experimental animal group that received red dragon fruit peel yogurt at a dose of 1.8 mL and 3.6 mL ( $p > 0.05$ ). The

experimental animal group that received red dragon fruit peel yogurt with a dose of 1.8 mL shows a significant difference when compared to the group that received yogurt with a dose of 2.7 mL ( $p=0.021$ ). Table 2 also shows that there is no statistical

difference when the dose is increased to 3.6 mL ( $p>0.05$ ).

**Table 1. Difference Mean**

Group	Deuteronomy1 (mm)	Deuteronomy2 (mm)	Mean±SD (mm)
K-	0	0	0.00±0.00
K+	12.0	11.0	11.50± 1.41
K1	11.25	11.75	11.50± 0.96
K2	10.0	10.25	10.13± 0.66
K3	10.5	10.25	10.38± 1.12

*K-* (DMSO); *K+* (chloramphenicol); *K1* (caecum derived from red dragon fruit peel yogurt at a dose of 1.8 mL); *K2* (caecum derived from red dragon fruit peel yogurt at a dose of 2.7 mL); *K3* (caecum derived from red dragon fruit peel yogurt at a dose of 3.6 mL)

**Table 2. Post Hoc**

Group	Group	p*
K-	K1	0.001*
	K2	0.001*
	K3	0.001*
K+	K-	0.001*
	K1	0.219
	K2	0.026*
	K3	0.084
K1	K2	0.021*
	K3	0.370
K2	K3	1.000

*p\** significant post hoc test ( $p<0,05$ ); *K-* (DMSO); *K+* (Chloramphenicol); *K1* (caecum derived from red dragon fruit peel yogurt at a dose of 1.8 mL); *K2* (caecum derived from red dragon fruit peel yogurt at a dose of 2.7 mL); *K3* (caecum derived from red dragon fruit peel yogurt at a dose of 3.6 mL)

## DISCUSSION

Red dragon fruit peel yogurt in this study was made using *Lactobacillus bulgaricus* and *Streptococcus thermophilus* as starters. Probiotic activity in yogurt is largely determined by nutrient availability, inoculation levels, incubation temperature, fermentation time, storage conditions, pH, sugar concentration, and content of milk solids. Lactic acid bacteria in yogurt can break down complex carbohydrates into simple carbohydrates thus producing the final product in the form of lactic acid. Lactic acid as a result of fermentation metabolites shows the ability to inhibit microbial growth.<sup>17</sup>

This study used DMSO in negative control and chloramphenicol in positive control. DMSO is a good extract solvent because it can dissolve without affecting the growth of the bacteria so that it does not interfere with the results of the observation. Polar and nonpolar compounds can be dissolved by DMSO.<sup>18</sup> Chloramphenicol gives an inhibitory area, so this

shows that chloramphenicol is still sensitive to *Bacillus subtilis*.<sup>19</sup> Gram-positive bacteria have better antibacterial sensitivity than gram-negative bacteria. The composition and structure of cell walls of gram-positive bacteria are more susceptible to chemical components than gram-negative bacteria.<sup>20</sup> This sensitivity is due to differences in the structure of cell walls. Gram-positive bacteria have a relatively simple cell wall structure, making it easier for antimicrobial compounds to enter the cell and find targets to work. The structure of the cell wall of gram-negative bacteria is relatively more complex and consists of three layers, the outer layer is a lipoprotein, the middle layer is a lipopolysaccharide, and the inner layer is peptidoglycan. The middle layer in the form of lipopolysaccharide acts as a barrier against various antibacterial bioactive materials while the inner layer in the form of peptidoglycan has a high-fat content.<sup>1</sup> The results in table 2 show that there is no difference in the three administration of dragon fruit peel yogurt with chloramphenicol positive control. There was no

significant difference in the group that received 2.4 mL and 3.6 mL of red dragon fruit peel yogurt, so it can be assumed that administering 1.4 mL of red dragon fruit peel yogurt can also be used as antibacterial because it has the same antibacterial activity as the 3.6 mL. The process of making yogurt can trigger the production process of proteolytic enzymes by lactic acid bacteria. This proteolytic enzyme can cut peptide bonds in milk protein to form free amino acids and peptides.

Some peptides show antibacterial activity. Potential peptides that have antibacterial activity derived from milk proteins include Leu-Arg-Leu-Lys-Lys-Tyr-Lys-Val-Pro-Gln-Leu. These peptides are produced by the hydrolysis of pepsin in cow casein.<sup>21</sup> Antibacterial activity of these peptides also depends on various properties such as peptide charges, amphipathicity, and hydrophobic and hydrophilic conditions. This antibacterial activity can also be caused by the presence of several cationic peptides which are believed to interact with lipopolysaccharide anion bonds in cell membranes. This peptide can replace divalent cations such as  $Ca^{2+}$  and  $Mg^{2+}$ , distorting to the cell's outer membrane bilayer because these two minerals are important to support the integrity of the cell's outer membrane. This distortion in the outer membrane results in membrane lysis and ultimately cell death.<sup>22</sup>

Antibacterial effectiveness of red dragon fruit peel yogurt is possible due to the presence of polyphenols in the peel of red dragon fruit, alkaloids, and terpenoids. The antibacterial activity of phenol compounds is carried out through a protein denaturation process and results in cell lysis. Flavonoids are derivatives of phenolic compounds and act as protein coagulators. The toxicity of phenol compounds to bacteria adjusts the number of hydroxyl groups and the concentration given. Phenol can bind to proteins through hydrogen bonds resulting in damage to the protein structure.<sup>23</sup>

Terpenoids have antibacterial activity by reacting with porin (a transmembrane protein) on the outer membrane of the bacterial cell wall to form strong polymer bonds that cause damage to porin. Porin is the entrance and exit of compounds so that when porin is damaged it will reduce the permeability of bacterial cell walls. This cell wall permeability will interfere with the entry of nutrients and other compounds, thus inhibiting bacterial growth and can even cause bacterial death. Luo stated that red dragon fruit peel contains  $\beta$ -amirin (15.87%) and  $\alpha$ -amirin (13.90%) which are the terpenoid group.<sup>22</sup> Other than terpenoids, the antibacterial activity of dragon fruit peel yogurt is also caused by alkaloids. Alkaloids carry out the antibacterial activity by disrupting the

peptidoglycan that makes up bacterial cells so that the cell wall layer is not formed completely and causes cell death. Another bioactive substance that is believed to have an antibacterial role in red dragon fruit peel yogurt is flavonoids. Mardiana's research stated that red dragon fruit peel yogurt using *Lactobacillus bulgaricus* and *Streptococcus thermophilus* bacterial isolates had higher levels of flavonoids compared to dragon fruit peel yogurt using commercial starters.<sup>12</sup> Flavonoids are known to have antibacterial activity due to their complex formation process with bacterial cell walls. Flavonoids work by damaging bacterial cell membranes in the phospholipid portion to further reduce membrane permeability. The decrease in membrane phospholipid permeability due to the activity of phenolic components causes lysis of bacterial cell membranes. Saponin activity mechanism as an antibacterial is possible because saponins can cause proteins and enzymes leakage in cells. Tannins also play a role in inhibiting bacterial growth by damaging bacterial cell proteins.<sup>20</sup> Other alkaloid compounds found in the peel of red dragon fruit are betacyanins (betanin and isobetanin). The fermentation process in making yogurt of red dragon fruit peel causes an increase in the content of betacyanin.<sup>24</sup> Alkaloids act as antibacterial through an inhibitory mechanism of DNA synthesis. Alkaloid compounds have alkaline groups that contain nitrogen which will react with amino acid compounds that make up the bacterial cell walls and DNA. This reaction will result in changes in the structure and arrangement of amino acids, causing changes in the genetic balance of the DNA chain. This will cause bacterial cell lysis which will then cause cell death in bacteria.<sup>25</sup> Alkaloids can also inhibit bacterial growth through the action mechanism of the dihydrofolate reductase enzyme so that it inhibits nucleic acid synthesis.<sup>26</sup>

The limitations of this study are that the phytochemical (tannins, alkaloids, and terpenoids) tests on red dragon fruit peel yogurt were not carried out.

## CONCLUSIONS

Red dragon fruit peel yogurt with a dose of 2.7 mL has the potential to inhibit the growth of *Bacillus subtilis* bacteria.

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