The effect of dadih in BALB/c mice on pro-inflammatory and anti-inflammatory cytokine productions

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ABSTRACT

The normal microflora formed as commensal bacteria have roles in maintaining homeostasis in the intestine tract. The reduction in the amount and on the diversity of the commensal bacteria lead to gastrointestinal dysbiosis which increase number of pathogens, induce inflammatory and can drive to colorectal cancer. Probiotics can be used to prevent, regulate, and modulate immune response by triggering the development of pathogen-specific memory. Currently, many foreign probiotic products are available in the market that cause the domestic products are less well known. Dadih is an original probiotic's products originally from West Sumatra, Indonesia. It is made from fermented buffalo milk containing lactic acid bacteria (LAB). The objective of the study was to investigate the effect of dadih pro-inflammatory and anti-inflammatory cytokine production. The study was conducted using male BALB/c mice aged 6-8 weeks with body weight (BW) 20-30 g. Mice were given dadih at doses of 112 mg/20g BW for eight weeks. The results indicated that LAB bacteria in dadih are coccus, Gram-positive bacteria with 3x10^7 colony-forming units (CFU) and dominated by Lactococcus lactis subsp. lactis. In addition, the increase of both the anti-inflammatory cytokines (IL-10) and pro-inflammatory cytokines (TNF-α and IL-1β) was observed. In conclusion, the dadih can be used to maintain the immune system of mice.

Keywords: dadih, probiotics, IL-1β, IL-10, TNF-α
In the digestive tract, there are approximately 400 species of bacteria that can produce 100 trillions of normal microflora. Survival of bacteria in the gastrointestinal tract depends on their endurance and human immune system. Gut microbiotas are non-pathogenic bacteria, which have roles in metabolizing the nutrition and drugs, preventing the invasion of pathogenic bacteria, and colonizing the intestinal lining. Thus, the gut microbiotas can against pathogens which can cause diseases such as inflammatory bowel disease, obesity, diabetes mellitus, and allergic diseases.

The composition and diversity of bacteria in the intestine causes to imbalance of the microbial ecosystems called dysbiosis that may be caused by antibiotics consumption, high-protein diets, and genetic factors. Dysbiosis creates a disruption of bacterial homeostasis and the immune system in the intestine. Homeostatic disorders can cause in both local and systemic inflammation which in the long term (chronic conditions) can develop into colorectal cancer.

World Health Organization (WHO) reported consumption of probiotics in sufficient quantities can give benefits to human health. Previous studies have proven the important role of the probiotics in maintaining immune system homeostasis. Thus, the consumption of probiotics can prevent various diseases. Bacterial strains contained in probiotics can adhere to the intestinal mucosal lining, which can prevent the colonization of pathogenic bacteria by increasing the body's immune system. The probiotics have an important role in improving the interaction between the innate and adaptive immune systems which will reduce the risk of intestinal inflammation.

Bacteria strains can be referred to probiotics if the number of bacteria in probiotic reaches $10^7$-$10^9$ CFU/g. They can metabolize quickly, colonize in the digestive tract, and produce organic acids. Currently, the awareness to consume probiotics among Indonesian people has increased. However, the probiotic's products available in the market are dominated by imported products. Only a few local probiotic products are available in the market consumed regularly, although their enormous benefits.

One of the local probiotics available in the market is dadih. Dadih is fermented buffalo milk, a product from West Sumatra. The buffalo milk was fermented in bamboo tubes and covered with banana leaves or plastic (spontaneous fermentation/back-slopped) for 2-3 days at room temperature. Microorganisms are possibly derived from bamboo tubes, banana leaves, and buffalo milk.

The people from the West Sumatera consumed dadih with rice as dishes or mixed with ampiang (cookies made from sticky rice) and brown sugar. Dadih is believed can increase appetite so it is often used for people who are recovering from illness.

Dadih has an antibacterial activity against pathogenic bacteria such as *Escherichia coli*. Collado et al. reported that dadih also has the potential activity to eliminate intestinal pathogen bacteria due to its ability to produce lactic acid. The lactic acid in fermented milk can reduce pH a thus only a few microbes survive. In addition, dadih also contains secondary metabolite such as lactic acid, acetic acid, and antibiotic compounds such as acidophylline, acidolin, nicin, and bulgarican which can prevent the growth of pathogenic microbes and destructive microbes. Surono et al. showed probiotic *Lactobacillus plantarum* IS-10506, supplementation isolated from dadih can stimulate TGF-β1, which in turn increases the production of IgA. The purpose of this
study was to investigate the effects of dadih on the intestinal immune response based on pro-inflammatory and anti-inflammatory cytokine production.

**MATERIALS AND METHODS**

**Gram staining and bacteria identification in dadih**

The dadih was obtained from local market and then Gram staining and bacteria identification were performed using bacterial colonies in the last dilution. The colony was isolated to determine the morphology and type of the bacteria. The examination was carried out by dipping the suspension of the test sample into a cassette; then it was analyzed using the Vitek@2 COMPACT for 8 h. This instrument specific developed for industrial microbiology (for electronic records and signatures) and a colorimetric reagents card used to identify the spore-forming Gram-positive, Gram-negative and yeast.\(^\text{18}\)

**The calculation number of colonies in dadih**

Number of bacteria colonies in dadih were calculated by the culture method. The number of bacterial colonies was diluted using sterile saline media and blood agar media, thus the dadih concentrations from \(10^{-1}\) until \(10^{-7}\) were obtained. Bacterial colonies in \(10^{-5}\), \(10^{-6}\), and \(10^{-7}\) dilutions were taken as much as 100 \(\mu\)L and being cultured on blood agar media using the spread method. The inoculums were incubated at 37°C for 72 h using an aerobic jar. After 72 h, a petri dish has been overgrown by bacterial colonies; then the colonies were counted using a BZG 30 colony counter. The total colony forming unit (CFU)/gram was calculated using the formula below:

\[\text{CFU/gram} = \text{colony count} \times (1/\text{dilution}) \times (1/\text{sample weight})\]

**Induction dadih to mice**

This study has been approved by the Research Ethics Committee, the Faculty of Medicine of the Universitas Indonesia (Number: KET-162/UN2.F1/ETIK/PPM.00.02/2019). The mice were acclimatized for 2 weeks and then divided into 2 groups, which are Group A as control group and Group B as treatment group. Mice in Group A were treated with aquadest, whereas mice in Group B were treated with 112 mg/20g/BW dadih. The dose administration is according to Ellyza’s study showed that E. coli colony was lower in mice treat with dadih than without dadih. Each group was given dadih or aquadest daily for 8 weeks. Aquades and dadih administrasion were performed via oral gavage. After 8 weeks, mice were terminated by injecting the ketamine and xylazine with the dose based on the BW of the mice. Blood collection was performed through the intracardiac puncture using a syringe.

**Measurement of cytokine mediators**

Serum from the blood was used to measure the levels of cytokines TNF-\(\alpha\), IL-1\(\beta\), and IL-10. The levels of cytokines were detected using the Luminex Assay RnD Magnetic System kit (Premixed Multi-Analyzer Kit) and read using Luminex Bead type 200.

**RESULTS**

**Morphology and identification of bacteria in dadih**

In the observation of bacteria from gram staining, it is found the round/coccus, chain-like bacteria, purple bacteria that has gram-positive properties (FIGURE 1.). The results in identification of bacteria using Vitek showed L. lactis sp lactis with a probability value of 94%.
Calculation the number of colonies and bacterial morphology

The number of bacteria colonies in *dadih* from dilutions of $10^5$, $10^6$, and $10^7$CFU/g are listed in TABLE 1. *Dadih* used in this study contained total bacteria $3 \times 10^7$CFU/g.

**TABLE 1. Number of bacterial colonies of various dilutions.**

<table>
<thead>
<tr>
<th>Colony count</th>
<th>Dilution</th>
<th>Total of bacteria (CFU/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>199</td>
<td>$10^4$</td>
<td>$199 \times 10^4$</td>
</tr>
<tr>
<td>31</td>
<td>$10^6$</td>
<td>$31 \times 10^6$</td>
</tr>
<tr>
<td>3</td>
<td>$10^7$</td>
<td>$3 \times 10^7$</td>
</tr>
</tbody>
</table>

Measurement of animal BW

The mean BW differences of mice in the control group (Group A) and treatment group (Group G) is presented in FIGURE 2.

The mice in Group A showed increment on body weight from the first week, with the difference of 2 g compared Group B. This weight gain continued to increase until week 7. At week 7 and 8, the weight of Group A tended to decrease. In contrast, in the Group B has higher weight gained compared to Group A, approximately 1 g/week.

Cytokines TNF-α, IL-1β and IL-10 in each group

The measurement result of cytokine levels between the control group (Group A) and treatment group (Group B) are shown in TABLE 2.
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FIGURE 2. Mean of difference in BW between control group (Group A) and treatment group (Group B)

TABLE 2. The level of cytokine i.e. TNF-α, IL-1β, and IL-10 of Group A and Group B

<table>
<thead>
<tr>
<th></th>
<th>TNF-α</th>
<th></th>
<th>IL-1β</th>
<th></th>
<th>IL-10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group A</td>
<td>Group B</td>
<td>Group A</td>
<td>Group B</td>
<td>Group A</td>
</tr>
<tr>
<td>Min</td>
<td>10.69</td>
<td>25.99</td>
<td>0.001</td>
<td>0.000</td>
<td>72.99</td>
</tr>
<tr>
<td>Max</td>
<td>48.05</td>
<td>65.89</td>
<td>0.005</td>
<td>0.010</td>
<td>157.64</td>
</tr>
<tr>
<td>Median</td>
<td>36.27</td>
<td>41.22</td>
<td>0.002</td>
<td>0.003</td>
<td>88.61</td>
</tr>
<tr>
<td>SD</td>
<td>12.671</td>
<td>13.37</td>
<td>0.002</td>
<td>0.001</td>
<td>31.55</td>
</tr>
</tbody>
</table>

The median TNF-α, IL-1β, and IL-10 between the two groups had a difference of 4.95, 0.001, and 2.1 pg/mL, respectively. To find out the differences in cytokines among groups, the proportion of tests were performed using high and low categories of cytokines. Categorizing was based on a cut of points using receiver operating characteristic (ROC) analysis method. Cut off point value of several cytokines including TNF-α (39.788 pg/mL), IL-1β (0.005 pg/mL) and IL-10 (101.589 pg/mL). The differences in the cytokines proportion between groups are shown in FIGURE 3.
FIGURE 3. Proportion of cytokine (%) i.e. TNF-α, IL-1β and IL-10 between control group (Group A) and treatment group (Group B).

The proportion of cytokine in Group B was higher than Group A respectively TNF-α 34% and 33% for IL-1β and IL-10. It could be seen that the levels of TNF-α, IL-1β, and IL-10 cytokines in mice treated with dadih were higher than control group.

The result showed that median of cytokine between 2 group are TNF-α (36.27-41.22 pg/mL; p=0.485), IL-1β (0.002-0.003 pg/mL; p=0.240) and (88.61-90.71 pg/mL, p=0.485). There was no significant effect of dadih administration on mice compare to without dadih administration. However, in mice with dadih administration showed the higher increase of cytokines level compare to that without dadih administration.

DISCUSSION

This study showed that the LAB found in dadih is from L. lactis subsp. lactis. The strain has morphology element in the coccus and gram-positive characteristics. Lactococcus bacteria are homofermentative, microaerophilic, catalase negative bacteria, non-motile, gram-positive, coccus-shaped, 0.5-1.5 µm in size and arranged like chains. They are able to survive at temperatures of 10°C with an optimal temperature of 30°C and pH of 9.6, and able to produce lactic acid. However, they can not produce endospores. Other than in fermented milk products, these bacteria can be found in the digestive tract of humans and animals.

Lactococcus species found in this study is corresponding with the previous studies. Harsanti found that bacteria in dadih from buffalo milk were dominated by Lactococcus. Moreover, Jatmiko used the isolate of L. lactis subsp. lactis from dadih in their study. This showed that the L. lactis subsp. lactis is one type of the bacteria contained in dadih that were evaluated in vitro for potential probiotic properties based on its salt resistance, low pH tolerance, antimicrobial activity, antibiotic susceptibility, and adherence to Caco-2 colon cancer cells.

There are approximately 36 strains of LAB microorganism that have been found in dadih which are Lactobacillus, Streptococcus sp., Lactococcus sp., Pediococcus sp., Enterococcus sp., and Weissella sp. This strain differences cause no standard protocol in dadih manufacturing, thus the quality and
composition of nutrients and bacteria in every dadih production are different.\textsuperscript{13}  
The availability amount of the bacteria in \textit{dadih} was $3 \times 10^7$ CFU/g. This amount is matched with the criteria of probiotics. According to the probiotic requirements, the number of bacterial colonies that can grow and develop well in the digestive tract should be $10^7-10^9$ CFU/g. This amount can decrease during storage and digestion. The number of bacteria declines due to environmental factors that do not support the survival of probiotic bacteria such as low pH and high bile salts.\textsuperscript{9} 

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The increase in BW of mice occurred in Group B due to mice treated with \textit{dadih} probiotic. It can increase physical growth.\textsuperscript{22} Shokyryazdan\textsuperscript{23} reported that probiotics administration not only has a beneficial effect on health but also has an impact on increasing BW. Probiotic is advantage to the host by synthesizing vitamin and realizing amino acids to support the host growth, and also able to improve food consumption of mice beside improve nutrient absorption by producing digestive enzyme.\textsuperscript{24} 

Intestinal microbiota contributes to epithelial cell function, energy balance, and host immune system.\textsuperscript{25} In normal conditions, pathogens will be suppressed by commensal bacteria, which can induce antimicrobial proteins such as IL-10 and REG3γ that can cause intestinal homeostasis. The homeostasis of intestinal immunity can be seen from the high number of T regulators rather than Th1 and Th17.\textsuperscript{26} 

Probiotics have the potential to be immunomodulator with their ability to interact with epithelium, dendritic cells, macrophages, and lymphocytes. The components of the \textit{L. lactis} bacterial wall can be recognized by dendritic cells in the digestive tract and affect the function of dendritic cells.\textsuperscript{27} 

LAB contained in probiotics can also induce an inflammatory response.\textsuperscript{28,29} This effect can be seen from the increase in TNF-α in \textit{dadih}-induced mice compared to the control group because the intestinal microbiota will induce macrophages and dendritic cells to produce IL-6, IL-1β and TNF-α which will stimulate Th17 differentiation. Th17 has a role in secreting pro-inflammatory cytokines such as IL-17. Some probiotic bacteria could increase the production of IL-6, IL-12, and TNF-α.\textsuperscript{28}  

Many other studies explained that probiotics could induce IL-10, cytokines produced by many cells such as Th-2, macrophages, dendritic cells, keratinocytes, and Treg cells. IL-10 has an anti-inflammatory role in inhibiting the response of Th-1.\textsuperscript{8} In this study, the levels of IL-10 cytokines in \textit{dadih}-induced mice were higher than those mice without \textit{dadih} administration. Some studies stated that the administration of probiotics could induce Foxp3+ Treg cells which produced TGF-β and IL-10.\textsuperscript{30} Probiotics interact with enterocytes, dendritic cells, Th1, Th2, and Treg in the intestinal tract and modulate adaptive immune cells to produce pro-inflammatory and anti-inflammatory cytokines. 

**CONCLUSION**  
The study indicates that \textit{dadih} can be referred as probiotics based on bacterial number. The bacteria found in \textit{dadih} is \textit{L. lactis} subsp. \textit{lactis}. \textit{Dadih} can increase the levels of pro-inflammatory and anti-inflammatory cytokine even though \textit{dadih} does not change of cytokine pattern.

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**REFERENCES**

1. Sunaryo D. Karakteristik ketahanan


