



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

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### ABSTRACT

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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  $3 \times 10^7$  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- $\alpha$  and IL-1 $\beta$ ) was observed. In conclusion, the *dadih* can be used to maintain the immune system of mice.

### ABSTRAK

Mikroflora normal bakteri komensal berperan untuk menjaga homeostasis dalam usus. Berkurangnya jumlah dan keragaman bakteri komensal dapat menyebabkan disbiosis saluran cerna diikuti dengan meningkatnya jumlah patogen sehingga menginduksi inflamasi yang dapat berkembang menjadi kanker usus. Probiotik berperan dalam pertahanan, regulasi dan memodulasi respon imun dengan memicu perkembangan memori spesifik terhadap patogen. Saat ini produk probiotik luar negeri banyak beredar sehingga, produk dalam negeri menjadi kurang dikenal. *Dadih* merupakan probiotik local dari Sumatera Barat berupa susu kerbau fermentasi yang mengandung bakteri asam laktat (BAL). Tujuan penelitian ini adalah untuk mengetahui karakterisasi bakteri dalam *dadih* dan efeknya terhadap kadar sitokin dalam serum mencit. Penelitian menggunakan mencit BALB/c jantan berumur 6-8 minggu dengan berat 20-30 g. Mencit diberi *dadih* sebanyak 112 mg/20 g BB selama 8 minggu. Hasil uji bakteri menunjukkan BAL yang terkandung dalam *dadih* merupakan bakteri gram positif berbentuk bulat memiliki jumlah koloni  $3 \times 10^7$  yang didominasi oleh *Lactococcus lactis* subsp. *lactis*. Selain itu, ada peningkatan sitokin anti-inflamasi (IL-10) dan sitokin pro-inflamasi (TNF- $\alpha$  dan IL-1 $\beta$ ). Dapat disimpulkan, *dadih* dapat mempertahankan sistem imun mencit.

### Keywords:

*dadih*  
probiotics  
IL-1 $\beta$   
IL-10  
TNF- $\alpha$

## INTRODUCTION

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.<sup>1</sup> 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.<sup>2</sup>

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.<sup>3</sup> Homeostatic disorders can cause in both local and systemic inflammation which in the long term (chronic conditions) can develop into colorectal cancer.<sup>4</sup>

World Health Organization (WHO) reported consumption of probiotics in sufficient quantities can give benefits to human health.<sup>5</sup> Previous studies have proven the important role of the probiotics in maintaining immune system homeostasis. Thus, the consumption of probiotics can prevent various diseases.<sup>6</sup> 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.<sup>7</sup> 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.<sup>8</sup>

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.<sup>9,10</sup> 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*.<sup>11</sup> *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.<sup>12,13</sup> 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.<sup>14</sup>

*Dadih* has an antibacterial activity against pathogenic bacteria such as *Escherichia coli*.<sup>15</sup> Collado *et al.*<sup>16</sup> 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.<sup>11</sup> Surono *et al.*<sup>17</sup> showed probiotic *Lactobacillus plantarum* IS-10506, supplementation isolated from *dadih* can stimulate TGF- $\beta$ 1, 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.<sup>18</sup>

### 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* administration 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%.

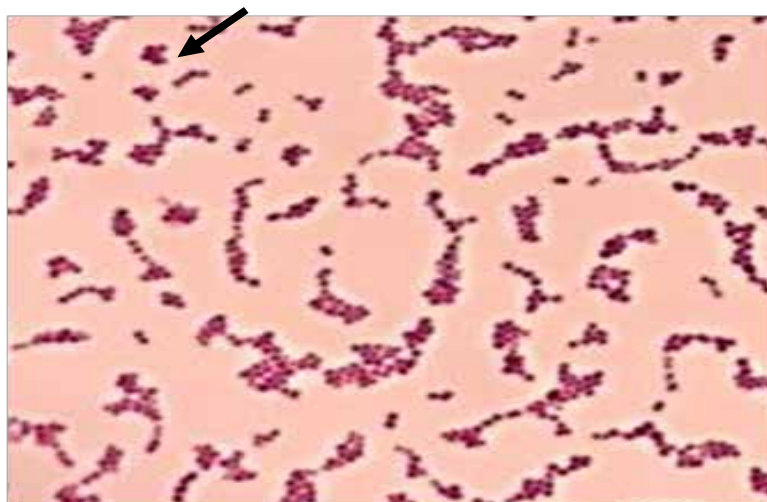


FIGURE 1. Isolate of bacteria from *dadih* (1000x) showed purple staining of bacteria with coccus

**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.

Colony count	Dilution	Total of bacteria (CFU/g)
199	$10^{-5}$	$199 \times 10^5$
31	$10^{-6}$	$31 \times 10^6$
3	$10^{-7}$	$3 \times 10^7$

**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- $\alpha$ , IL-1 $\beta$  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.

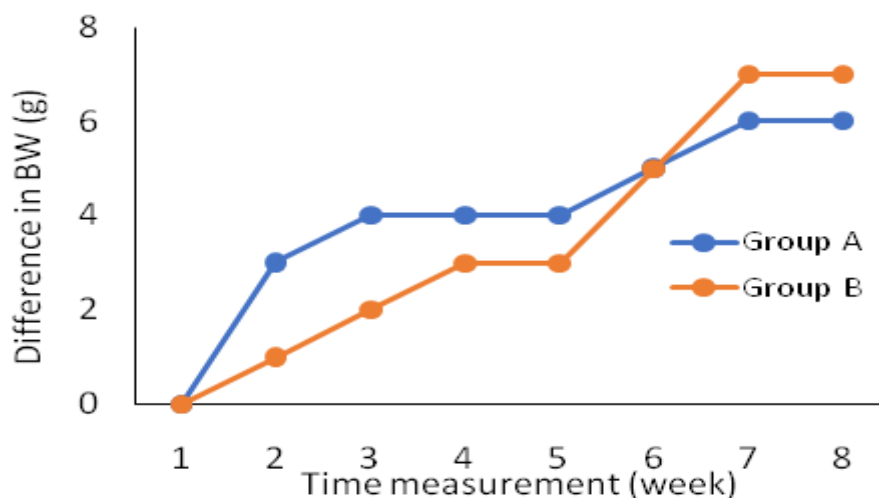


FIGURE 2. Mean of difference in BW between control group (Group A) and treatment group (Group B)

TABLE 2. The level of cytokine i.e. TNF- $\alpha$ , IL-1 $\beta$ , and IL-10 of Group A and Group B

	TNF- $\alpha$		IL-1 $\beta$		IL-10	
	Group A	Group B	Group A	Group B	Group A	Group B
Min	10.69	25.99	0.001	0.000	72.99	82.18
Max	48.05	65.89	0.005	0.010	157.64	133.66
Median	36.27	41.22	0.002	0.003	88.61	90.71
SD	12.671	13.37	0.002	0.001	31.55	18.34

The median TNF- $\alpha$ , IL-1 $\beta$ , 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- $\alpha$  (39.788 pg/mL), IL-1 $\beta$  (0.005 pg/mL) and IL-10 (101.589 pg/mL). The differences in the cytokines proportion between groups are shown in FIGURE 3.

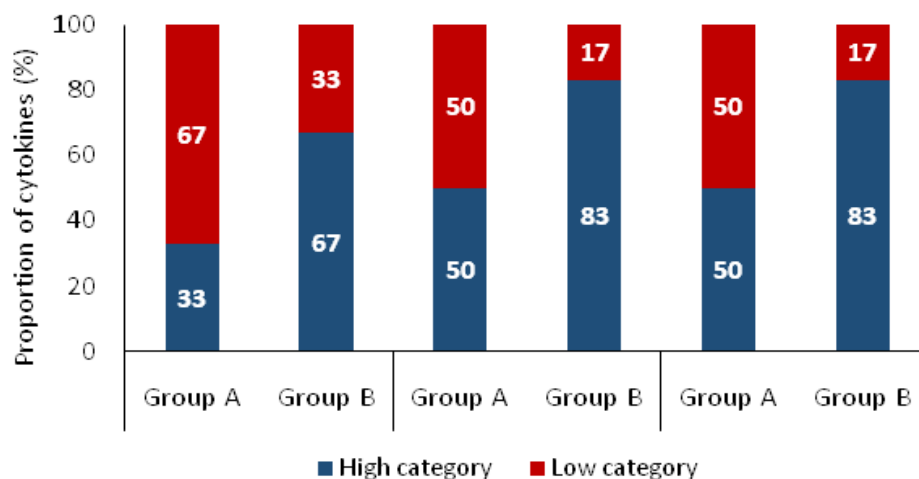


FIGURE 3. Proportion of cytokine (%) i.e. TNF- $\alpha$ , IL-1 $\beta$  and IL-10 between control group (Group A) and treatment group (Group B).

The proportion of cytokine in Group B was higher than Group A respectively TNF- $\alpha$  34% and 33% for IL-1 $\beta$  and IL-10. It could be seen that the levels of TNF- $\alpha$ , IL-1 $\beta$ , 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- $\alpha$  (36.27-41.22 pg/mL; p=0.485), IL-1 $\beta$  (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  $\mu$ m 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.<sup>19</sup>

*Lactococcus* species found in this study is corresponding with the previous studies. Harsanti<sup>20</sup> found that bacteria in *dadih* from buffalo milk were dominated by *Lactococcus*. Moreover, Jatmiko<sup>21</sup> also 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.*<sup>8</sup> 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.<sup>13</sup>

The availability amount of the bacteria in *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.<sup>9</sup>

The increase in BW of mice occurred in Group B due to mice treated with *dadih* probiotic. It can increase physical growth.<sup>22</sup> Shokyryazdan<sup>23</sup> 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.<sup>24</sup>

Intestinal microbiota contributes to epithelial cell function, energy balance, and host immune system.<sup>25</sup> In normal conditions, pathogens will be suppressed by commensal bacteria, which can induce antimicrobial proteins such as IL-10 and REG3 $\gamma$  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.<sup>26</sup>

Probiotics have the potential to be immunomodulator with their ability to interact with epithelium, dendritic cells, macrophages, and lymphocytes. The components of the *L. lactis* bacterial wall can be recognized by dendritic cells in the digestive tract and affect the function of dendritic cells.<sup>27</sup>

LAB contained in probiotics can also induce an inflammatory response.<sup>28,29</sup> This effect can be seen from the

increase in TNF- $\alpha$  in *dadih*-induced mice compared to the control group because the intestinal microbiota will induce macrophages and dendritic cells to produce IL-6, IL-1 $\beta$  and TNF- $\alpha$  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- $\alpha$ .<sup>28</sup>

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.<sup>6</sup> In this study, the levels of IL-10 cytokines in *dadih*-induced mice were higher than those mice without *dadih* administration. Some studies stated that the administration of probiotics could induce Foxp3+ Treg cells which produced TGF- $\beta$  and IL-10.<sup>30</sup> 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 *dadih* can be referred as probiotics based on bacterial number. The bacteria found in *dadih* is *L. lactis* subsp. *lactis*. *Dadih* can increase the levels of pro-inflammatory and anti-inflammatory cytokine even though *dadih* does not change of cytokine pattern.

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