Immunomodulatory Activity of Black Tea Kombucha (Camellia sinensis) and Arabica Coffee Leaves Tea Kombucha (Coffee arabica) for Salmonella typhi-infected mice

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Abstract. This study aimed to compare between black tea kombucha, and Arabica coffee leaves tea kombucha on immunomodulatory activity on Salmonella typhi infected mice. The trial animals were divided into two groups; the infected and the non-infected group. Each group consisted of a control group, the black tea kombucha group, and the Arabica leaves tea kombucha group. Both types of tea kombucha increase the adaptive immune response, as shown by an improvement on CD4+, CD8+, CD4+IFNƔ, CD4+TNFα, CD8+IFNƔ, and CD8+TNFα. It was also suggested that the immunomodulatory activity of black tea kombucha was higher than that of Arabica coffee leaves tea kombucha.

1. Introduction
Typhoid fever caused by Salmonella typhi bacteria is a disease that is a health issue in developing countries. Based on WHO data, 16 million cases and 600 thousand cases were found to cause death. In Indonesia, the incidence of this disease is estimated to be around 300-810 cases per 100,000 populations per year [1, 2]. This bacterial infection is caused by the decreased body's immune system activity where so that the body's immune is unable to kill and destroy the S. typhi bacteria. This causes the bacteria in the bloodstream to survive, develop, invade, and damage the body's cells [3, 4, 5].

The occurrence of S. typhi bacterial infection can be prevented by increasing the body's immune system, especially CD4+ and CD8+ T cells by using immunomodulatory agents derived from natural ingredients with fermentation treatment, namely kombucha. The fermentation of kombucha mushroom in rosella tea has immunomodulatory activity in the form of immunostimulatory activity against lymphocyte cell proliferation in Balb/C mice in vitro [6].

Kombucha starts as a sugary tea, which then fermented by using SCOBY (Symbiotic Culture of Bacteria and Yeast), not only consisting of various acetic acid bacteria but also yeast. The tea was then incubated for 14 days. This product contains polyphenols, organic acids, fibre, ethanol, amino acids
including lysine, micronutrients namely Cu, Fe, Mn, Ni, and Zn, water-soluble vitamins such as vitamin C, some B vitamins, carbon dioxide, antibiotic substances, and the hydrolytic enzymes that make these products famous for having several health effects, namely as antioxidants, antibacterials, improving intestinal microflora, lowering blood pressure, and increasing endurance [7, 8, 9].

Kombucha generally comes from black tea; it can be replaced with other ingredients [8]. One of the raw materials that can replace tea is coffee leaves. Coffee leaves are one of the plantation wastes and can be used as tea because they contain antioxidant alkaloids, saponins, flavonoids, and polyphenols [10].

Therefore, this study compared the potential of kombucha tea from Arabica coffee leaves and kombucha black tea as immunomodulators in mice infected with S. typhi by measuring CD4⁺, CD8⁺, CD4⁺IFNγ, CD4⁺TNFα, CD8⁺IFNγ, and CD8⁺CD4⁺ levels. TNFα was obtained from the spleen.

2. Materials and Methods

2.1. Materials
The Arabica coffee leaf tea was obtained from Dampit, Malang, Indonesia and the black tea were from the Tong Tji brand from the local shopping centre. Commercial kombucha starter was purchased from a local distributor. The sugar was purchased at a local shopping centre. Experimental animals, female mice aged 4-8 weeks that have bodyweight 20-30 grams with healthy conditions marked by active movements obtained from a local distributor of certified experimental animals in Malang, Indonesia. Mice are fed with BR2 which can be purchased at the experimental animal store in Malang and water ad libitum. The Ethics Commission has approved the experimental protocols and procedures used in this study.

2.2. Kombucha production
The kombucha was prepared by making 4 grams of coffee tea leaves brewed in 1000ml water at 100°C for 2-3 minutes. Brewed water was filtered to separate tea and tea water, resulting in clean tea water. A 10% sucrose (w/v), was added, dissolved, and the mix was put in a glass jar. The mix was cooled to ±25°C, and the cooling time should not be more than 4 hours. Before 4 hours, the tea water had cooled and was added with a 10% (v/v) liquid kombucha starter, then covered with a sterile fabric and tied with rubber and fermented at room temperature for 10 days [11].

2.3. Kombucha's properties analysis
The pH was measured using a Micro Bench TI 2100 Manual pH meter. Total sugar was analysed using the anthrone method using spectrophotometry with a wavelength of 630 nm. Total acid was analysed using 0.1 N NaOH titration. Total phenols were analysed using Folin Ciocalteau and Na₂CO₃ reagents using 750 nm spectrophotometry. Flavonoids were measured by NaNO₂ and AlCl₃ reagents using 510 nm spectrophotometry. Tannin was measured with Na₂CO₃ using 775 nm spectrophotometry. Furthermore, analysis of antioxidant activity with DPPH solution using 517 nm absorbance measurements and expressed in IC₅₀.

2.4. Immunomodulatory assay activity
In this experimental study, mice were divided into 6 experimental groups of 4 animals each. Each group is given specific treatment as follows. Normal healthy control mice and control S. typhi infections are referred to as the groups [Healthy Non-Treatment / P0] and [Non-Treatment / P3]. Healthy mice that receive kombucha from Arabica coffee leaf tea are called group P1. Healthy mice that receive black tea kombucha are called the P2 group. The mice infected with S. typhi, and received kombucha from Arabica...
coffee leaf tea is called group P4. Mice infected with S. typhi who received black tea kombucha were called group P5. All rats were fed with standard food (BR2) and drinking ad libitum water. Kombucha was administered orally to rats using intragastric tubes daily for 28 days at a dose of 0.26ml / 20gr BW of mice. S. typhi infection was carried out on day 22 in groups P3, P4, and P5 via intraperitoneal at 108 doses. On day 29, mice were sacrificed by neck dislocation. Spleen samples were collected to measure CD4+, CD8+, CD4+IFNƔ, CD4+TNFα, CD8+IFNƔ, and CD8+TNFα using flow cytometry.

2.5. Statistical analysis
Data were analysed by complete random design with analysis of variance (ANOVA) and further analysis by Tukey at α = 5%.

3. Results and Discussion

3.1. The kombucha's quality
During the kombucha fermentation process, the growth of lactic acid bacteria occurs, which produces specific properties in the kombucha product. Analysis of the quality of kombucha's black tea and kombucha tea Arabica coffee leaves is presented in Table 1.

| Parameter (Unit)         | Kombucha Type       |          |          |          |          |          |
|--------------------------|---------------------|----------|----------|----------|----------|----------|
|                          | Black Tea Kombucha  | Change   | Tea Arabica Coffee Leaves | Change |
|                          | Day 0               | Day 10   | Kombucha | Day 0   | Day 10   |
| pH                       | 3.62 ± 0.01         | 3.06 ± 0.02 | -0.56    | 3.68 ± 0.03 | 3.13 ± 0.01 | -0.54   |
| Total Acid (%)           | 0.15 ± 0.00         | 0.24 ± 0.02 | 0.09     | 0.11 ± 0.01 | 0.17 ± 0.02 | 0.06    |
| Total sugar (%)          | 8.86 ± 0.12         | 8.64 ± 0.01 | -0.22    | 8.83 ± 0.08 | 8.65 ± 0.07 | -0.14   |
| Fenol (mg/L GAE)         | 251.97 ± 17.68      | 305.38 ± 10.76 | 53.41    | 162.37 ± 9.38 | 196.06 ± 33.69 | 33.69   |
| Flavonoid (mg/L QE)      | 83.39 ± 6.01        | 97.49 ± 4.70 | 14.10    | 72.87 ± 3.20 | 90.05 ± 3.11 | 17.18   |
| Tanin (mg/L TAE)         | 26.68 ± 0.65        | 31.34 ± 0.95 | 4.66     | 10.02 ± 0.79 | 26.17 ± 1.15 | 16.15   |
| Antioxidant Activity / IC50 (ppm) | 138.27 ± 1.51 | 88.01 ± 0.13 | -50.27   | 166.38 ± 1.22 | 96.13 ± 0.70 | -70.25 |

Table 1 shows the changes in kombucha's properties during the fermentation process. The analysis using the paired t-test, the results in Table 1 have a significant change in each parameter. The kombucha fermentation process shows the use of sugar as a microbial nutrient. This was as seen from the decreased total value of sugar and the formation of acids so that the total acid in both kombuchas increased. The increase in total acid caused a decrease in pH. The concentration of bioactive compounds, namely phenols, flavonoids, and tannins, also increased due to the fermentation process. In various kombucha studies, the results may be inconsistent concerning the microbiological conditions in the kombucha [12]. This microbiological condition is related to the composition of microbes that live in kombucha, which are also influenced by the raw materials used in kombucha making. Black tea contains sugar, organic acids, and caffeine that support the growth and development of microbes [13]. Black tea contains polyphenol and alkaloid compounds that are higher than that of coffee leaves and can influence microbial activity in the fermentation by producing thicker Nata [14].
3.2. Kombucha immunomodulator activity

Figure 1 shows the immune response analysis that was carried out on the response of CD4 + T cells and CD8 + T cells.

Note: (P0) group of healthy mice, (P1) group of healthy mice treated kombucha arabica coffee leaf tea, (P2) group of healthy mice treated black tea kombucha, (P3) group of infection mice, (P4) group of mice infection treated kombucha tea leaves Arabica coffee, (P5) group of mice infected with black tea kombucha

Figure 1. The results of the analysis of flowcytometry CD4+ [A] and CD8+ [B] on spleen.

Figure 1 shows the CD4+ and CD8+ values. The panel (panel [A] and panel [B]) divide into several graphs; each graph is divided into 4 boxes. To see the CD4+ value can be seen in the image in the lower right box, while the CD8+ value can be seen in the upper left box on the graph. The increase in the percentage of CD4 + T cells for both healthy mice and infectious mice groups was caused by the presence of active substances in kombucha both kombucha Arabica coffee leaf tea and black tea kombucha which functioned as immunostimulants against the immune system. The active substance that has a role as an immunostimulant is flavonoids. Flavonoids can induce an increase in the secretion of cytokines involved in the process of CD4 + T cell activity. Flavonoids play a role in triggering the regulation of T helper cells by increasing the production of interleukin 2 cytokines (IL-2) [15, 16]. These IL-2 cytokines are needed by CD4 + T cells to differentiate in a subset of helper 2 (Th2) and Th1 cells [17]. This increase in IL-2 also allows an increase in the number of CD8 + T cells. IL-2 can trigger the activation of CD8 + T cells, so that activated CD8 + T cells produce perforin and Granzin [18]. Perforin and grazing can destroy infected cells, and S. typhi antigens in the cytoplasm, so in this study, the number of CD8 + T cells in mice infected by S. typhi was higher. At the time of infection, CD8 + T cells can increase several times compared to before infection [17]. Immune response analysis was carried out on the adaptive immune response of T cells with CD4 +, CD8 +, IFN-γ +, TNF-α + cytokines. The results are presented in Table 2.

Kombucha from Arabica coffee leaf tea and black tea kombucha has the characteristics of a functional drink. However, there are no studies that report potential effects as immunomodulators. In this research, the potential of kombucha arabica coffee tea leaf and black tea kombucha in mice with S. typhi infection was studied.
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Table 2. Immunomodulatory activity of kombucha black tea and kombucha tea arabica coffee leaves.

| Kelompok | CD4⁺IFN γ⁺ (%) | CD4⁺TNF α⁺ (%) | CD8⁺IFN γ⁺ (%) | CD8⁺TNF α⁺ (%) |
|----------|---------------|---------------|---------------|---------------|
| Negative Control - Healthy Mice (P0) | 0.26⁺ ± 0.04 | 0.47bc ± 0.01 | 0.43d ± 0.17 | 0.70d ± 0.17 |
| Healthy Mice with Kombucha Arabica Coffee Leaf Tea (P1) | 0.42c ± 0.10 | 0.65bc ± 0.05 | 0.67cd ± 0.27 | 1.19d ± 0.28 |
| Healthy Mice with Black Tea Kombucha (P3) | 0.53c ± 0.06 | 0.75bc ± 0.05 | 0.78c ± 0.20 | 1.34cd ± 0.51 |
| Positive Control - Infection Mice (P3) | 0.62bc ± 0.10 | 0.79bc ± 0.17 | 1.39b ± 0.06 | 1.96bc ± 0.03 |
| Mice Infection with Kombucha Arabica Coffee Leaf Tea (P4) | 0.97b ± 0.34 | 1.75ab ± 0.22 | 1.89b ± 0.01 | 2.49ab ± 0.51 |
| Mice Infection with Kombucha Black Tea (P5) | 1.84a ± 0.22 | 2.13a ± 1.30 | 2.07a ± 0.02 | 3.15a ± 0.03 |

Note: 1. Different notations show significant differences (α = 0.05)
2. Data obtained from the mean of 4 replications ± SD

Infection of S. typhi bacteria to mice will cause innate and adaptive immune responses. After the bacteria enter the body, it will be phagocyte, stimulate macrophages, and increase Interleukin 12 to activate NK cells. With the activation of NK cells, Interferon-gamma will be produced, which functions as bacterial elimination [19, 20].

The mechanism of increasing CD4⁺, CD8⁺, CD4⁺IFNγ, CD4⁺TNFα, CD8⁺ IFNγ, and CD8⁺TNFα cells can occur because of the phenolic compounds present in kombucha arabica coffee leaf tea and black tea kombuchas such as flavonoids and tannins. These bioactive compounds can increase the production of interleukin-2, which can stimulate the Th1 response by activating the differentiation of CD4⁺ cells, when the Th1 response, the production of gamma Interferon was increased, which will activate macrophages by secreting reactive O2 compounds that become toxic to microbes [17, 15, 21, 16, 22].

Flavonoid compounds have been shown to increase IL-2 and lymphocyte proliferation [23]. The administration of black tea kombucha can increase lymphocyte cell proliferation in Falur BALB/c mice in vitro. In this study, it was found the effect of giving kombucha from arabica coffee leaf tea and black tea kombucha to increase CD4⁺, CD8⁺, CD4⁺IFNγ, CD4⁺TNFα, CD8⁺ IFNγ, and CD8⁺TNFα cells. Significant improvement occurred in the group giving black kombucha. The increase in alpha TNF is due to the ongoing infection process [24].

The mechanism of the compounds contained in kombucha in providing an immunomodulatory effect is similar to a mitogen attached to the surface of lymphocyte cells. Bioactive compounds such as flavonoids are ligands that bound to the surface of T and B cell receptors. The activation of signal transduction through second messengers, including IP3 (Inositol trisphosphate), is resulting in the stimulation of Ca²⁺ to the cytoplasm so that the concentration increases. Such an increase will stimulate the activation of protein kinase C. Activation of this protein kinase C will trigger cellular level gene expression such as the expression of Interleukin 2 products which cause activation of B cells and T cells for proliferation [11, 25, 26, 27].

In this study, the best treatment was found in the group of infected mice with black tea kombucha based on the Zeleny method. This is presumably because black tea kombucha is the best kombucha treatment according to the Zeleny method with parameters pH, total acid, total sugar, flavonoids, total phenol, tannin, and IC50. Microbiological conditions can increase the advantage of black tea kombucha [12]. The type of raw material and the type of content contained in the raw material will also affect the kombucha product which can be seen from the total levels of acid, flavonoids, polyphenols, and other contents [28]. The difference in content can be influenced by the place of growth and the availability of
sufficient sunlight for photosynthesis and microbial activity in the degradation of polyphenols during the fermentation process [10, 29].

The result showed that black tea has several higher bioactive components compared to that of Arabica coffee leaf tea. The difference in chemicals exist in kombucha can occur due to the raw material, types of raw materials, temperature and duration of fermentation, the number and type of microbial starter, source of starter, total sugar, and carbon [30].

4. Conclusions
In conclusion, this study shows that black tea kombucha was adequate to be used as an immunomodulatory agent in mice infected by S. typhi. Among the experimental group, mice treated with kombucha tea in Arabica coffee leaves, and kombucha black tea showed an increase in levels of CD4+, CD8+, CD4+IFNγ, CD4+TNFα, CD8+ IFNγ, and CD8+TNFα. However, mice with black tea kombucha treatment showed a significant increase. Most of the properties of kombucha black tea are related to the composition of phenolic compounds from drinks and the presence of organic acids might also have contributed. Further research is needed to explain in more detail about the effective dose for kombucha Arabica coffee leaf tea so that it has the potential as a potential immunomodulator for black kombucha tea as an effective functional food.

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