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INVESTIGATING CHEMICAL CHANGES DURING SNAKE FRUIT AND BLACK TEA KOMBUCHA FERMENTATION AND THE ASSOCIATED IMMUNOMODULATORY ACTIVITY IN *SALMONELLA TYPHI*-INFECTED MICE

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ABSTRACT
This study uncovered the chemical changes during kombucha's fermentation process and revealed the associated immunomodulatory activity in *Salmonella typhi*-infected mice. The snake fruit juice and black tea extract were processed into kombucha (a beverage known for its health benefits) by fermentation with SCOBY culture at room temperature for 14 days. Snake fruit kombucha showed high changes in fermentation parameters (total acidity, pH, and total sugar), as well as bioactive compounds and antioxidant activity. *Salmonella typhi* demonstrated a reduction in the population of CD8+TNFα+ and CD4+IFNγ+ of infected experimental animals. Both snake fruit kombucha and black tea kombucha have the potential to be utilized as an immunomodulator to circumvent unstable conditions of the immune system caused by *Salmonella typhi*. Black tea kombucha and snake fruit kombucha cancan raise the production of CD8+TNFα+ and CD4+IFNγ+ in mice infected with *Salmonella typhi*. In the group of normal mice, black tea and snake fruit kombucha were able to lower down the production of CD8+TNFα+, which is a potent mechanism to modulate the immune system. Further research is required to highlight the mechanism and role of black tea kombucha and snake fruit kombucha in the immune response that modulates and treats infection by *Salmonella typhi*.

Keywords: kombucha; snake fruit; black tea; immunomodulator; *Salmonella typhi*

INTRODUCTION
Snake fruit (*Salacca zalacca* (Gaerth.) Voss) is a popular tropical fruit in South East Asian countries. In addition to its appetizing taste, snake fruit provides many health benefits due to its sugar content, dietary fiber, selected vitamins and minerals, and antioxidant compounds **(Aralas, Mohamed and Abu Bakar, 2009; Suica-Bunghez et al., 2016)**. In our previous studies, we demonstrated that snake fruits have the potential to be processed into Kombucha **(Zubaidah et al., 2018a)**.

Kombucha is a fermented tea beverage, black tea is commonly used which is fermented by a symbiotic culture of bacteria and yeast (SCOBY) **(Jayabalan et al., 2014)**. Kombucha has shown several beneficial effects, such as inhibit pathogenic bacteria growth **(Sreeramulu, Zhu and Knol, 2000)**, acts as an antioxidant, protects hepar, in addition to its anti-cancer property **(Dufresne and Farnworth, 2000)**. Furthermore, it reduces inflammation severity, prevents arthritis, and enhances the immune system as an 'immunomodulator' **(Jayabalan et al., 2014)**. An immunomodulator is a compound that can modulate the immune system, which is needed to overcome the unstable condition of health complications caused by antigen. Clinically, immunomodulation mechanisms are categorized as immunoadjuvant, immunostimulant, and immunosuppressant. On the other hand, instability in the immune system caused by bacterial invasion increases the occurrence of serious disease, e.g. typhoid **(Abbas, Lichtman and Pilai, 2007)**. *Salmonella typhi* is a pathogenic bacteria that causes typhoid fever – a serious health issue globally **(Crump, 2019; Thung et al., 2017)**. It spreads through non-hygienic consumption of water and food. The bacteria can invade gut mucosal through microfold cells and infects the area without resulting in any clinical symptoms. Lack of inflammation response caused a late treatment and worsened the condition of the patient **(Khan et al., 2012)**.

Studies reported that snake fruit kombucha can lower fasting blood glucose, increases superoxide dismutase, reduces malondialdehyde level, and promotes pancreatic beta-cell- regeneration in the hyperglycemic rat. Furthermore, snake fruit kombucha was proven to have a similarly significant effect compared to metformin in treating diabetic rats with a dosage of 5 $mL.kg^{-1}$ bodyweights per day given orally for 28 days. These positive effects of snake fruit kombucha known to be related with its chemical composition such as phenol, tannin, hexane, 1-methyl-2, 2-furancarboxaldehyde, glucopyranose, and caffeine, which are produced during the fermentation process **(Zubaidah et al., 2018b; Zubaidah et al., 2018c)**.

These beneficial effects of snake fruit kombucha, which have been reported, lacked scientific evidence to ascertain its potential immunomodulatory effect. Thus, this study aimed to investigate the chemical changes during fermentation of kombucha and its immunomodulatory activity in *Salmonella typhi*-infected mice, which will be ascertained through the population of CD4+TNFα+, CD4+IFNγ+, CD8+TNFα+, and CD8+IFNγ+.

Scientific hypothesis

The fermentation affects the chemical characteristics of the kombucha. The kombucha administration raises the production of CD8+TNFα+ and CD4+IFNγ+ in mice infected with *Salmonella typhi*.

MATERIAL AND METHODOLOGY

Material

Snake fruit (*Suwaru salak* cultivar) was obtained from a local farmer in Malang, East Java, Indonesia. Black tea was purchased from the local market. SCOBY culture was bought from Wiki Kombucha, Bali, Indonesia. *Salmonella typhi* was obtained from a national culture collection.

Snake fruit kombucha and black tea kombucha preparation

Peeled snake fruit was separated from its seed, cut, and washed with distilled water. Snake fruit was juiced in a food processor with distilled water at a ratio of 1:1 (w:v), then filtered. The juice was added with 10% sucrose (w/v) and brought to boil. While black tea extract was prepared by eight grams of black tea immersed in 1 L of boiling water, added with 10% sucrose (w/v), and let sit for 15 minutes. The prepared snake fruit juice or black tea extract was poured aseptically into a sterilized glass container, cooled until it reached room temperature, and then inoculated with 10% SCOBY culture (v/v). The container was covered with a sterile cloth and let aside to undergo fermentation at room temperature for 14 days. The cellulose layer was aseptically separated and the solution was subjected to chemical and immunomodulatory activity evaluation.

Chemical Analysis

Total acidity, total sugar, total dissolved solids [TDS] was analyzed according to **AOAC (1995)**. pH was measured by using a pH meter. Total phenolic content was determined according to **Yang, Paulino and Janke-Stedronsky (2007)**. Total flavonoid content was evaluated according to **Atanassova, Georgieva and Ivancheva (2011)**. Antioxidant activity (DPPH scavenging activity) was analyzed according to **Pinsirodom, Rungcharoen and Liumminful (2010)**. All analyses were carried out on a day 0 and day 14 of the fermentation process to ascertain any changes in both black tea kombucha and snake fruit kombucha.

Immunomodulatory activity evaluation

Thirty female Balb-C mice aged 12 weeks were adapted for 7 days given food and water ad libitum, then randomly categorized into 6 groups: Normal (N, healthy group), N-BTK (normal + black tea kombucha), N-SFK (normal + snake fruit kombucha), Infected with *Salmonella typhi* (I), I-BTK (infected + black tea kombucha), and I-SFK (infected + snake fruit kombucha), with each group, consists of 5 mice. The experimental protocols and procedures of care and use of animals used in the present study were approved by the Ethics Committee (ethical clearance No. 1059-KEP-UB). The National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978) was followed in this experiment. Kombucha was given orally as much as 0.007 $mL.g^{-1}$ body weight per day for 21 days. On day 22, *Salmonella typhi* infection was carried out intraperitoneally with a dosage of 0.1 mL per mice with a concentration of 10^8 cells per mL. On day 29, lymph organ was taken for flow cytometry analysis to assess the population of CD4+TNFα+, CD4+IFNγ+, CD8+TNFα+, and CD8+IFNγ+.

Statistical analysis

The chemical characteristics data were analyzed with ANOVA at a significance level of 0.05% with SPSS. Flow cytometry data were analyzed with BD cell quest ProTM and statistically analyzed with ANOVA at a significance level of 0.05% with SPSS. A significant result was furtherly analyzed with Tukey.

RESULTS AND DISCUSSION

Chemical changes during fermentation

The fermentation process is a metabolic process that triggers simultaneously changes to characteristics of the medium including its nutritional contents and antioxidant activity. Changes in both black tea kombucha and snake fruit kombucha are presented in Table 1. The increase in total acid at the end of fermentation is the result of the culture metabolism which converts sugar into organic acids, mainly acetic acid as the primary metabolite. Other acids were also produced during bacteria metabolisms such as acetic acid, gluconic acid, glucuronic acid, L-lactic acid, malic acid, tartaric acid, and citric acid.

Table 1 Changes in chemical characteristics of black tea kombucha and snake fruit kombucha during fermentation.

Parameter	Black Tea Kombucha		Snake Fruit Kombucha	
	Day 0	Day 14	Day 0	Day 14
Total Acid (%)	0.21 ±0.02*	0.42 ±0.08*	0.83 ±0.07*	1.11 ±0.01*
pH	5.06 ±0.05*	4.90 ±0.02*	4.01 ±0.01*	3.07 ±0.01*
Total Sugar (%)	10.99 ±0.01*	8.27 ±0.04*	13.00 ±0.11*	8.09 ±0.03*
TDS (°Brix)	13.79 ±0.01*	11.79 ±0.01*	14.45 ±0.01*	12.23 ±0.01*

Note: Data is the average of 3 replications ±*SD*. A notation of * shows significant different at each parameter in the same day at significant level of p >0.05.

High total acid increment in Snake fruit kombucha was predicted and caused by native acid in salak such as ascorbic acid **(Jayabalan, Marimuthu and Swaminathan, 2007; Jayabalan et al., 2014; Malbasa et al., 2011; Supapvanich, Megia and Ding, 2011)**.

Higher accumulation of organic acids during fermentation is related to lower pH value owing to acid ability to release H+ and cause a drop in pH level. By the end of the fermentation process, total sugar and total dissolved solid levels in the medium were lower compared to their levels at the beginning of fermentation as sugar is considered the primary carbon source for microorganisms that facilitates metabolism during fermentation. The reduction of TDS might be also caused by sedimentation of protein, pectin, pigment, and minerals.

The fermentation process not only changed the chemical characteristics of a medium, but also its bioactive components such as phenolic content, flavonoid content, and antioxidant activity **(Jayabalan, Marimuthu and Swaminathan, 2007; Bhattacharya, Gachhui and Sil, 2013)**. Changes in bioactive characteristics of black tea kombucha and snake fruit kombucha are presented in Table 2. Kombucha fermentation has been known to produce several enzymes such as invertase, cellulase, and amylase that catalyzes the breakdown of the chain between phenolic and medium complex that contributed to the increase of phenolic content after fermentation. On the other hand, epicatechin in tea and salak is known to undergo isomerization and depletion form microbes cell during fermentation resulting in in an increase of total flavonoid by the end of fermentation **(Essawet et al., 2015; Jayabalan, Marimuthu and Swaminathan, 2007; Supapvanich, Megia and Ding, 2011; Apriyadi, 2017)**. The antioxidant activity also increased during fermentation as the phenolic and flavonoid contents increased.

Animal Observation

The effect of treatment on mice body weight was monitored and evaluated on day 0, 7, 14, 21, and 28 the data were presented in Table 3.

Weight gain was observed in the healthy group, black tea kombucha, and Snake fruit kombucha (Normal, N-BTK, N-SFK). On the contrary, *Salmonella typhi* infection has led to

Table 2 Changes in bioactive characteristics of black tea kombucha and snake fruit kombucha during fermentation.

Parameter	Black Tea Kombucha		Snake Fruit Kombucha	
	Day 0	Day 14	Day 0	Day 14
Phenolic content (mg.L^{-1} GAE)	181.18 ±0.98	407.14 ±1.43*	280.48 ±0.55	534.94 ±0.24*
Flavonoid content (mg.L^{-1} QE)	3388.03 ±58.93*	3916.34 ±31.70*	3762.81 ±12.22*	4618.82 ±85.84*
DPPH scavenging activity (%)	76.62 ±0.13*	80.92 ±0.11*	77.22 ±0.42*	83.90 ±0.19*

Note: Data is the average of 3 replications ±*SD*. A notation of * shows significant different at each parameter in the same day at significant level of p >0.05.

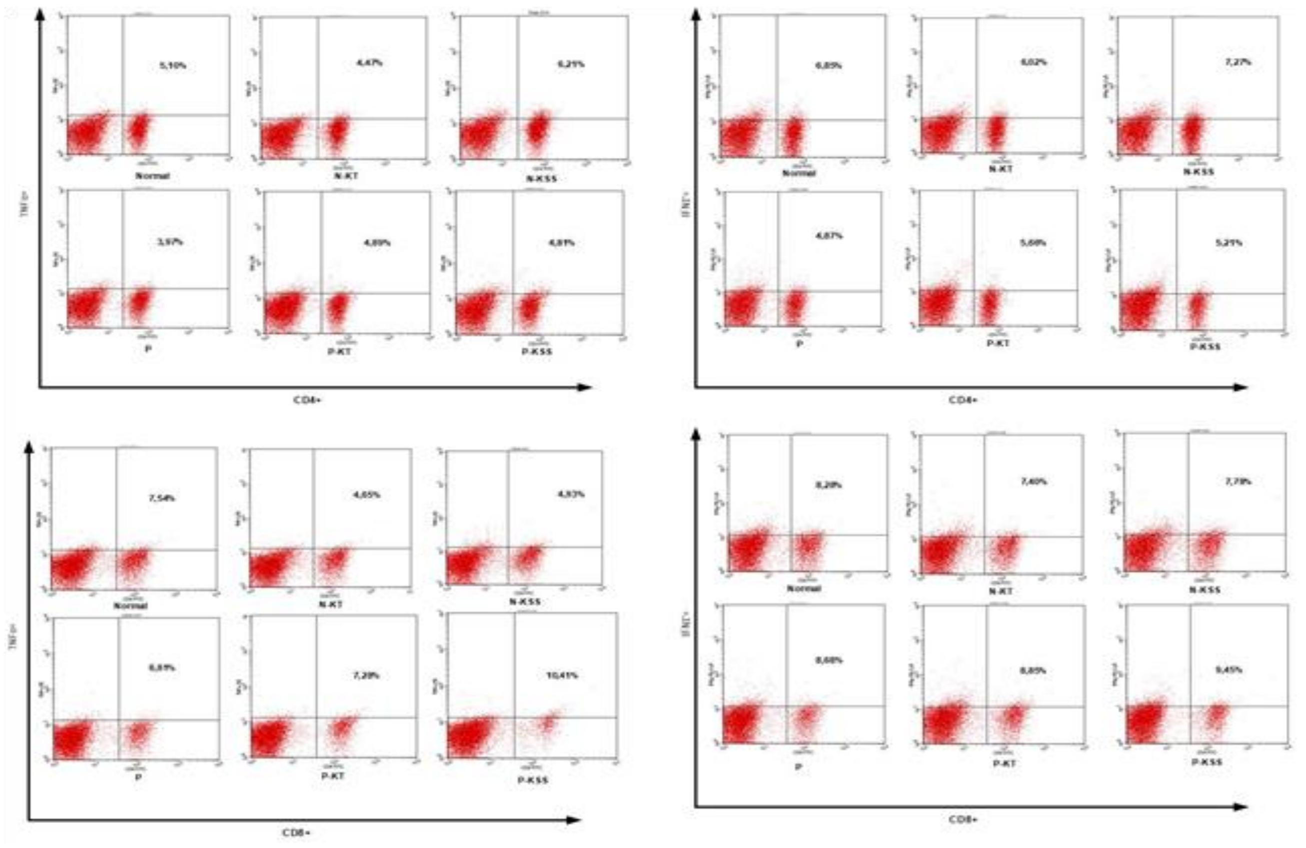

Figure 1 Flow cytometry analysis of CD4+TNFα+, CD8+TNFα+, CD4+IFNγ+, and CD8+IFNγ+.

Table 3 Changes in mice body weight during treatment (n = 5).

	Day 0	Day 7	Day 14	Day 21	Day 28
Normal	32.00 ±2.92	32.00 ±2.92	33.00 ±2.55	33.00 ±2.92	33.20 ±2.77
N-BTK	32.40 ±0.89	32.40 ±0.89	32.40 ±1.14	32.80 ±1.48	32.80 ±1.92
N-SFK	31.40 ±2.61	31.60 ±2.51	32.00 ±4.42	32.20 ±4.32	34.00 ±3.39
Infected	35.00 ±3.00	34.80 ±3.35	34.40 ±3.29	33.60 ±6.02	31.00 ±2.92
I-BTK	28.60 ±2.88	28.60 ±2.88	28.20 ±4.49	28.60 ±3.97	25.20 ±3.42
I-SFK	26.40 ±2.07	26.40 ±2.07	28.00 ±3.81	28.60 ±2.79	25.20 ±2.49

weight loss among an infected group, black tea kombucha, and snake fruit kombucha (Infected, I-BTK, I-SFK). Weight gain in the healthy group was related to the efficiency of gut activity in absorbing nutrients.

Moreover, high phenol, flavonoid, and antioxidant activity enhanced the body's metabolism to positive energy balance **(Fuller, 1989)**, thus healthy mice treated with snake fruit kombucha noted the highest weight gain (34.00 g).

Weight loss was a clear indication of *Salmonella typhi* infection. *Salmonella typhi* invade the gut mucosal surface and impaired the gastrointestinal tract absorption activity causing diarrhea, nausea, and vomit. The bacteria have also produced enterotoxin which stimulates gut epithelium to metabolize adenyl cyclase enzyme and c-adenosine monophosphate, which facilitated the secretion of chloride, natrium, and water from the gut lumen into the cell. In response to such conditions, hyperperistaltic occurred reduce excess water in the intestine thus diarrhea case has been established **(Ukhrowi, 2011; Nurhalimah, Wijayanti and Widyaningsih, 2015)**. Phenolic and flavonoid are known to have a bactericidal activity which is important to minimize the severity of diarrhea through inhibiting the growth of pathogenic bacteria **(Damayanti and Suparjana, 2007; Clinton, 2009; Loresta, Murwani and Trisunuwati, 2012)**. Acetic acid as the result of kombucha fermentation also correlates with inhibition of *Salmonella typhi* growth thus increases the efficiency of nutrient absorption, leading to weight gain **(Sreeramulu, Zhu and Knol, 2000)**.

Immunomodulatory effect of Black Tea Kombucha and Snake Fruit Kombucha

Figure 1 demonstrated the relative percentage of CD4+TNFα+, CD8+TNFα+, CD4+IFNγ+, and CD8+IFNγ+. Statistical tests noted that both *Salmonella typhi* infection and kombucha treatment did not reveal a significant effect on the relative percentage of CD4+TNFα+ and CD8+IFNγ+.

TNFα is an important cytokine produced in response to acute inflammation response stimulated by lipopolysaccharide. TNFα is needed to reduce pathogenic bacteria infection by inhibiting cell replication and destroying the infected cell. In the case of *Salmonella typhi* infection, TNFα+ mainly produced by CD8+ **(Oppenheim and Ruscetti, 2003; Bhuiyan et al., 2014)**. **Buttler and Girard (1993)** reported that TNF, IL-1, and IL-6 were increased as the response to *Salmonella typhi* infection. But, in this study we noticed that TNFα producing CD8+ has decreased. It is predicted that 3 – 7 days post-infection, macrophage effectively kill *Salmonella typhi* and eliminate dead cell **(Keuter, 1998)**, thus the expression of CD8+TNFα+ were lower (for instance, in the infected group), thus it can be inferred that both kombuchas have immunostimulant activity toward CD8+TNFα+.

Also, we revealed an immunosuppressant activity by both black tea kombucha and snake fruit kombucha. On the other hand, the non-infected group of mice treated with kombucha showed a lower CD8+TNFα+ relative percentage than the normal group. This may be due to bioactive components like flavonoid that causes lower expression of NF-kB transcription, followed by lower pro-inflammation cytokine production such as IL-17, IFNγ, and TNFα **(Saini, Sivanesan and Keum, 2016)**.

IFNγ is mainly produced by T- lymphocyte cells (CD4+ and CD8+) and natural killer cells which are activated as a response to antigen. High production of IFNγ increased the efficiency of macrophage to scavenge and kill microbes, initiate Th1 development, increase natural killer cells activity to lyse infected cell, increase MHC I expression which is needed by CD8+ to identify antigen, and increase MHC II expression to enhance the antibacterial activity **(Oppenheim and Ruscetti, 2003; Samuel, 2001)**.

Several immunological studies noted an increased level of IFNγ especially by CD4+ cells as a response to *Salmonella typhi* infection **(Sheikh et al., 2011)**. In this study, the population of IFNγ was decreasing compared to the normal group. Both black tea kombucha and snake fruit kombucha reported raising the population of CD4+IFNγ+ in which can be an alternative way to overcome the infection of *Salmonella typhi* since increasing IFNγ+ correlates to increasing activity of macrophage **(Abbas, Lichtman and Pilai, 2007)**.

Flavonoids also are known to have the ability to induce secretion of cytokines related to CD4+ cells and modulate its regulation by IL-2 production and increase CD8+ production **(Lyu and Park, 2005)**. Moreover, IL-2 is known to trigger CD8+ activation to produce perforin and grazin to support CD8+ function in destroying infected cells and *Salmonella typhi* antigen. Flavonoids are recognized to have immunostimulant activity by affecting macrophage and T cell and eliminate an infection. Flavonoids were able to activate the natural killer cell to trigger the production of IFNγ and increase the phagocytosis activity of macrophage. Also, phenols were proclaimed to initiate the production of IL-12 which activate natural killer cells to produce IFNγ and furtherly activate macrophage to kill antigen through the mechanism of oxygen-dependent- and oxygen-independent- **(Abbas, Lichtman and Pilai, 2007; Amit et al., 2017; Sulistiani and Rahayuningsih, 2015; Ramadhan, Mahfudh and Sulistyani, 2020)**.

CONCLUSION

Snake fruit kombucha triggers higher changes in chemical parameters during the fermentation process when compared to black tea kombucha. Moreover, snake fruit kombucha has higher bioactive components at the end of fermentation compared to black tea kombucha. Both products have the potential to be utilized as an immunomodulator to circumvent the unstable conditions of the immune system caused by *Salmonella typhi*.

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