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## Kefir Stimulates Anti-Inflammatory Response in TB-AFB (+) Patients

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**Abstract:** Regular consumption of kefir promotes beneficial physiological and therapeutic effects including stimulation of immune response. In this study, we investigated the immunomodulatory effects of kefir on peripheral mononuclear cells (PBMCs) derived from patients diagnosed with pulmonary tuberculosis (TB). PBMCs were isolated, treated with different concentration of kefir (1/20, 1/50, 1/100 and 1/200) and cultured for 4 days. The proportion of CD4<sup>+</sup> and CD8<sup>+</sup> cells was analyzed by flow cytometry and the production of T<sub>H</sub>1 cytokines IL-2 and IFN- $\gamma$  and T<sub>H</sub>2 cytokines IL-4 and IL-10 was determined by ELISA. The results indicate that kefir slightly stimulated CD4<sup>+</sup> as well as CD8<sup>+</sup>, but the effect was insignificant. Low doses of kefir significantly induced the secretion of IL-10 but not IL-2, IL-4, or IFN- $\gamma$ . We conclude that kefir in a low concentration stimulates T<sub>H</sub>2 rather than T<sub>H</sub>1-type immune response and therefore works as an immunosuppressant for early diagnosed TB patients.

**Key words:** Kefir, anti-inflammatory cytokines, TB patients

### INTRODUCTION

Kefir is a dairy product fermented from cow or goat milk using specific kefir grains containing a variety of microorganisms such as *Lactobacillus* spp., yeast and fungi trapped in a complex matrix of polysaccharides and proteins (Farnworth, 2006). Regular consumption of kefir promotes beneficial physiological and therapeutic effects, including stimulation of both specific and non-specific immune responses (Gill *et al.*, 2001) partly due to the activity of microorganisms and their product called kefiran (Isolauri *et al.*, 2001). Kefir has been shown to stimulate the secretion of T<sub>H</sub>1-type cytokines and suppress that of T<sub>H</sub>2-type cytokines in people with allergies (Hong *et al.*, 2010). An *in vivo* study has shown that kefir exerted immunostimulatory effects in the intestinal mucosa glands of rats (Vinderola *et al.*, 2005). Kefir is said to have been used in the past as a therapeutic food supplement to sustain tuberculosis (TB) patients in Eastern Europe, although there is no publication to support this information. However, anti-TB therapeutic effect of kefir is possible and may be caused by its immunomodulatory activity, which may boost the immune system of TB patients. Resistance to *Mycobacterium tuberculosis*, the causative agent of TB, critically depends on CD4<sup>+</sup> T-cell mediated activation of macrophages, which kill intracellular mycobacteria. Two subsets of CD4<sup>+</sup> T-cells, T helper 1 (T<sub>H</sub>1) and T helper 2 (T<sub>H</sub>2), are characterized by different profiles of secreted cytokines: T<sub>H</sub>1 cells typically produce interferon (IFN)- $\gamma$

and interleukin (IL)-2, which activate macrophages and T<sub>H</sub>2 lymphocytes produce anti-inflammatory IL-4 and IL-10 (Orme *et al.*, 1993). T<sub>H</sub>1 and T<sub>H</sub>2 cytokines can regulate and even inhibit each other's activity and the balance between the secreted cytokines is important for the host resistance against *M. tuberculosis*.

In this study, we investigated the potency of kefir as an immunomodulatory agent using peripheral blood mononuclear cells (PBMC) isolated from adult patients newly diagnosed with pulmonary AFB-positive TB. Since it is not known yet whether kefir worked as immunostimulant or immunosuppressant, we measured the expression of both T<sub>H</sub>1 and T<sub>H</sub>2 cytokines.

### MATERIALS AND METHODS

**Kefir samples:** Unflavored goat kefir was purchased from a small kefir-producing firm (Malang, Indonesia). Kefir was rapidly cooled and kept refrigerated until use (not more than 1 month). Kefir was centrifuged at 6,000 x g for 15 min and the supernatant was added to the PBMC culture. At first, four different doses were applied: 1/10, 1/20, 1/50, 1/100 and 1/200 (v/v, kefir supernatant/medium); cells not treated with kefir served as negative control. However, as kefir supernatant was very acidic (pH 3-4), in subsequent experiments we excluded the 1/10 dose because it exceeded the buffering capacity of PBMC medium, which became very yellow.

**Patients' characteristics:** This study recruited 19 patients who had been newly diagnosed with pulmonary TB by the pulmonary consultant of Saiful Anwar General Hospital, Malang, Indonesia. Inclusion criteria were as follows: newly diagnosed TB with positive AFB (+), moderate or far-advanced lesions, positive tuberculin X-ray tests and no previous treatment. Exclusion criteria were as follows: extra pulmonary TB, pregnancy, or immune diseases such as diabetes mellitus or HIV/AIDS.

Experiments were performed after the approval of the Ethical Committee of Faculty of Medicine, Brawijaya University and Saiful Anwar General Hospital. TB patients who met the inclusion criteria joined the study.

**Blood collection and PBMC isolation:** Blood samples (approximately 10 ml) were collected from each patient and transferred into EDTA-containing vacutainer tubes at 25°C. As the patients objected to blood collection of more than 10 mL, the number of isolated PBMCs was limited. Ideally, each test well requires  $1 \times 10^6$  cells; however, we could only obtain  $3-7 \times 10^5$  cells/well. PBMCs were cultured without PMA normally added to stimulate maximal cell proliferation because we considered it a confounding factor.

PBMCs were isolated within 2 h after blood collection. Briefly, 10 ml of sterile heparinized blood was layered slowly over 10 ml of Ficoll (Biolegend, San Diego, CA, USA) and centrifuged for 30 min at 1,000 rpm with the brakes off. The PBMC-containing ring layer was carefully removed into a new tube. PBMCs were washed with PBS and centrifuged at 1,300 rpm for 10 min; the washing process was repeated. The supernatant was discarded and PBMC content in the pellet was calculated using a hemocytometer. Isolated PBMCs were suspended at the concentration of  $1 \times 10^5-10^6$  cells/ml in sodium bicarbonate-free RPMI 1640 (SIGMA) supplemented with L-glutamine, streptomycin (50 µg/ml) and penicillin (50 µg/ml). A total of  $10^5-10^6$  cells/ml were suspended in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and seeded into the 96 well plates pre-coated overnight with  $\alpha$ CD3 (35 µl/ml) one day before use.

**Cytokine production:** Cytokine production by PBMCs was assessed as previously described (Elsasser-Beile *et al.*, 1989). PBMCs were incubated with kefir supernatant added at the doses of 1/20, 1/50 and 1/100 (v/v, supernatant/medium) or without kefir supernatant (in duplicate) for 4 days at 37°C and 5% CO<sub>2</sub>. Blood culture was performed at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. After the incubation, the supernatant from each well was removed and analyzed for IL-2 and IL-10 levels by solid double antibody sandwich ELISA (Biolegend, USA) as described in the manufacturer's protocol. The optical density was

measured at 450 nm using an automated ELISA reader ELX 800 (BioTek, Winooski, VT, USA) and cytokine levels were determined based on a semi-logarithmic reference curve generated using standards. All samples were assayed in duplicate and the results are presented as the mean±SD.

To assess the proportion of cells expressing CD4<sup>+</sup>, CD8<sup>+</sup>, IFN- $\gamma$  and IL-4, the cells treated with kefir for 4 days were harvested by centrifugation at 2,500 rpm for 3 min. The supernatant was removed and kept in -20°C until use to measure IL-2 and IL-10 by ELISA, while the pellet was stained with anti-human CD4<sup>+</sup>, CD8<sup>+</sup>, IFN- $\gamma$  and IL-4 antibodies and directly analyzed by flow cytometry (BD, San Jose, CA, USA).

**Data analysis:** To determine whether cytokine production of PBMCs isolated from TB patients was affected by kefir supernatant, statistical analysis was performed by one-way analysis of Variance (ANOVA). The difference at  $p < 0.05$  was considered statistically significant.

## RESULTS

**Patients:** TB patients were directed to Saiful Anwar General Hospital from other hospitals or local health clinics; most of them came from Malang countryside. All the patients were newly diagnosed with AFB-positive TB, displayed moderate to far-advanced lesions and had not received prior treatment. Among the 19 TB patients, 8 had multidrug-resistant (MDR) TB.

**Activation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells upon stimulation with kefir:** We investigated the stimulation of immune cells in response to kefir in PBMCs isolated from 19 TB patients. The addition of kefir supernatant did not cause significant difference in both CD4<sup>+</sup> and CD8<sup>+</sup> populations, which were decreased in PBMCs of TB patients compared to the positive control (Fig. 1).

**Extracellular cytokine production by kefir-stimulated PBMCs of TB patients:** We assessed the secretion of T<sub>h</sub>1 cytokine IL-2 and T<sub>h</sub>2 cytokine IL-10 by PBMCs isolated from 19 newly diagnosed TB patients upon addition of kefir. The addition of kefir at the doses 1/50, 1/100 and 1/200 slightly but not significantly induced IL-2 production compared to negative control ( $p = 0.162$ ); the highest stimulation was achieved by the treatment with kefir supernatant diluted 1/200 (Fig. 2, Table 1).

The production of IL-10 in kefir-stimulated PBMCs of TB patients in all groups markedly exceeded that in unstimulated cells without kefir. The highest level of IL-10 (1.094 pg/ml) was observed after the treatment with 1/200 dilution of kefir supernatant ( $p = 0.002$ ).

**Intracellular cytokine levels in PBMCs stimulated with kefir:** Next, we studied the ability of kefir to stimulate the

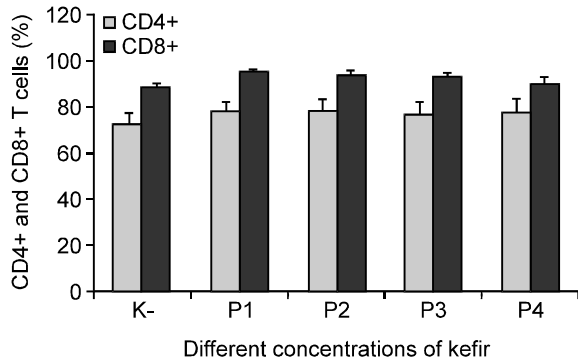


Fig. 1: CD4<sup>+</sup> and CD8<sup>+</sup> populations in PBMCs of TB patients stimulated with different kefir doses of kefir: 1/20 (P1), 1/50 (P2), 1/100 (P3) and 1/200 (P4); K-, without kefir

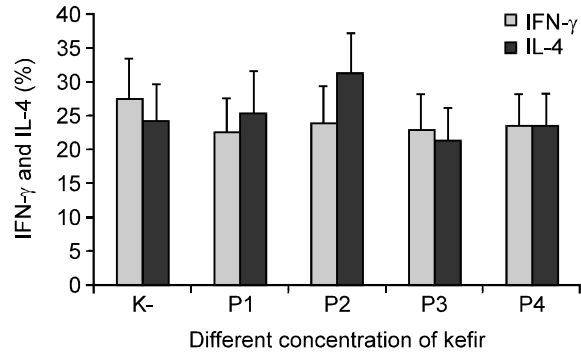


Fig. 3: T<sub>H1</sub> cytokine IFN $\gamma$  T<sub>H2</sub> cytokine IL-4 synthesis in PBMCs derived from TB patients and after stimulated with different doses of kefir: 1/20 (P1), 1/50 (P2), 1/100 (P3), 1/200 (P4). K-, no stimulation

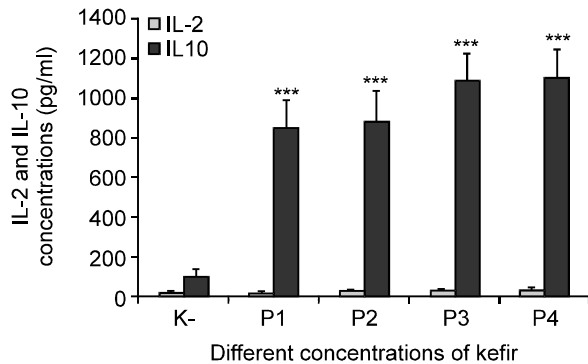


Fig. 2: Production of T<sub>H1</sub> cytokine IL-2 and T<sub>H2</sub> cytokine IL-10 by PBMCs isolated from TB patients and stimulated with different kefir doses: 1/20 (P1), 1/50 (P2), 1/100 (P3), 1/200 (F4P4). K-, without kefir. \*\*\* p = 0.002 compared to K-

Table 1: Production of T<sub>H1</sub> and T<sub>H2</sub> cytokines by PMBCs isolated from TB patients and application treated with kefir supernatant for 72 h

Dose of kefir	--- EC (pg/mL) ---		----- IC (%) -----	
	IL-2	IL-10	IFN- $\gamma$	IL-4
0 (K-)	22	22	27.5	24.3
1/20	22	851	22.4	25.2
1/50	28	876	23.5	31.2
1/100	28	1085	22.4	21.2
1/200	36	1091	23.7	23.4

EC: Extracellular cytokines, IC: Intracellular cytokines

expression of intracellular T<sub>H1</sub> cytokine IFN- $\gamma$  and T<sub>H2</sub> cytokine IL-4 by PBMCs derived from TB patients. The results demonstrated that IFN- $\gamma$  synthesis was repressed in all kefir-treated groups compared to unstimulated control, but the difference was not statistically significant. On the other hand, the intracellular levels of T<sub>H2</sub> cytokine IL-4 $\gamma$  were higher after stimulation with 1/20 and 1/50 concentrations of kefir but slightly decreased when stimulated with lower kefir doses (Fig. 3); however, the effect was also statistically insignificant.

## DISCUSSION

It has been known for decades that kefir, the fermented milk product, exerts immunomodulatory effects. Kefir has been shown to support immune system by stimulating production of a number of T<sub>H1</sub> response-related cytokines, including IFN- $\gamma$  and IL-2 known to mediate immunity against bacterial pathogens (Isolaure

*et al.*, 2001). Most investigations have been conducted in animal models and only few studies used PBMCs isolated from human patients suffering from bacterial infections. In our study, we investigated the immunomodulatory effect of kefir on the activation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells and the production of T<sub>H1</sub>- as well as T<sub>H2</sub>-type cytokines in PBMCs isolated from AFB-positive TB patients.

The control of *M. tuberculosis* infection highly depends on CD4<sup>+</sup> and CD8<sup>+</sup> cells. The main role of CD4<sup>+</sup> T helper lymphocytes is to activate other types of immune cells, including CD8<sup>+</sup> killer cells, which then destroy the infectious bacteria. The lack of CD4<sup>+</sup> cells may result in acute infection as well as re-infection (van Crevel *et al.*, 2002). Our results show that kefir only insignificantly raised the numbers of CD4<sup>+</sup> as well as CD8<sup>+</sup> lymphocytes. CD4<sup>+</sup> T helper cells and CD8<sup>+</sup> killer cells play a critical role in the host defense against *M. tuberculosis*. Previous studies have demonstrated that PBMCs isolated from TB patients contain lower levels of CD4<sup>+</sup> but higher levels of CD8<sup>+</sup> lymphocytes compared to healthy people (Deveci *et al.*, 2006; Afzal *et al.*, 2010). The reduction in CD4<sup>+</sup> would in turn lead to a decrease in the levels of T<sub>H1</sub> cytokines such as IL-2 and IL-15. However, after three months, the amount of CD4<sup>+</sup> but not CD8<sup>+</sup> cells increased. In our study, the

supplementation with low concentrations of kefir could increase the number of CD4<sup>+</sup> as well as CD8<sup>+</sup> cells, although the effect was not statistically significant. We did not try higher doses of kefir since they can critically reduce medium pH, which may be a confounding factor. This is the first study of the effect of Indonesian kefir on human PBMCs isolated from TB patients. We found that the secretion and intracellular production of cytokines after stimulation with kefir showed similar profiles, i.e., T<sub>H</sub>1 cytokines were inhibited, while T<sub>H</sub>2 cytokines were upregulated. In mycobacterial infection, T<sub>H</sub>1-type cytokines play an essential role in the protective immune responses against the pathogen. In contrast, T<sub>H</sub>2-type cytokines inhibit the production of IFN- $\gamma$  *in vitro* and activation of macrophages and therefore weaken host immune defense (van Crevel *et al.*, 2002). In our study, the production of T<sub>H</sub>1-type cytokines IFN- $\gamma$  and IL-2 was downregulated upon the addition of kefir, which can be attributed to low content of CD4<sup>+</sup> lymphocytes. IFN- $\gamma$  is closely associated with TB severity (Jamil *et al.*, 2007). Garcia *et al.* (2002) have found that PBMCs derived from TB patients produced much less IFN- $\gamma$  than those from healthy people. At the same time, the increase in IFN- $\gamma$  level significantly correlates with therapeutic progress, while T<sub>H</sub>2 cytokine production is linked to the anti-inflammatory cascade.

The results of our study are not consistent with those of other studies, which found that kefir can stimulate T<sub>H</sub>1-type cytokines in healthy mice (Vinderola *et al.*, 2005). This difference could be due to the immune status of the research subjects: we used PBMCs from TB patients, while Vinderola *et al.* (2005) used healthy mice. *M. tuberculosis* infection can quickly induce the secretion of TNF- $\alpha$  and IL-10 by monocytes and macrophages (Sendide *et al.*, 2005). IL-10 delays the production of pro-inflammatory cytokines, deactivates macrophages and prevents the release of nitrogen oxide and reactive oxygen species. It is not surprising that the expression of IFN- $\gamma$  and IL-2 was suppressed, because the elevated levels of IL-10 correlate with the decrease of resistance to infection (Redpath *et al.*, 2001). On the other hand, IL-10 can act as an important regulator of the body responses against infection, not only adaptive immune reactions but also innate immune mechanisms, including apoptosis in macrophages due to *M. tuberculosis* infection (Feng *et al.*, 2002). IL-10 works in concert with T<sub>H</sub>1-type cytokines such as IL-12 and regulates T<sub>H</sub>2 responses through stimulation of IL-4, IL-5 and IL-13, which can contribute to the development of chronic fibrosis observed in TB patients (Couper *et al.*, 2008). Hence, kefir supplementation should not be given at the beginning but rather several months after the start of anti-TB therapy when fibrosis has already been detected.

An increase of IL-2 in a TB patient has been reported (Wang *et al.*, 2012), which is in contrast to the

unchanged IL-2 level in TB patients after kefir treatment. One explanation could be iron deficiency normally occurring in TB patients because of chronic anemia. Thus, Afzal *et al.* (2010) have reported a decrease in hemoglobin concentration in TB patients. It has been suggested that iron deficiency may inactivate the proliferation of T lymphocytes and reduce the production of IL-2 (Suega, 2006). Since we did not check the level of anemia in our patients, this question remains open. A study of van Crevel *et al.* (2002) has shown an elevated level of T<sub>H</sub>2-type cytokine IL-4 in a TB patient with cavities. Although we did not examine this parameter, 8 of our 19 patients have been diagnosed with the MDR TB, which indicates disease severity, because the MDR patients typically show moderate to far-advanced lesions.

A number of studies have demonstrated that kefir can exert immunomodulatory effects *in vivo*. Kefir-derived polypeptides exhibited a significant antimicrobial activity in several animal models, not only through direct bactericidal effects but also through their immunomodulatory activity via inducing the production of antimicrobial proteins and mucin by host cells (Silva *et al.*, 2009). Some of kefir-derived peptides have been shown to stimulate T<sub>H</sub>1 cytokine production upon interaction with glycosylated Toll-like receptors 2 and 4 on macrophages and dendritic cells, respectively. Our study indicates that kefir exerts its immunosuppressant effects in smaller doses. Since kefir is normally consumed without dilution, further studies are required to explore the effect of high kefir concentrations using TB animal models.

**Conclusion:** In conclusion, kefir at low doses acts as immunosuppressant for PBMCs of newly diagnosed TB-AFB (+) patients.

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