

The effect of isolated probiotics from Indonesian *Passiflora edulis* Sims. on interferon gamma levels in peripheral blood mononuclear cell of adult tuberculosis patients *in vitro*

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Abstract

Background: Mycobacterium tuberculosis (M.tb) is unique because the bacteria live intracellularly and hide in macrophages so that they can escape from phagocytosis. This is one of the factors that complicate the treatment of M.tb infections. Interferon-gamma (IFN- γ) is a compound that plays an important role in macrophage activation to control intracellular pathogens.

Objective: The purpose of this research is to analyze the concentration of IFN- γ in peripheral blood mononuclear cells of adult Tuberculosis patients (TB) after *in vitro* administration of multi-strain probiotics from indigeneous Indonesian red passion fruit (*Passiflora edulis* Sims.).

Method: The probiotics isolated from red passion fruit were introduced into Peripheral Blood Mononuclear Cell (PBMC) adult TB patients who were undergoing Anti-tuberculosis Drug Therapy (ATD) category one at the Trosobo Primary Health Center (Sidoarjo, East Java, Indonesia), and the patients who were undergoing ATD category one treatment were willing to be involved in this study.

Result: The probiotics isolated from fermentation-broth of Indonesian red passion fruit were able to increase IFN- γ levels in PBMC of adult TB patients.

Conclusion: The red passion fruit probiotics isolated can increase IFN- γ of adult TB patients increased from 0.82% to 23.17% after *in vitro* administration.

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Introduction

Tuberculosis still is the major health problem in Indonesia. Based on the Global TB Report 2021, Tuberculosis (TB) is an infectious disease that causes the most deaths, about a quarter of the people in the world has been infected with M.tb. This condition is exacerbated by the Corona Virus disease-19, TB is the main cause of death due to infectious diseases. Indonesia is ranked second in the world in the largest number of tuberculosis cases based on the 2021 Global TB Report. This provides a high rate of morbidity and mortality.¹

M. Tuberculosis is able to live intracellularly, so that the most important role in the eradication of these bacteria is the cellular immune system. T-cells are important components of the cellular immune response to M.tb, especially the lymphokine Interferon-gamma (IFN- γ) which has been shown to be an important role for activating macrophages in limit the number of intracellular infections of pathogens.²

The increasing of IFN- γ strengthens the phagocytic potential of macrophages by stimulating phagolysosomal fusion and the formation of ROI and RNI which can destroy M.tb bacteria.³ *Mycobacterium tuberculosis* on macrophages will give a signal, so that the endoplasmic reticulum produces Major Histocompatibility Complex class II molecules. This molecule will carry the M.tb fragment, which is processed by macrophages to the macrophage surface and exposed, so that it is recognized and bound by the CD4+ T-lymphocyte receptor. Activated CD4+ T-lymphocytes will produce cytokines that has main role in destroying or controlling

the growth of *M.tb*. The IFN- γ cytokine strengthens the phagocyte potential of *M.tb*-infected macrophages, namely by stimulating the formation of phagolysosomes and stimulating the formation of free radicals that can destroy *M.tb* components. IFN- γ will stimulate macrophages containing *M.tb* to increase RNI which is needed to destroy *M.tb*.⁴ Therefore, the evaluation of IFN- γ levels in TB patients as a parameter of the immune system is very important.

Immunomodulators has begun to be developed in treating TB, especially resistant *M.tb*. Probiotics are significant components of the microbiome in the gastrointestinal tract. They have been reported to act as immunomodulators through their antimicrobial activity and as mediators of the pro-inflammatory activity of Th 1-cytokines and stimulates local and systemic adaptive immunity.⁵

Previous research, administration of probiotics (*L.acidophilus*, *L.casei*, *L.bulgaricus*, *B.bifidum*, *B.animalis*, *L.plan-tarum*, *S.thermophilus*) was reported to be able to increase the secretion of IFN- γ in PBMC of pediatric TB patients.⁶ Oral administration of probiotics will enhance the immune system through interaction with intestinal epithelial cells (IECs) thru Toll-like receptors (TLRs), and induce the production of various cytokines or chemokines. Probiotics strengthen the gut barrier by increasing mucin and Paneth Cells, so they can modulate the gut microbiota by suppressing the growth of potential pathogenic bacteria in the gut. Therefore, the interaction of probiotics with IECs, phagocytes and dendritic cells (DCs) plays an important role in this immune response without causing an inflammatory pattern.⁷

Passion fruit contains many secondary metabolites including flavonoid glycosides⁸ and alkaloids, carotenes and g-lactones are also found in passion fruit.⁹ Passion fruit contains high nutrition and multimineral, vitamins, carbohydrates and water.⁸ Passion fruit with sufficient nutritional content so that it is a good substrate or growing place for probiotics. Passion fruit, which is rich in health benefits, has not been developed further in the pharmaceutical field. Therefore, in this research was to analyze the levels of IFN- γ in PBMC of adult tuberculosis patients after in vitro administration of multistrain probiotics (isolate MM1 and MM2) from *Passiflora edulis* Sims. These probiotics which are resistant to the antibiotic erythromycin and vancomycin, resistant to phenol compounds and NaCl, as well as resistant to acidic pH and compatible when mixed each other.¹⁰

Peripheral blood mononuclear cell is consisting of lymphocytes, T cells, B cells, Natural Killer (NK) cells and monocytes. Erythrocytes and platelets do not have a nucleus, and granulocytes (neutrophils, basophils, and eosinophils) have a nucleus that has many lobes.¹¹ Therefore, to identify T cell cytokines, namely IFN- γ can be done by isolating PBMCs.

Materials and Methods

Red passion fruit

Passion fruit with ripe red skin (marked with perfect red skin) obtained from the Krembung area, Sidoarjo Regency, East Java, which was harvested in April-June and determined at Herbarium Malangensis with identification number 09/25.07.18/herb. malg as *Passiflora edulis* Sims.

Cell biomass production of probiotic bacteria by fermenting in media de man rogosa and sharpe broth (MRS-broth)

Probiotics isolated from red passion fruit (MM1 and MM2) were suspended in MRS-broth media and fermented for 24 hours. Sell probiotic biomass is then used as a further test.¹² After the probiotic biomass cell was obtained, simple identification was carried out which included Gram staining test.

Preparation of mycobacterium tuberculosis (m.tb) h37rv

Suspension *M.tb H37Rv ATCC-27294* 3-4 weeks old in Lowenstein-Jensen media (L-J) taken one ose, suspended in 5 mL RPMI medium (+3% glycerin + 10% HIPHS serum) in a tube + glass beads (2 mm diameter); 6-8 beads, homogenized using a vortex, then left for 30 minutes, so that the coarse lumps are below. The top part of the H37RV *M.tb* content equivalent to 7.5×10^5 per mL (S) was taken 0.2 mL suspended in 3.8 mL RPMI (S1) so that the *M.tb* content was equivalent to 1.5×10^5 CFU/mL.¹³

Procedure for taking venous blood of research subjects

Adult TB patients (adult pulmonary TB patients who were still sensitive to anti-tuberculosis drugs (OAT) with category 1 therapy regimens and agreed to be involved in the study and were willing to participate in the study) will have 10 mL of venous whole blood taken, then put into 2 vacutainer tubes with EDTA (5 mL) and together with an ice pack sent to ITD UNAIR. Examination of IFN- γ using enzyme-linked immunosorbent assay (ELISA) using the Elabscience Human IFN- γ immunoasy kit Catalog. No. E-EL-H0108 96 tests.

Separation of PBMC from peripheral blood cells

Venous whole blood (5 mL) is then put in EDTA tube and add with an ice pack sent to Institute of Tropical Diseases Universitas Airlangga. Venous blood cells were added buffer with a volume of 4 times. The suspension was taken and put into a 50 mL conical tube which already contained 15 mL of ficoll (inserted slowly so that the blood did not mix with ficoll), centrifuged 400 x g for 30-40 minutes at 20°C. The blood that has been centrifuged will be separated into four layers, the first layer is plasma, the second layer is PBMC with grayish white called buffy coat, the third layer is ficol and the bottom is erythrocyte. Separated as much as PBMC into 50 mL conical tube, then diluted 1:3 in buffer solution, then centrifuged 300 x g for 10 minutes at 20°C. The fraction was transferred into a sterile centrifugation tube and washed with 50 mL buffer solution (2 x number of cells), centrifuged at 200 x g for 10 minutes. Monocyte cell viability was determined using trypan blue exclusion (>95%). The total population of mononuclear cells obtained was calculated using a cubicle count per mL of blood. Peripheral blood mononuclear cells obtained with a concentration of $\pm 2 \times 10^6$, then stored in deep freeze until a culture is performed.¹¹

PBMC cell culture and treatment

PBMC that has been isolated is pipette 200 μ L put into wells, then 500 μ L RPMI-1640 (Sigma) has been added to which 10% FBS has previously been added (referred to as PBMC). Incubated for 1 hour at 37°C and 5% CO₂, replaced with 500 μ L of RPMI by throw away the previous medium and replace with the new medium. Multistrain probiotic biomass of red passion fruit isolate (MM1 and MM2) as much as 100 μ L was put into well-2, 100 μ L of *M.tb* in to well-3 and well-4, then incubated with 5% CO₂ for 2 x 24 hours at 37°C. The use of RPMI was carried out again, then probiotic bacteria were added to well-4. Incubated again for 2 x 24 hours at a temperature of 37°C and 5% CO₂. Next is the harvesting process, with 1600 rpm PBMC centrifugation for 5 minutes. The cell-free supernatant was then sterilized by filtration through a 0.4 m pore size filter membrane, then saved at -80°C until used for IFN- γ analysis.¹⁴

Analysis of IFN- γ with ELISA kit

Elisa kit used is Elabscience® Human IFN- γ with a total of 96-wells. A mark is given to each well. 100 μ L standard, 100 μ L blank

(diluent), and 100 μL of sample in each well that have been marked. The well-plate is closed with the sealer provided by the kit. Incubated for 90 minutes at 37°C . Decanted liquid from each well and do not wash. Immediately added 100 μL of Biotinylates Detection Ab working solution in each well. Incubated for 1 hour at 37°C . Liquid was decanted from each well and 350 μL of wash buffer was added. After one minute, the liquid was aspirated or decanted from each well and then washed three times. Add 100 μL of HRP conjugate working solution to each well. The well-plate is closed with a new sealer. Incubated for 30 minutes at 37°C . The liquid was decanted in each well and washed five times. Added 90 μL of reagent substrate in each well, incubated for 15 minutes at 37°C and protected from light. Add 50 μL of stop solution to each well. Determination at a wavelength of 450 nm.

Results

The cell biomass was carried out, namely to determine its effect in increasing interferon gamma levels in adult tuberculosis patients in vitro with the results shown in the Table 1 that can be seen that IFN- γ levels increased after being given probiotic isolate from red passion fruit (group 3) and increased with M.tb induction when compared to group 1 (PBMC culture without probiotic administration) and group 2 (which induced M.tb only). The percentage increase in IFN- γ levels after being given probiotics is presented in Table 2. In this study the percent increase in IFN- γ levels was only for group 3 and group 4 because to know the percent increase in IFN- γ levels incubated with probiotics only with those incubated with probiotics and induced M.tb. The % value of IFN- γ levels was calculated through $\{(Treatment\ 4 - Treatment\ 3) / Treatment\ 3\} \times 100\%$.

Discussion

In this study, probiotics isolated from red passion fruit were from the Bacillus group, identified as *Bacillus subtilis* (MM1) and *Bacillus wiedmannii* (MM2).¹⁰ The ability of the antibacterial activity of *Bacillus* species depends on the type of strain. Bacillus is widely used as a probiotic because of its good stability to heat, gastric pH, and humidity.¹⁵ The genus *Bacillus* has about 377 species and most of them contain antimicrobial compounds. Antimicrobial compounds produced by *Bacillus spp.* including volatile compounds, ribosomal peptides/RPs (bacteriocins and enzymes (lactonase, decarboxylase, acylase, deaminase), polyketides (PKs), and non-ribosomal peptides/NRPs (lipopeptides/LPs, fatty acid chains, and siderophores).¹⁶

Bacillus sp. is in great demand in the functional food sector because of its benefits for human health and its ability to withstand the pH of the digestive tract as well as being more stable to heat during processing and storage.¹⁷ Although the use of *Bacillus* as a probiotic is still controversial regarding its pathogenic properties and great benefits, there are several groups of bacilli that are excluded from the group of pathogens, and have antimicrobial, antioxidant, and immunomodulatory activities. Several species of Bacillus have also been used for the production of additional nutraceuticals including vitamins (e.g., riboflavin, cobalamin, inositol) and carotenoids for the synthesis of several health supplements for human consumption.¹⁸

The probiotic isolates from indigenous Indonesian red passion fruit mixed isolates MM1 and MM2 which in the previous study identified as *Bacillus subtilis strain IAM 12118* and *Bacillus wiedmannii strain FSL W8-0169*.¹⁰ That mixed probiotics were induced into peripheral blood mononuclear cell (PBMC) adult TB patients who were undergoing anti-tuberculosis drug therapy (OAT) category one at the Trosobo Health Center. Sidoarjo Regency Park District (East Java).

Based on the results obtained, probiotic isolates from red passion fruit were able to increase IFN- γ of adult TB patients. IFN- γ

Table 1. Measurement results of IFN- γ levels in PBMC of adult tuberculosis patients after being given probiotics isolate from indigenous Indonesian red passion fruit.

Duration of Therapy (Week)	Average IFN- γ Level in Serum	Average IFN- γ Level in PBMC			
		Group 1	Group 2 (+M.tb)	Group 3 (+ Prob*)	Group 4 (M.tb + Prob)
1-2	0.06275	-	-	68.7135	78.114
Standard deviation	0	-	-	3.634451988	10.55331389
3-4	2.5502	4.5074	3.5414	86.89	87.6018
Standard deviation	0	5.501906478	5.172858999	2.445796476	6.709565065
5-6	16.8676	3.2721	4.0486	85.2143	104.9602
Standard deviation	0	1.3051	1.9662	7.8736	15.1883

*Prob = Probiotic mixculture (MM1 and MM2).

Table 2. The percentage increasing IFN- γ levels in PBMC of adult TB patients after administration of probiotic isolates from red passion fruit.

Duration of therapy, week	Average levels of IFN- γ		Increasing IFN- γ levels, %
	Group 3	Group 4	
1-2	68.7135	78.114	13.68
3-4	86.89	87.6018	0.82
5-6	85.2143	104.9602	23.17

levels in PBMC of adult TB patients increased from 0.82% to 23.17% after in vitro administration of indigenous Indonesian red passion fruit isolate probiotics.

PBMC cells are infected by *M.tb*, which results in the secretion of cytokines. Cytokines are immune system proteins that regulate interactions between cells and stimulate immune reactivity, both in innate and adaptive immunity. Cytokines is a chemical messenger protein or intermediate in communication between cells that is very potent. Cytokines play a role in cell activation. B, monocytes, macrophages, inflammation and induction of cytotoxicity.⁴

IFN- γ is able to activate several signaling pathways and transcription factors. Signals from the TLR and CD40 will activate the transcription factor NF- κ B and activation protein 1 (AP1). Transcription factors stimulate the expression of macrophage phagolysosomal enzymes, namely the phagocytic oxidase enzyme, which induces the production of reactive oxygen species, inducible nitric oxide synthase that stimulates the production of nitric oxide and lysosomal enzymes. These various substances will destroy *M.tb* that has been ingested (inserted) in macrophages and activate macrophages so that they are able to phagocytose *M.tb* (Mashruwala *et al.*, 2011). The macrophage phagocytosis mechanism is an innate defense mechanism that is very important in eliminating intracellular pathogenic bacteria. The induction of macrophage phagocytosis will deliver *M.tb* into lysosomes which will kill the bacteria.¹⁹

The phagocytosis mechanism of macrophages is an innate defense mechanism that is very important in eliminating intracellular pathogenic bacteria. The induction of macrophage phagocytosis will deliver *Mtb* into lysosomes which will kill the bacteria.¹⁹ Based on the results obtained, probiotic isolates from red passion fruit have the potential to be developed as a health supplement for adult TB patients to boost the immune system, so it is necessary to carry out toxicity tests and determine their safety status as probiotics (generally recognized as safety/GRAS). Because probiotic isolates from red passion fruit seeds are *Bacillus* species, some of which are capable of producing toxins (pathogenic in nature), so it is very important to carry out toxicity tests.

Conclusions

Probiotic isolate from red passion fruit (*Passiflora edulis* Sims.) (MM1 and MM2 isolates) was effective in increasing IFN- γ levels in PBMC of TB patients in vitro with a percent increase of 0.82% to 23.17%.

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