Synbiotic and nutrients supplement improved of Secretory Immunoglobulin A (sIgA) in treated pulmonary tuberculosis patients

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Abstract:
Basically, bacterial populations in the gut ecosystem of healthy people who eat a balance diet are generally stable. Used of anti–tuberculosis drugs that consist of several antibiotics was altering the human gut microbiota. To analyze efficacy of milk–based protein supplement, synbiotic and micronutrients supplements on maintaining of microflora balance and increasing of sIgA level of treated pulmonary tuberculosis patient. A double–blind randomized treatment–control trial design. Data of microflora population of Lactobacillus sp. and Bifidobacterium sp. in human fecal was enumerated using plate count method and counting chambers method. Determination of secretory IgA (sIgA) in saliva was using sIgA ELISA Kit. Both subjects of treatment and control groups got same standard package of anti–tuberculosis drugs and MBP supplementation, except of treatment subjects were given synbiotic and micronutrients. Wilcoxon signed ranks test and Mann–WhitneyU–test. After first month of intervention, total colony of Lactobacillus sp. and Bifidobacterium sp. were higher in treatment group than control groups (p < 0.05). sIgA concentration in saliva of treatment group did not changes (p > 0.05) in all phase, contrary in the control group sIgA titers after 6 mo was declined (p < 0.05). There was positive effect of these supplements to maintain balance of gut microflora and stimulating sIgA secretion during TB chemotherapy.

Keywords: Bifidobacterium, microflora, micronutrients, milk, mucosal, Lactobacillus.

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Introduction
In adults, composition of microflora species in human intestinal is relatively stable since there are no pathological condition such as diarrhea and use of antibiotics, but differ between individuals due to enterotypes, body mass index (BMI) level, exercise frequency, lifestyle, and cultural and dietary habits (1). Basically, bacterial populations in the gut ecosystem of healthy people who eat a balance diet are generally stable. Change in lifestyle, diet and ill conditions altered stability of this ecosystem (2). Impaired of microflora balance caused of dominant of aggressive bacterial and low of concentration protective bacteria, and it’s associated to inflammation state and pouchitis (3). Used of anti–tuberculosis drugs that consist of several antibiotics (rifampicin, isoniazid, pyrazinamid and ethambutol), for treating pulmonary tuberculosis disease has negatively effect to intestinal. Although, currently there is no supported research data showed the negative effect of anti–tuberculosis drugs to balance commensal bacteria, but several studies using other antibiotics showed that there is negative effects. Study was conducted by De Lacochetieri and colleagues, showed that there was effect of treatment using antimicrobial agent on alteration of the micro biota (4). Disruption of normal microflora could be caused by antimicrobial. Several antibiotics are specifically active against anaerobic bacteria that dominate in the human intestinal microbiota (5). Treatment with antibiotics and immunocompromised status (immune response is weak) disturbed of microbial colonies. In most cases of antimicrobial therapy, the bacterial populations in some genera are reduced in numbers while those in other genera increase (6). The use of probiotic for healthy people showed that this bacterial has effect to composition of microbiota in the gut tract (7). Consumption of probiotics, prebiotics and synbiotic demonstrated ability to modify of microflora composition and restore microbial balance (especially, Bifidobacteria sp. [Orla–
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Jensen, 1924) and Lactobacillus acidophilus [Moro, 1900 Hansen & Mocquot, 1970]), therefore potentially has health benefits (3, 8). Specific strain of probiotic influence directly and indirectly modulating of immunological system in digestive tract through increased of local immunity. Stimulation effect of probiotic is strain specific regarding to protein profile of cell wall belong to this probiotic (9).

Genera of Lactobacillus sp. and Bifidobacterium sp. bacteria most commonly used as a probiotic microorganism (10). It is mainly related to that both bacteria present in the normal microflora (5). Lactobacillus sp. is major microflora in the intestinal tract and Bifidobacterium sp. is main microflora in the colon (11). Historical data indicated that the use of Lactobacillus sp. and Bifidobacterium sp. as probiotic in food and capsule is safe for human use (12). Maintaining and improving of microflora balance can be done directly by consumption of exogenous bacteria and another use of indigenous bacteria. The first way is known as a probiotic approach and the last is prebiotic approach. A combination using both approach is known as synbiotic. There is high prevalence of vitamin A and zinc deficiency among pulmonary tuberculosis patients who has low plasma retinol (< 0.70 µmol L⁻¹) and plasma zinc concentration (< 10.7 µmol L⁻¹). The proportions of vitamin A, zinc and the combination of the two micronutrient deficiencies were 47.9 %, 96.5 % and 47.8 %, respectively (13). Vitamin A and zinc play important role in immunological system both innate and adaptive immune response and also maintaining integrity of mucosal cells (14). These micronutrients are also required in cellular gene expression in transcription and translation level (15).

In contrast to serum Immunoglobulin A (IgA) in monomeric form, secretory Immunoglobulin A (sIgA) in the mucosal of intestinal tract in dimeric form (16). Secretory IgA is effectors component of mucosal adaptive immune system and it is the main immunoglobulin of humoral immune response that protects mucosal surfaces from microbial antigens. Production of sIgA needs presence of commensal microflora (17). The aim of this research is to analyze efficacy of milk–based protein supplement, synbiotic and micronutrients supplements on maintaining of microflora balance and increasing of sIgA level of treated pulmonary tuberculosis patients.

Materials and methods
Subject:
Population target was pulmonary tuberculosis outpatients of Bandung Tuberculosis Center Indonesia and Garut Tuberculosis Center Indonesia who attended on November 2009 to August 2010. Subjects were part of population target who met the inclusion criteria: newly diagnosed with pulmonary tuberculosis (regarded to microscopy and clinical examination and radiography), aged 20 yr to 45 yr and exclusion criteria: type two Diabetes Mellitus (DM), impaired renal function (blood creatinine test) and HIV (Human Immunodeficiency Virus) and for women, if pregnant or lactated. Furthermore, selected subjects who fulfilled inclusion and exclusion criteria were placed randomly allocation technique into treatment and control groups (1:1). Subjects of treatment group received milk–based protein, synbiotic and micronutrients supplements, and subjects of control group received milk–based protein without synbiotic and micronutrients.

Materials:
Both subjects of treatment and control groups got same standard package of anti–tuberculosis drugs. Supplementation was delivered for 56 d or during intensive phase of tuberculosis therapy. In the next followed phase, nutrients supplementation was stopped in all subjects in two groups, they only got anti–tuberculosis drugs.

Milk–Based Protein Supplement (MBP) containing of whey protein isolate, instantfull–cream milk, maltodextrins, sucrose and chocolate and vanilla flavor was distributed for treatment group. Improving daily nutrition intake of both subjects were advised by nutritionist counselor to consume balance diet using Indonesia Recommended Dietary Allowances. Its supplement was given approximately 70 g d⁻¹ (contained of: 294 kcal energy, 13.9 g protein, 18.2 g sucrose, 12.6 g lactose, 133 mg stachyose, 147 mg galactose, 203 g glucose, 210 mg raffinose, 847 mg fructose). The MBP supplement is consumed daily (four sachets @17 g d⁻¹ to @18 g d⁻¹). This carbohydrate composition may stimulate selectively and unselectively the growth of probiotic bacteria. Subjects of treatment group received recommended daily dose consist of: two capsules of synbiotic (@17.6 log¹⁰ CFU/capsule L. acidophilus and 2.5 log¹⁰ CFU/capsule Bifidobacterium longum [Reuter, 1963] and 60 mg (15 %) fructo–oligosaccharide) and one capsule of micronutrients (@ 5 000 IU of vitamin A as a retinylpalmitate and 15 mg zinc elemental as a zinc sulphate). Recommended daily dose of probiotic referred to best result of clinical trials that suggested to use minimal amount of therapeutic dose of 10⁸ to 10¹⁰ CFU day⁻¹ and upper level recommended daily dose of micronutrients (18).

This research was a field experimental and used double–blinded design, randomized treatment–control trial design. The researchers, field research team and research subjects did not recognized and identified subjects of treatment.


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and control groups. Applied real random allocation technique and not visually distinguished among two supplements (treatment and placebo) kept this research from internal and external biased.

Calculation of sample size used minimum sample size formula for estimating the mean difference between groups [19]. Used formula (1):

\[ \alpha = 0.05, 1 - \beta = 0.842 \]

and population mean different of *Lactobacillus* sp. in the previous study was 1.12 and standard deviation was 1.4 [20]. Obtained 25 subjects as sample for each group. Anticipated of high drop out among out patients tuberculosis and considered of exclusion criteria in the following phase of intervention were recruited 47 subjects per group.

**Data collection and analysis:**

Data of socioeconomic condition was collected by skilled interviewers using interview technique based on socioeconomic questionnaire. Data of microflora population was enumerated regarding colony of commensal bacteria of *Lactobacillus* sp. and *Bifidobacteria* sp. in human fecal. Used method of stool culture that appropriate to calculate population of microflora and applied good laboratory procedure that specimen was immediately processed [21]. Stool specimens of each subject were collected four times (baseline, after 1 mo, after 2 mo and after 6 mo). Enumeration of *Lactobacillus* sp. colony used plate count method. Suspension of stool specimen is putted in plastic and then it was taken and adapted at 37 °C for 1 h to 2 h. Stool suspension was dispersed into petry–dishes containing diluted MRS medium (De Man, Rogosa, Sharpe medium) of 10^1, 10^5, 10^6. Moreover, all diluted suspension in the petry–dishes was incubated at 37 °C for 2 d to 3 d. Finally, total colony of *Lactobacillus* sp. that grow on the surface of petry–dished was visually counted [22]. Total colony of *Bifidobacterium* sp. was examined using counting chambers method. Stool specimen in semi–solid medium was adapted at temperature of 37 °C for 1 h to 2 h. Stool specimen was shaken to make sure that colony spread out in the medium properly. Amount 100 mL of homogenous stool suspension was taken and diluted into 10^1, 10^5 and 10^6 of NaCl Phys solvent. Amount one to two drops of this suspension was dropped into surface of haemocytometer glass and it was covered by glass cover and putted it under a microscope. Visual colony of *Bifidobacterium* sp. was counted in five chambers. Determination of quantitative (titer) secretory IgA (sIgA) in saliva was using sIgA ELISA Kit (product of Immunodagnostik AG), Enzyme Linked Immuno Sorbent Assay (ELISA) method and λ spectrophotometer at 405 nm [23]. Data sIgA titer were obtained from converting of optical density values to micro g L\(^{-1}\) (µg L\(^{-1}\)) using standard conversion. The ethical approval of this research was published by the Research Ethical Committee No. LB.03.02/KE/505/2009 of National Health Research and Development Board. Explained informed consent was delivered to all accepted subjects before the study began.

**Figure 1.** Recruitment of subjects in treatment and control groups
During standard treatment period, subjects in two groups (within group) showed declining population of *Lactobacillus* sp. bacteria. However, in control group *Lactobacillus* sp. population significantly decreased earlier than treatment group (figure 2 and figure 3). There was also different in microflora balance of two probiotic bacteria. Unlike declined of *Lactobacillus* sp. population during intervention, within group population of *Bifidobacterium* sp. showed significant increased (figure 4). Increased population of *Bifidobacteria* sp. was earlier in the intensive phase (after 1 mo and 2 mo) than control group (after 2 mo) (figure 5). After 1 mo phase (figure 5), population of *Bifidobacterium* sp. in treatment group was significantly higher than control group (*p < 0.05*). However, in the intensive and following phase, population of *Bifidobacterium* sp. between two groups were not showed significant different (*p < 0.05*).

![Figure 2](image1.png)

**Figure 2.** Change in bacterial colonies of *Lactobacillus* sp. in each group. ns: not significantly, *p < 0.05, **p < 0.01, ***p < 0.001*, plot: median and range.

![Figure 3](image2.png)

**Figure 3.** Colonies of bacteria *Lactobacillus* sp. difference between the two groups of subjects. ns: not significantly; *p < 0.05; plot: median and range.
Figure 4: Changes in colonies of bacteria *Bifidobacterium* sp. subjects in each group. ns: not significantly; *p < 0.05; plot: median and range.

Figure 5: Colonies of bacteria *Bifidobacterium* sp. difference between the two groups of subjects. ns: not significantly; *p < 0.05; plot: median and range.

Secretory IgA in the initial phase of both groups was within normal limits (102 µg mL⁻¹ to 470 µg mL⁻¹). The average titer of sIgA in treatment group was 300.1 µg mL⁻¹ compared to 223.1 µg mL⁻¹ in control group. Figure 6 showed that sIgA concentration in saliva of treatment group did not cause significant changes (p < 0.05) in all phase, contrary in the control group sIgA titers after 6 mo was declined (p < 0.05). sIgA titers within treatment group did not significant decline (p < 0.05), but in control group was decrease fluctuated. However, after 1 mo phase, sIgA of treatment group significantly higher that control group (figure 7). The adherence to standar TB treatment of treatment dan control groups were 97.7 % and 100 %, respectively. Both groups showed positive trend to recovery in 1st mo, 2nd mo, 6th mo phase were 52.4 % and 48.7 %, 74.4 % and 61.5 %, 96.2 % and 97.1 %, respectively.
Changes in bacterial populations *Lactobacillus* sp. and *Bifidobacterium* sp.:

Symbiotic and micronutrients supplements showed positive influence on maintaining of microflora balance among subjects in treatment group who received standard anti-tuberculosis treatment (figure 2 and figure 3). In the 1\textsuperscript{st} mo, there was early positive effect of symbiotic and micronutrients supplementation to *Lactobacillus* sp. growth in treatment group compare to subjects of control group, that showed significantly declined of *Lactobacillus* sp. population (p < 0.05). It was showed that symbiotic capable restoring *Lactobacillus* sp. in gut. Meanwhile, after 2\textsuperscript{nd} mo period there was not significantly difference between two groups, it showed that administered of MBP supplement alone although slower stimulated microflora growth had positively effect to maintained *Lactobacillus* sp. balance. There was different effect of anti-tuberculosis drugs on impairing microflora balance of *Lactobacillus* sp. and *Bifidobacterium* sp. In addition, *Lactobacillus* sp. was most affected by antibiotics and it was characterized by declining microflora balance of its probiotic in treatment group and control group (figure 2). *Lactobacillus* sp. is a resident at area of distal small intestine, therefore interaction between this bacteria and antibiotics was more happen (most of antibiotics was absorbed in that area) than *Bifidobacteria* sp. that has habitat at area of proximal to distal colon (24). Meanwhile, the cause of higher population of both bacteria in the treatment group than control group (p < 0.05), was likely due to restoration effect of administration of probiotic *Lactobacillus acidophilus* and *B. longum* from exogenous symbiotic supplement. Previous study had supporting our suggestion that healthy people who taken Amoxicillin antibiotic for 1 wk and multi–species probiotic showed that *Lactobacillus* sp. population can be maintained, while the control group decreased (25). The exogenous bacteria have mechanism capacity to replacement loss of indigenous bacteria in the gut lumen (26).

There was not different in both of bacterial population (*Lactobacillus* sp. and *Bifidobacterium* sp.) between treatment group and control group after 2 mo phase. Amount one of possibility was due to strong prebiotic effect of milk based protein supplement on the growth of both indigenous probiotic bacteria. These results are consistent with other studies, the influence of prebiotic inulin administration of a small increase in population of *Bifidobacterium* sp. after 14 d of delivery (27). In this research, milk based protein supplement only (control group) was as effective as symbiotic supplement on improving and increasing population of *Lactobacillus* sp. and *Bifidobacterium* sp. after 2 mo. In vitro biological activity test showed that probiotic bacteria in milk based protein supplement increased metabolic activity of probiotic (production of short–chain fatty acids) five times higher than control group (data not show). Composition of milk based protein supplement containing various type of carbohydrate, namely monosaccharides (glucose, fructose, galactose), disaccharides (sucrose and lactose) and oligosaccharides (raffinose and stachyose) which has prebiotic properties to support growth of probiotic bacteria (28–30). Another factor showed that interaction capacity of indigenous and exogenous bacterial was not significantly different (31). Decreasing of *Lactobacillus* sp. and *Bifidobacterium* sp. population after 6 mo period probably due to negative effect of antibiotic and temporary (transient) adhesion of exogenous probiotics in digestive tract. This phenomena also found in previous studies regarding the used of *Lactobacillus plantarum* [Orla–Jensen, 1919] in healthy subjects (26).

Figure 6: Changed of sIgA titers in saliva of subjects in each group. ns: not significantly, * p < 0.05, plot: median and range.
Secretory immunoglobulin A (sIgA):

High titers of secretory IgA at baseline (compared of later phases) was likely caused by normal response to infection. The results were consistent with the another study that increased levels of sputum IgA in response to Moraxella catarrhalis [Frosch and Kolle, 1896] in adults with COPD (32). Local protection at the mucosal surface epithelium is facilitated by products such as mucin, defenses and secretory antibodies, especially IgA (33). In this study, saliva specimen for measurement of sIgA was used for reflecting immune response of the digestive tract. The mechanism of systemic immune response, can be stimulated secretory immune, whereas the cells that produce IgA precursors migrate from Galt to several locations of secretory (colostrum, lacrimal and salivary), including intestinal lamina propria (34). The occurrence of significant difference of sIgA titer between two groups after the first month was due to a decreased of sIgA titer in control group. This data showed that synbiotic and micronutrients supplement has positive effect kept increasing of sIgA titer from 1 mo to 6 mo (figure 6). Therefore, it was effect of the changes in different sIgA titers between treatment groups (more stable) and the control group (fluctuated). Studies in animals indicate that secretion of sIgA can be triggered by the consumption of Lactobacillus sp. (32)

Oral administration of L. acidophilus and Lactobacillus casei [Orla–Jensen, 1916] in animal experiments, increase the production of IgA and sIgA in the small intestine and enhance phagocyte of peritoneum macrophage and antibody production (35). Previous studies used mouse experiment showed that fed exogenous L. acidophilus and Bifidobacterium bifidum [Tissier, 1900] A12 A9, significantly capable on increasing number of IgA cells. Several other studies also showed similar result that increased production of IgA antibodies in response to supplementation Bifidobacterium sp. (36). In the each group there was not significantly increase of sIgA titer during supplementation, may be due to the body's homeostatic response to a decrease in the formation of Th2 (boost the growth cells of sIgA secretion) in response to rising titers of IFN–γ of Th1 cells (77). Another result showed there was a difference relationship of sIgA titers and population of probiotic bacteria. Population of Bifidobacterium sp. weakly positive associated with titers of sIgA in the first and sixth monts pheriod *(t5=0.226 and p > 0.05; t6= 0.314 and p > 0.01). On the other hand, there was not relationship population of Lactobacillus sp. and sIgA titers (p > 0.05). The lack of correlation between population of Lactobacillus sp. and titer sIgA, could be result of declining populations of these bacteria, so it is not sufficient to induce the secretion of sIgA. Synbiotic supplements are not the only factor triggering mucosal immune response, micronutrients such as vitamin A and zinc has capacity to modulate the immune response. These micronutrients were capable enhancing mucosal immunity. Pro-inflammatory cytokine production by effectors cells and the activity of phagocytic cells is enhanced by vitamin A and zinc. Zinc involved in the modulation of the proinflammatory response (38). Vitamin A has both promoting and regulatory roles in both the innate immune system and adaptive immunity (39). Both of these micronutrients are involved also in gene expression. Vitamin A and zinc have an important role in cell–mediated immune functions and they also function as anti–inflammatory and antioxidant agents in tuberculosis (40).

Conclusion

A combination of synbiotic supplements, micronutrients (vitamin A and zinc) and milk base protein was capable to maintaining the balance of gastrointestinal microflora, generating immunogenic in secretory IgA immune response of treated pulmonary tuberculosis patients. This result supporting that supplement being as part of complement in TB treatment.

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