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Effect of probiotic *L. plantarum* IS-10506 and zinc supplementation on humoral immune response and zinc status of Indonesian pre-school children

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ABSTRACT

A 90-day randomized, double-blind, placebo-controlled, pre-post trial was conducted in four groups of Indonesian children aged 12–24 months: placebo, probiotic, zinc, and a combination of probiotic and zinc ($n = 12$ per group). Microencapsulated *Lactobacillus plantarum* IS-10506 of *dadih* origin was supplemented at a dose of 10^{10} CFU/day as a probiotic. Zinc was supplemented as 20 mg zinc sulfate monohydrate (8 mg zinc elemental). Blood and stool samples were collected at baseline and at the end of the study period. Fecal sIgA was assessed by ELISA and serum zinc concentrations by ICP-MS. Fecal sIgA increased significantly in the probiotic group (30.33 ± 3.32 μg/g; $p < 0.01$) and in the combination probiotic and zinc group (27.55 ± 2.28 μg/g; $p < 0.027$), as compared with the placebo group (13.58 ± 2.26 μg/g). Changes in serum zinc concentrations in the combination probiotic and zinc group showed the highest elevation at the end of the study period. A combination of probiotic *L. plantarum* IS-10506 at a dose of 10^{10} CFU/day and 8 mg of elemental zinc supplementation showed a potential ability to improve the zinc status of pre-school children. Taken together, supplementation with the probiotic *L. plantarum* IS-10506 and zinc for 90 days resulted in a significantly increased humoral immune response, as well as improved zinc status, in young children.

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Introduction

Dietary supplementation is an attractive, non-invasive means of enhancing and optimizing important physiological functions, including the immune system [1]. The ability of dietary supplementation to optimize immune function is seen as particularly important among those groups of individuals who may have an under-developed or poorly functioning immune system, such as infants, young children, immunocompromised subjects and the elderly [2,3].

Recent advances in research have greatly increased understanding of the importance of the gut microbiota. Bacterial colonization of the intestine in a gut-microbiome system is critical to the normal development of many aspects of physiology. Further understanding

of the mechanisms underlying the gut-microbiome will provide new insight into the symbiotic relationship between gut microbiota and their mammalian hosts, and help to identify the potential for microbial-based preventive strategies to aid in health promotion.

Probiotics are defined as living microorganisms that when administered in adequate amounts, confer a health benefit to human health [4]. *Lactobacillus plantarum* IS-10506 is a novel probiotic strain isolated from a yogurt-like product, *dadih*, an Indonesian traditional buffalo milk of West Sumatera origin [5]. *In vitro* probiotic properties have been proven, such as acid and bile tolerance, adhesion properties, and competitiveness against pathogens [6,7]; *in vivo* [8,9] and human studies have been conducted in serial studies [10–12].

The primary functions of the gastrointestinal tract are digestion and absorption of nutrients, and electrolyte and water homeostasis; hence, the integrity of the intestinal brush border is a key element in preventing systemic absorption of enteric toxins and bacteria. Gastrointestinal lymphoid tissue plays an important role in controlling transepithelial passage of bacteria across the intestinal

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mucosa by synthesizing more antibody molecules than any other lymphoid tissue. Gastrointestinal antibodies are produced in the form of immunoglobulin and are key players in efficient humoral mucosal immunity, in particular the maintenance of the integrity of the epithelial barrier.

Zinc is an essential trace element of exceptional biologic and public health importance. Zinc is an essential component of a large number of enzymes, and plays a central role in cellular growth and differentiation of tissues with rapid turnover, including the immune system and the gastrointestinal tract [13,14]. Zinc deficiency involves impaired immune function, sub normal growth and development, and in children causes growth retardation, delayed sexual maturation, and infection susceptibility. Intervention trials have demonstrated that the supplementation with zinc can also enhance the immune response [15]. There is no evidence that zinc causes harm when given to non-septic, immunocompetent children [16].

Materials and methods

Preparation of diets

The tested powder contained maltodextrin as placebo and probiotic *L. plantarum* IS-10506 of *dadih* origin was identified by 16S rRNA gene sequencing as *L. plantarum* (Gene Bank accession no. DQ860148). For experimental purposes, microencapsulated bacteria were incorporated at a dose of 2.3×10^{11} CFUs/g powder, and has been shown to exhibit neither significant loss of viability nor contamination because of storage during the entire study period. Zinc was supplemented as zinc sulfate powder. Placebo, probiotic or zinc were given in microencapsulated form and were identical in appearance.

Subjects and trial criteria

Forty-eight apparently healthy children within the age range of 12–24 months (median 16.8 months) were selected for this study. Prior to commencement of the trial, the selection criteria were generated from the records of participating health care providers. Inclusion criteria were apparently healthy children, between the ages of 12 and 24 months and agreement to conform to the trial guidelines or provide notification of non-compliance. Exclusion criteria were congenital abnormality or disease, gastrointestinal disease, regular use of products with probiotic bacteria, receiving antibiotic therapy within two weeks prior to the intervention study and non-agreement to avoid potentially conflicting nutritional or trace element supplements during the 90 days of the trial. Compliance with supplementation was confirmed by the subjects by direct report to the health care provider.

Protocol

The trial protocol was approved by the Faculty of Medicine, University of Indonesia Ethics Committee. Participation in the study was voluntary, and written informed consent was obtained before the start of the study from the parents or guardian of children for their child's participation in the study. The study period was from August 2009 to March 2010 in Larangan, Ciledug districts, Tangerang, Banten Province, Indonesia. The study was a double-blind, randomized, placebo-controlled pre-post intervention trial. Material Transfer Agreement was obtained from Ministry of Health for analyses of blood samples in Japan.

Subjects were randomly assigned consecutively into four groups: placebo, probiotic, zinc, and combination of probiotic and zinc. At enrollment, children were randomized using sealed envelopes containing a note assigning the subject to one of the

four study arms. There were 36 children (19 boys) in the study groups (supplemented), and 12 (7 boys) in the control group. The probiotic *L. plantarum* IS-10506 was supplemented at a dose of 2.3×10^{10} CFUs per day; zinc was supplemented at a dose of 20 mg/day as zinc sulfate. Supplementation was conducted for 90 days. Age, sex, weight, adverse events were recorded for each child, and a physical examination was conducted by a physician every one month during the intervention study. Two children in the zinc group and one child in the combination probiotic and zinc group were dropped from the study because they moved out of town and the blood sample was not sufficient.

Stool and blood sample collection

Peripheral blood samples were withdrawn from subjects by venipuncture and stool samples were collected at baseline and at the end of the study period. Fecal secretory IgA (sIgA) was assessed by ELISA kit at the Institute of Human Virology and Cancer Biology (IHVCB) laboratory, Faculty of Medicine, University of Indonesia, and serum zinc was assessed by ICP-MS in the Trace Element Laboratory, Department of Public Health, Gunma University Graduate School of Medicine, Japan.

Analysis of fecal sIgA

Fresh samples of stool were collected in the morning and transported immediately to the laboratory and stored at −80 °C. Measurements of sIgA were carried out using ELISA kits (K8870 and K6500, Immundiagnostik, Bensheim, Germany) according to the manufacturer's instructions.

Stool samples weighing 80–120 mg were diluted with appropriate amounts of buffer provided in the kit to give constant dilutions. The stool were suspended in diluent buffer, vortexed, and centrifuged at 13,000 rpm (=13,000 × g) for 5 min in 1.5 mL tubes. Supernatants were diluted 1:250 in wash buffer. Standards, controls and stool samples were simultaneously transferred to microplates coated with antibodies specific for sIgA. Anti-sIgA antibody conjugated with peroxidase was used for development. For each well, the optical density was measured at 450 nm on a microplate ELISA reader (Dynex, Heidleberg, Germany). The results of the test samples were calculated from the standard curve and expressed as concentration (µg/g) by wet weight of stool.

Serum blood preparation and zinc analysis

Venous blood samples were collected from children and centrifuged at 3000 rpm for 10 min to obtain serum and kept at −80 °C until analysis. Each serum sample (0.1 mL) was digested with 1 mL of 60% ultrapure nitric acid and 0.1 mL of 30% ultrapure hydrogen peroxide in a perfluoroalkoxy polymer vessel using the following procedure: heating at 90 °C for 0.5 h, heating at 120 °C for 2.5 h, and finally heating at 150 °C for 2 h on a hot plate. After cooling, the digested solutions of serum were diluted up to 5 mL with Milli-Q water in polypropylene sample tubes. Blank and certified reference materials (CRMs) were subjected to the same treatment as the samples. To minimize contamination from devices, all containers were soaked in an acid bath (a solution of 6% (V/V) of nitric acid) for a minimum of 24 h, followed by final rinses with Milli-Q water.

Serum zinc concentrations were analyzed by inductively coupled plasma mass spectrometry (ICP-MS) with an ELAN DRC II (Perkin Elmer, Waltham, MA, USA). The accuracy of the analysis was checked with CRMs: Seronorm Trace Element Serum Level 2 (No.203105, Billingstad, Norway). Results were in agreement with the reference values.

Table 1
Post hoc statistical analysis of changes in fecal sIgA levels after 90 days of supplementation.

(I) Treatment/placebo	(J) Treatment/placebo	Mean difference $(I - J)$	Std. error	P value	95% Confidence interval	
					Lower bound	Upper bound
Placebo	Probiotic	−0.08044[a]	0.02262	0.001[a]	−0.1261	−0.0348
	Zinc	−0.04452	0.02373	0.068	−0.0924	0.0034
	Probiotic_Zinc	−0.05293[a]	0.02313	0.027[a]	−0.0996	−0.0062
Probiotic	Placebo	0.08044[a]	0.02262	0.001	0.0348	0.1261
	Zinc	0.03592	0.02373	0.138	−0.0120	0.0838
	Probiotic_Zinc	0.02751	0.02313	0.241	−0.0192	0.0742
Zinc	Placebo	0.04452	0.02373	0.068	−0.0034	0.0924
	Probiotic	−0.03592	0.02373	0.138	−0.0838	0.0120
	Probiotic_Zinc	−0.00841	0.02421	0.730	−0.0573	0.0405
Probiotic_Zinc	Placebo	0.05293[a]	0.02313	0.027	0.0062	0.0996
	Probiotic	−0.02751	0.02313	0.241	−0.0742	0.0192
	Zinc	0.00841	0.02421	0.730	−0.0405	0.0573

[a] The mean difference is significant at the 0.05 level.

Statistical analysis

Differences between before and after supplementation were analyzed using paired *t*-tests. Differences between groups were analyzed by one-way ANOVA, Mann–Whitney *U*, Kruskal–Wallis and Chi-square tests, as appropriate. Data were checked for normality by visual inspection of the normal probability curves and with the Shapiro–Wilk test of normality. In the statistical analysis, children in probiotic, zinc and probiotic-zinc groups were compared with children that received placebo for changes in fecal sIgA and serum zinc after 90 days of supplementation. *P*-values of less than 0.05 were considered significant.

Results

Forty-eight children of median age 17 months (range 12–24 months) were recruited for the study. The median socioeconomic status was categorized as lower middle class. No significant change in bowel frequency (median daily frequency = 1) or stool consistency was reported during consumption of the probiotic. There was no significant complaint of abdominal pain or bloating or other abdominal symptoms during supplementation.

Changes in fecal sIgA levels of the placebo, probiotic, zinc and combination of probiotic and zinc groups in the subjects after 90 days were 13.58 ± 2.26, 30.33 ± 3.32, 20.5 ± 1.73, and $27.55 \pm 2.28 \, \mu g/g$ feces, respectively. The changes in fecal sIgA levels in the subjects after 90 days of probiotic *L. plantarum* IS-10506 supplementation ($p = 0.001$) and the combination of probiotic *L. plantarum* IS-10506 and zinc supplementation ($p = 0.027$) were significantly higher than in the placebo group (Table 1, Fig. 1).

Serum zinc concentrations in children of the placebo, probiotic, zinc and combination of probiotic and zinc groups before supplementation were 699.94 ± 164.81, 764.02 ± 157.90, 704.27 ± 152.07, and $662.16 \pm 183.13 \, ng/mL$, respectively. Serum zinc concentrations in children after 90 days supplementation were 668.62 ± 94.87, 744.52 ± 128.80, 748.81 ± 170.38, and $739.79 \pm 163.14 \, ng/mL$, respectively (Fig. 2). Serum zinc concentrations in children supplemented with the combination of probiotic *L. plantarum* IS-10506 and zinc after 90 days was significantly higher than before supplementation ($p < 0.05$). Furthermore, changes in serum zinc concentrations of children supplemented with the combination of probiotic *L. plantarum* IS-10506 and zinc were significantly higher than the placebo group ($p < 0.05$).
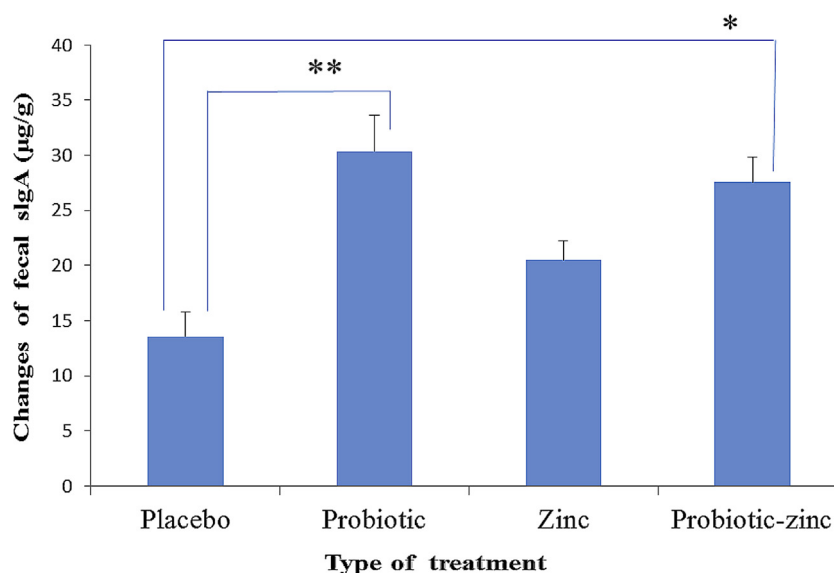

Fig. 1. Changes in fecal sIgA (μg/g) levels in the placebo, probiotic, zinc and combination probiotic and zinc groups after 90 days of supplementation.*$p < 0.05$, **$p < 0.001$.
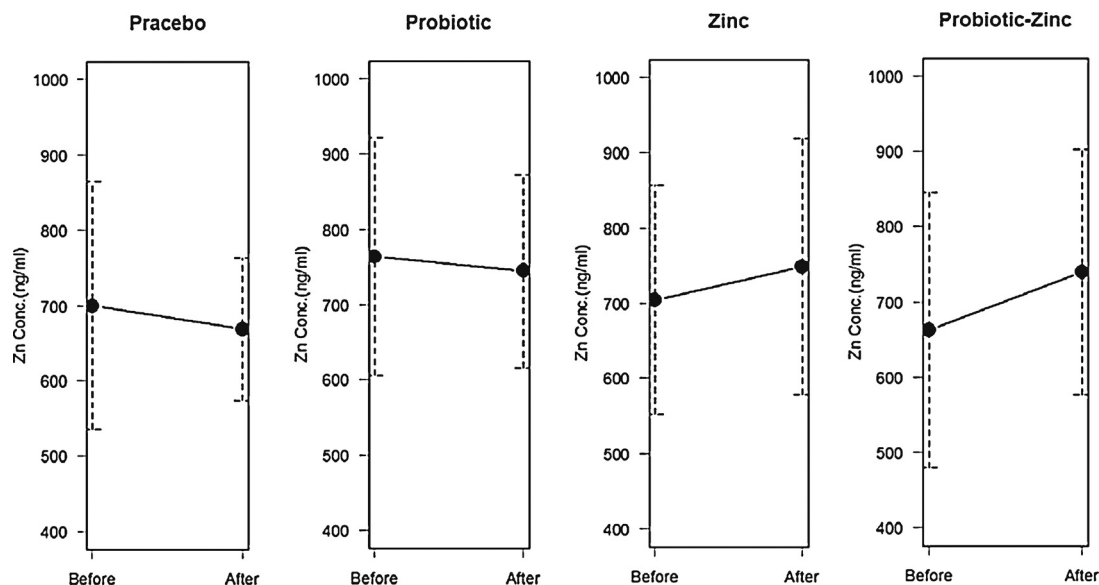
Fig. 2. Comparison of serum zinc levels (ng/mL) before and after 90 days of supplementation in each group.

Discussion and conclusion

Studies in healthy children and preterm infants have shown an increase in fecal IgA excretion in response to probiotic interventions [17,18]. A study in animals showed that probiotic yogurt containing *Lactobacillus casei* increased IgA secretion in mice [19]. In another study, salivary sIgA in Indonesian children younger than five was increased significantly in response to the probiotic *Enterococcus faecium* IS-27526 of *dadih* origin, supplemented at a dose of 10^8 CFU/day for 90 days [20].

Ingested probiotic bacteria interact with gut epithelial cells in a strain-dependent manner. The effects of *L. plantarum* IS-10506, as well as *E. faecium* IS-27526, on the humoral immune response could be the result of colonization and adhesion to epithelial cells, an efficient inducer of sIgA production, by the cell wall component of probiotics, such as lipoteichoic acids and peptidoglycan [7].

Both strong immune responses and gut integrity play important roles in the repair of intestinal brush border damage as a result of the balance of microbiota. Probiotic microorganisms positively change the intestinal microbiota, inhibit the growth of pathogenic bacteria, promote adequate digestion, stimulate local immune function, and increase resistance to infection.

The immunological properties of probiotic bacteria have been studied previously [21] and have shown that certain lactic acid bacteria, such as *L. casei, Lactobacillus rhamnosus*, and *L. plantarum*, enhance both systemic and mucosal immunity. Food containing probiotic bacteria are able to stimulate the IgA immune response [22].

In addition to probiotics, intervention trials have demonstrated that supplementation with zinc can also enhance the immune response [23]. The rationale for the beneficial effects of zinc supplementation is based on the depletion of zinc because of malnourishment and the deleterious effects of zinc deficiency on the immune system, leading to more severe enteric infections. There is also no evidence that zinc causes harm when given to non-septic, immune competent children [16].

Because zinc and probiotics work via different mechanisms, it is possible that adding both would have a synergistic effect, as shown by the significant increase in fecal sIgA ($p = 0.027$) in the children supplemented with the combination of probiotic *L. plantarum* IS-10506 and zinc after 90 days (Fig. 1).

Children are at risk for zinc deficiency. Infants and young children are the most vulnerable as shown by the decline of serum zinc levels of those children in the placebo and probiotic groups (Fig. 2), which may lead to zinc deficiency. The lack of reliable, widely accepted, and adequate sensitive indicators of zinc status has resulted in uncertainty about the global prevalence of zinc deficiency. ICP-MS is an accurate and sensitive instrument for measuring zinc levels in the blood plasma and can detect zinc at concentrations in the ng/mL range.

Supplementation with zinc and its combination with the probiotic *L. plantarum* IS-10506 showed significant increases in zinc levels after 90 days in young children. This might be the result of a synergic effect between the probiotic and zinc, or might be because of the integrity of the intestine, which in turn will be able to optimize the absorption of zinc.

Zinc is necessary for the activity of some immunity mediators. The human body contains 2–4 g of zinc, but in the plasma or serum blood zinc only occurs in a concentration of 12–16 μmol/L. Although very small, it is highly mobile and immunologically important [24]. Zinc is necessary for normal function of the immune system [25] as shown by thymulin, anonapeptidic hormone (Glu–Ala–Lys–Ser–Gln–Gly–Gly–Ser–Asn) secreted by thymic epithelial cells, that requires the presence of zinc for its biological activity [26].

Fermented dairy products with added probiotics are interesting targets for zinc fortification. Probiotics are well known for their positive impact on intestinal health and stimulation of immune response [27]. Because both zinc and probiotics have beneficial effects on nutritional and immunological status, the effect when taken together could be amplified, resulting in improved mineral absorption or a higher cellular immune response.

Supplementation with the probiotic *L. plantarum* IS-10506 significantly increased fecal sIgA after 90 days at a dose of 10^{10} CFU/day ($p = 0.01$). A combination of the probiotic *L. plantarum* IS-10506 at a dose of 10^{10} CFU/day and 8 mg of elemental zinc supplementation showed the potential ability to improve zinc status of pre-school children as shown by the highest elevation of serum zinc ($p = 0.05$), as well as high changes in fecal sIgA ($p = 0.027$). No adverse events were observed in the children.

Conflict of interest

The corresponding author to be inventor of patent ID P0026922 (Indonesian patent granted on November 9, 2010 by Ministry of Justice Republic of Indonesia), on the "*Lactobacillus plantarum* strain

IS-10506 dan strain IS-20506, *Enterococcus faecium* Is-27526 asal Dadih Bersifat Probiotik" (*Lactobacillus plantarum* strain IS-10506 and strain IS-20506, *Enterococcus faecium* Is-27526 of dadih origin have Probiotic properties'). However, the author does not receive any payment for this patent from any company. All the other authors declare to have no conflict of interest.

Acknowledgments

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