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Lactobacillus plantarum IS-10506 supplementation increases faecal sIgA and immune response in children younger than two years

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RESEARCH ARTICLE

Abstract

The immature intestinal immune system in young children develops as it comes into contact with dietary and microbial antigens in the gut. Intestinal microbiota plays a significant role in host defence mechanisms as shown by inflammatory responses towards potential pathogens. We investigated the probiotic function of *Lactobacillus plantarum* IS-10506 of '*dadih*' origin in modulating immune response in young children. We aimed to assess its effect on their immune response by assessing transforming growth factor-β1 (TGF-β1) and tumour necrosis factor-α (TNF-α) responses and faecal secretory immunoglobulin A (sIgA) titre in a randomised, double-blinded placebo-controlled trial in 12-24-month-old children (n=38). We used four treatment groups for a 90-day supplementation period: placebo (n=11), probiotic (n=9), zinc (n=8) and probiotic and zinc (n=10). Faecal sIgA, plasma TGF-β1 and TNF-α titre were evaluated using the enzyme-linked immunosorbent assay standard technique. Statistical analysis divided the results (pre/post treatment) into high (>1) and low (<1) ratios. The results showed that faecal sIgA titre increased in all treatment groups compared with the control (placebo) and significantly increased in the probiotic group (P=0.05). In addition, the TGF-β1 ratio in the zinc group was significantly higher (P=0.05) than that in the placebo group. We observed a significant positive correlation between TGF-β1/TNF-α and faecal sIgA (r=0.27, P=0.04). *Post hoc* test results revealed that zinc supplementation has a significant effect on body-weight gain. Taken together, probiotic *L. plantarum* IS-10506 supplementation stimulates TGF-β1, which in turn increases the production of sIgA, in line with the significant correlation between TGF-β1/TNF-α and faecal sIgA.

Keywords: probiotic *Lactobacillus plantarum*, sIgA, TGF-β1, TNF-α, young children

1. Introduction

Probiotics are defined as live microorganisms that when administered in adequate amounts provide a health benefit to the host (FAO/WHO, 2002). *Dadih* is manufactured from buffalo milk via spontaneous fermentation involving indigenous lactic acid bacteria derived from buffalo milk. Hence, different strains of lactic acid bacteria are involved in each fermentation depending on the presence of initial lactic acid bacteria (Surono *et al.*, 2009). *Lactobacillus plantarum* IS-10506 has originally been isolated from *dadih* and has good *in vitro* and *in vivo* probiotic properties (Surono *et al.*, 2010). Adequate adhesion ability has been reported (Collado *et al.*, 2007) that allows (transient) colonisation by this strain, which in turn modulates the adaptive immune response of the host.

Secretory immunoglobulin A (sIgA) is abundantly present at mucosal surfaces of the gastrointestinal and respiratory tracts. Several strains of lactobacilli and bifidobacteria have been shown to enhance total and pathogen-specific IgA production in intestinal mucosa, thereby preventing pathogens from binding to epithelial cells and maintaining homeostasis in the gut (Ahmed *et al.*, 2012; Collado *et al.*, 2007; Gloudemans *et al.*, 2013). The down-regulation of pro-inflammatory cytokine production in response to bacterial lipopolysaccharide in the intestine, liver, plasma and lung has been demonstrated using *Lactobacillus*

rhamnosus GG treatment in rats. Lipopolysaccharide-induced pre-necrotic changes in intestinal mucosa were partially prevented by *L. rhamnosus* GG. Recent studies have suggested that probiotic supplementation may reduce endotoxemia and chronic low-grade inflammation (Reddy *et al.*, 2013).

Innate immune system responses to low-grade inflammation are key to inducing more tightly regulated post-natal adaptive immune responses. Malnutrition occurs because of deficiencies in the transport or use of nutrients and affects infants and pre-schoolers due to their greater vulnerability. Deficiencies in vitamin A, iron, zinc and iodine are most prevalent in Indonesian children and play a central role in immune system development. Probiotic are frequently used for improving the digestibility and uptake of nutrients by intestinal cells (Agustina *et al.*, 2013). In the present study, zinc was supplemented in combination with probiotic for enhancing the immune response in young children. Malnutrition interferes with the growth and development of children and compromises all organs and systems, including the immune system. In malnourished children, the number of leukocytes, the mobilisation of phagocytes to the inflammatory focus, the stage of phagocytosis and intracellular digestion are affected, which compromise the first line of defence and increase the susceptibility to infections. In 2010, a worldwide systematic review revealed that of the 7.6 million deaths of children under five years, 64% were caused by infectious diseases and 40.3% occurred in neonates, with a high prevalence of pneumonia (14.1%) and diarrhoea (9.9%). High mortality rates associated with malnutrition in developing countries result from potential effects of the disease, inappropriate practices of diagnosis and the treatment management of malnourished children (Peixoto *et al.*, 2015).

The probiotic function of microencapsulated *L. plantarum* IS-10506 (2.3×10^{10} cfu/g/d) was investigated for assessing the immune response of young children quantified by faecal sIgA, plasma transforming growth factor-β1 (TGF-β1) and tumour necrosis factor-α (TNF-α) titres after 90 days of supplementation.

2. Materials and methods

Preparation of supplementation

The tested powder contained maltodextrin as placebo and microencapsulated probiotic *L. plantarum* IS-10506 (Gene Bank accession no. DQ 860148). For experimental purposes, microencapsulated probiotic was incorporated at a dose of 2.3×10^{10} cfu/g/d and shown to exhibit neither significant loss of viability nor contamination during the study period. *L. plantarum* IS-10506 was cultivated on De Man, Rogosa and Sharpe (MRS) broth at 37 °C for 18 h and harvested by centrifugation (4 °C). *L. plantarum* IS-10506 with viable counts of 10^{11} cfu/ml was washed with distilled water and microencapsulated with Na alginate and $CaCl_2$ in a Fluid Bed Dryer at 35 °C. The placebo (empty alginate beads) was prepared in a similar manner but without the probiotic culture. The placebo and probiotic had appearances similar to fine powders. After microencapsulation, viability was tested using MRS agar. The probiotic powder was also tested for *Escherichia coli* contamination and was found to be below *E. coli* detection levels. Zinc was supplemented as 20 mg zinc sulphate powder. The placebo, probiotic and zinc were provided in a capsule and were identical in appearance.

Subjects, location and study design

The experiment was conducted in South Larangan, Cileduk, Tangerang Banten Province, Indonesia. The majority of the population in this location lives at low economic levels with poor sanitation conditions. Thirty-eight apparently healthy children within the age range of 12-24 months (average 18 months) were selected for the present study. Prior to commencement of the trial, selection criteria were generated from the records of participating healthcare providers. On the basis of the initial selection process, 49 subjects met the inclusion criteria which included the following: apparently healthy children aged 12-24 months living in the community, no consumption of fermented milk or probiotic supplementation one week before and during supplementation, willingness to participate in the 90-day study and signed informed consent forms. The exclusion criteria included the following: congenital abnormality or disease, gastrointestinal disease, regular use of products with probiotic bacteria, receiving antibiotic therapy within two weeks prior to the intervention study to avoid potentially conflicting nutritional or trace element supplements and other probiotics.

A pre-post randomised, double-blinded, placebo-controlled clinical trial was conducted on 38 children under two years of age. The children were divided into four groups (placebo, probiotic, zinc, and probiotic-zinc groups) and were supplemented for 90 days with 700 mg maltodextrin, 700 mg probiotic *L. plantarum* IS-10506 in the equivalent of 2.3×10^{10} cfu/g/d, 20 mg zinc sulphate equivalent to 8 mg zinc elemental, or 700 mg probiotic and 20 mg zinc sulphate, respectively. Majority of the children were living in poor sanitary conditions. The respondent parents and guardians were instructed to give one capsule per day before or after daily meals. Phone calls to check for compliance were also conducted by community health workers. Every 30 days, a physical examination was conducted by a physician, who checked the children's general condition (anamnesis and physical examination, anthropometric measurement) by enumerators.

Methods

The research procedure was approved by the Ethics Committee of the Faculty of Medicine of the Universitas Indonesia. Participation in the study was voluntary and written informed consent was obtained before commencing the study from the parents or guardian of children. The study period lasted from August 2009 to March 2010. The subjects remained anonymous.

Peripheral blood samples were withdrawn from subjects via venipuncture and stool samples were collected at the start and end of the study period. sIgA was assessed using an enzyme-linked immunosorbent assay (ELISA) kit (Wuhan, China P.R.) and plasma TGF-β1 (R&D System, Minneapolis, MN, USA) and TNF-α (R&D Systems) titres were conducted at the Institute of Human Virology and Cancer Biology laboratory, Faculty of Medicine, Universitas Indonesia.

Blood and stool samples from each volunteer were collected before and after supplementation. The physician and laboratory staff collected 3 ml blood samples in ethylene diamine tetra acetic acid (EDTA) tubes. After centrifugation, the plasma was stored in a refrigerator at -80 °C. TGF-β1 and TNF-α were measured in accordance with manufacturer's instructions.

Stool samples were collected and stored in a sterile bottle. Shortly after collection, the stool samples were stored in a freezer at -20 °C in the first location before being stored in a freezer at -80 °C. Approximately 1 g (wet weight) of stool was taken with a sample scoop from the stool collection tube. The wet weight of collected stool ranged from 0.83 to 2.16 g (median 1.24 g). The extraction buffer (0.01 M phosphate-buffered saline [PBS] [pH 7.4], 0.5% Tween [Sigma-Aldrich, Poole, Dorset, UK] and 0.05% sodium azide) was added at a ratio of 10 ml of buffer to 1 g (wet weight) of stool, and samples were thoroughly homogenised by a combination of manual shaking and mechanical homogenisation in a vortex mixer. Stool suspensions were centrifuged at 1,500×*g* for 20 min at 5 °C. A portion of the supernatant (2 ml) was transferred to a sterile Eppendorf tube containing 20 µl of protease inhibitor cocktail (Sigma-Aldrich) and the tube was briefly vortexed to mix the contents. Samples were centrifuged at 10,000×*g* for 10 min in a micro-centrifuge and the supernatants were transferred to clean Eppendorf tubes and stored at -20 °C (Fergeuson *et al.*, 1995).

ELISA was performed for sIgA according to manufacturer instructions. For 100 µl standard, control and sample was added to the wells of a microtiter plate, incubated for 1 h while shaking on a horizontal mixer at room temperature. Then wells were aspirated and washed 5 times with 250 µl ELISA wash buffer, and 100 µl conjugate (mouse anti-human sIgA Peroxidase-labelled) added and incubated for 1 h shaking on a horizontal mixer at room temperature. The content of the plate was decanted and the wells were washed 5 times with 250 µl wash buffer. After that 100 µl TMB substrate was added and incubated for 10-20 min at room temperature. Next, 50 µl Elisa STOP solution mix was added shortly. Finally, absorption was determined with an ELISA reader at 450 nm.

Statistical calculations were performed using SPSS version 22 (IBM Corp, Armonk, NY, USA). Differences before and after supplementation and differences between groups were quantified using Chi-squared (Fisher's exact) tests. Relationships between TGF-β1/TNF-α and sIgA were analysed using two-tailed Spearman-ρ correlation coefficients (at a significance level of $P<0.05$). All results of faecal sIgA, TGF-β1 and TNF-α were analysed with ratio (i.e. the post-treatment titre compared with the pre-treatment titre). A high ratio (>1) indicated that concentration in the post-treatment titre was higher than that in the pre-treatment titre; conversely, a low ratio (<1) indicated that concentration in the post-treatment titre was lower than that in the pre-treatment titre.

3. Results

Humoral and cellular immune response

The median socio-economic status was categorised as lower middle class. There were no significant complaints of abdominal pain, bloating or other abdominal symptoms during supplementation. The drop-out rate was 20% because of blood lysis, non-compliance and sickness (one child was hospitalised because of tuberculosis infection).

Ratio of secretory immunoglobulin A

At the end of the supplementation period, a high ratio of faecal sIgA was observed in the probiotic group (88.9%) of children, significantly higher than in the placebo group ($P=0.05$). Ratios in the zinc (12.5%) and probiotic-zinc (70.0%) groups tended to be higher than in the placebo group (45.5%) (Figure 1).

Ratio of transforming growth factor-β1

The ratio of TGF-β1 at the end of the supplementation period increased in all treatment groups compared with the placebo group. There were significant changes noted in children supplemented with zinc ($P=0.05$) with a ratio of TGF-β1 of 75% compared with the placebo group (29%). Ratios were relatively high in the probiotic group (68%) and the probiotic-zinc group (55%) (Figure 2).

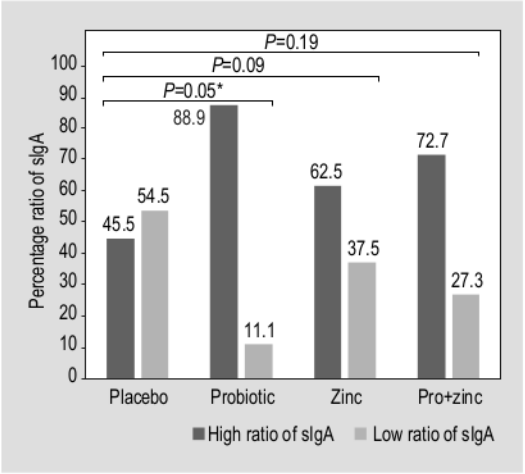

Figure 1. Proportion of high and low ratio of secretory immunoglobulin A (sIgA) levels in placebo, probiotic, zinc, and probiotic+zinc groups. The high ratio of sIgA in the probiotic group was significantly more than in the placebo group (*P*=0.05).

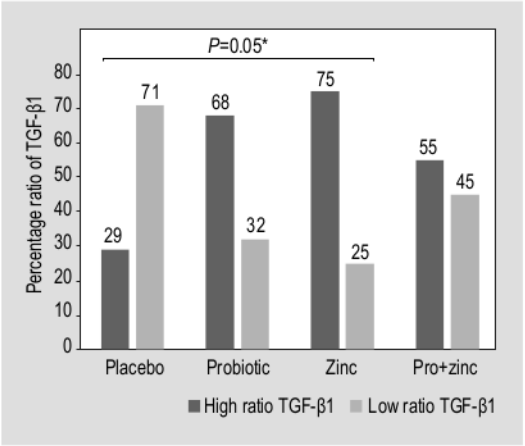

Figure 2. Proportion of high ratio of transforming growth factor-β1 (TGF-β1) levels in placebo, probiotic, zinc and probiotic+zinc groups. High ratio of TGF-β1 levels in the zinc group was significantly more than in the placebo group (*P*=0.05).

Ratio of tumour necrosis factor-α

At the end of the supplementation period, the titre ratios of TNF-α in the probiotic (44.4%), zinc (75%) and probiotic-zinc (50%) groups did not statistically differ (*P*=0.59) from the ratio in the placebo treatment group (45.5%) (Figure 3).

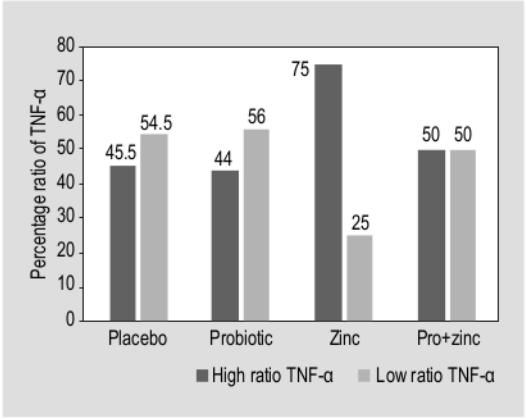

Figure 3. Proportion of high ratio of tumour necrosis factor-α (TNF-α) levels in placebo, probiotic, zinc and probiotic+zinc groups. The high ratio of TNF-α levels in all treatment groups was not significantly different as compared to placebo group (*P*=0.59).

Correlation between TGF-β1/TNF-α ratios and sIgA

TGF-β1 is an anti-inflammatory mediator, while TNF-α is an inflammatory mediator. The TGF-β1/TNF-α ratio shows a stronger anti-inflammatory response. TGF-β1 induced by probiotic supplementation is responsible for sIgA production. The ratios of TGF-β1/TNF-α are shown in Figure 4. There was a significant positive correlation (r=0.27, *P*=0.04) between TGF-β1/TNF-α and sIgA.

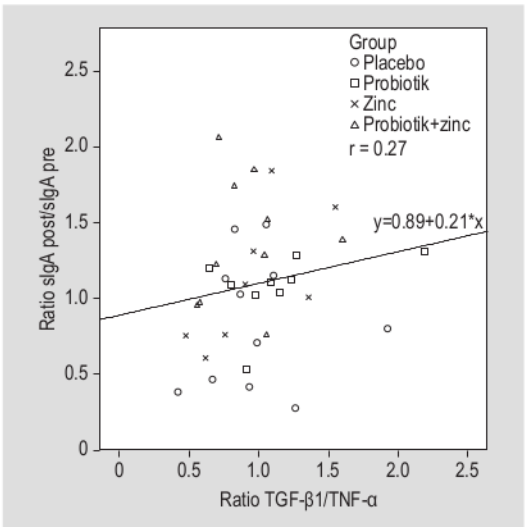

Figure 4. Correlation between the transforming growth factor-β1 (TGF-β1)/tumour necrosis factor-α (TNF-α) ratio and secretory immunoglobulin A (sIgA) levels of all subjects.

Body-weight gain

Body-weight increased in all four groups after 90 days of supplementation. Average body-weight gain in the placebo, probiotic, zinc and probiotic-zinc groups were 0.46±0.40 kg, 0.86±0.58 kg, 0.99±0.68 kg and 0.33±0.4 kg, respectively. *Post hoc* tests showed that compared with the placebo group, only the zinc group significantly increased body-weight (P=0.03), while the probiotic and probiotic-zinc groups showed no significant increase (P=0.10 and P=0.53, respectively).

4. Discussion

Probiotic and immune response

Probiotic influence the composition and activity of the gut microbiota, modulate the immune response, improve the non-specific intestinal barrier and reinforce and modulate the mucosal and systemic immune response. The long-term administration of fermented milk containing the probiotic strain *Lactobacillus casei* DN-114001 reportedly has immunomodulatory effects and maintained intestinal homeostasis without adverse secondary effects in mice (LeBlanc *et al.*, 2008). *Bifidobacterium bifidum* increases local IgA levels in the intestine and prevents diarrhoea and shedding of rotavirus (Xiao *et al.*, 2017). *Lactobacillus helveticus* inhibits *Campylobacter jejuni* invasion of intestinal epithelial cells. Its surface-layer protein may exert anti-inflammatory effects by reducing the activation of NF-kB on intestinal epithelial cells. The complex intestinal microbiota harboured by individuals has long been proposed to contribute to intestinal health and disease and considered to be a symbiotic partner in human health (Xiao *et al.*, 2017).

Gut microbiota plays an important role in the development of Th17 cells, Treg cells and memory T cells. At birth, nearly all T cells carry the CD45RA glycoprotein typical of naive T cells, which have never encountered foreign antigens. There are also relatively abundant Treg cells within the CD45RA-negative CD4 T cells. During childhood, Treg cell numbers decline and memory Th1, Th17 and Th2 cells gradually increase compared to the number of naive T cells. Even though some of these memory T cells could have been stimulated by infections with specific pathogens and by vaccinations, numerous may be primed by the microbiome not only in the gut but also in the respiratory tract and skin. These primed memory T cells may respond to subsequent infections through cross-reactions (Simon *et al.*, 2015).

Secretory immunoglobulin A

The supplementation of probiotic significantly increased faecal sIgA in children younger than two years after 90 days of supplementation at a dose of 2.3×10^{10} cfu/g/d compared with placebo. Zinc and probiotic-zinc also tended to increase faecal sIgA (Figure 1). Bosch *et al.* (2012) reported that the consumption of *L. plantarum* CECT7315 and CECT7316 for three months increased influenza-specific IgA levels and IgG after influenza vaccination. In the intestinal tract, sIgA is the most abundant immunoglobulin isotype with up to 3 g/d of sIgA secreted in the human intestinal lumen. IgA plays an important role in host defence against mucosal-transmitted pathogens, preventing pathogenic bacteria from binding to epithelial cells and neutralising toxins to maintain homeostasis at the mucosal surfaces. These functions are beneficial for the host in reducing the risk of infection and maintaining the intestinal environment (Sakai *et al.*, 2014).

The effect of probiotic on the expression of TGF-β1 and TNF-α

There was a significant increase in TGF-β1 in children supplemented with zinc, with 75% of the subjects showing increased TGF-β1 compared with the placebo. Ratios in the probiotic and probiotic-zinc groups tended to be higher than that in the placebo (Figure 2). SIgA within the mucus establishes distance with commensals and forms a barrier between invading and commensal microorganisms. Intestinal Dendritic Cells (DCs) support IgA isotype class-switching and differentiation into IgA-secreting plasma cells, either together with the assistance of Th cells or in a T cell-independent manner by expressing B cell-activating factors and a proliferation-inducing ligand. The lamina propria CD 103 + CD11b + DCs, Tip DCs and TLR5 + DCs are expressed in aldehyde dehydrogenase type 2 RALDH2 and produce retinoid acid which in turn can cause IgA production together with TGF-β1 (Ko and Chang, 2015). Cross-talk with commensal microorganism in the intestine results in the production of IgA antibodies which provide protection by binding and excluding microbial and dietary antigens on the mucosal surfaces. TGF is the initial trigger for IgA production and expression of TGF is directly correlated with the mucosal IgA antibody response (Rautava *et al.*, 2006).

Correlating with that signalling pathway, Kozakova *et al.* (2016) analysed the immunomodulatory properties of three *Lactobacillus* strains (*L. rhamnosus* LOCK0900, *L. rhamnosus* LOCK0908 and *L. casei* LOCK0919) and the impact of their mixture on allergic sensitisation to Bet v 1 using a gnotobiotic mouse model L-mix colonisation improved the gut epithelial barrier and reduced allergic sensitisation to Bet v 1. Furthermore, the L-mix treatment was accompanied by the increased production of circulating and secretory IgA and the regulatory cytokine TGF-β, which suggests that L-mix colonisation induced immune regulatory mechanisms (Kozakova *et al.*, 2016). According to the TNF-α expression, we see that there are no significant changes in the ratio of TNF-α between placebo and

treatments (probiotic, zinc and probiotic-zinc). However, we observed a correlation between a low ratio of TNF-α and a high ratio of TGF-β1. TNF-α-induced IĸBα was inhibited by TGF-β1 pre-treatment (Shiou *et al.*, 2013). In the zinc treatment, the high ratio of TGF-β results in an inversely proportional low ratio of TNF-α, suggesting a positive role of zinc finger protein ZPR9 functions (Seong *et al.*, 2017).

Correlations between the ratios TGF-β1/TNF-α and sIgA

Among numerous hypothesised action mechanisms, bifidobacteria and lactobacilli have been extensively studied for their immunomodulatory activities. Bacterial cells and components interact with a wide variety of cells present in the intestines, such as epithelial, dendritic and macrophage cells which further induce the secretion of pro-and anti-inflammatory cytokines. A significant positive correlation between TGF-β1/TNF-α and sIgA in children supplemented with the probiotic *L. plantarum* IS-10506 for 90 days at a dose of 2.3×10^{10} cfu/g/d supports that TGF-β1 was stimulated by the probiotic supplement as an anti-inflammatory response responsible for sIgA production, which suggests that a higher value of the TGF-β1/TNF-α ratio leads to a higher value of sIgA. A number of mechanisms can explain how certain probiotic strains can improve the structure and function of intestinal epithelial barriers, including stressing the physical barrier, increasing mucin production, promoting the production of antimicrobial peptides and heat shock proteins, attenuating the negative effects of pathogenic microorganisms and modulating signalling pathways that affect cell proliferation and survival. Of particular interest is the ability of specific probiotic strains to significantly decrease TNF-α pro-inflammatory cytokines that causes leaky barriers by directly impairing the integrity of the tight junctional complexes between epithelial cells. Different populations of regulatory T cells exert their inflammation-modulating effects by secreting specific cytokines including IL-10 (which strongly suppresses the Th-1 dominant pro-inflammatory response) or more regulatory cytokines such as TGF-β (which promotes the development of regulatory T cells). Certain probiotic strains help regulate inflammation with their enhanced ability to influence the secretion of these important cytokines (Lescheid, 2014).

Beneficial microbes may modulate immune responses in the host and may consequently provide strategies for the amelioration of paediatric immune-mediated disorders. Interestingly, several probiotic immunomodulatory effects are not only species specific but may be strain-specific as well. TGF-β1 is a strong immune suppressor and a factor in breast milk that has been shown to be protective against necrotising enterocolitis (NEC). According to Shiou *et al.* (2013), the oral administration of the isoform TGF-β1 in an NEC animal model activated the downstream effector Smad2 in the intestine and significantly reduced NEC incidence, showing that TNF-α-induced IĸBα was inhibited by TGF-β1 pre-treatment. According to this experiment, there is a correlation with the phenomenon that TGF-β1 suppresses TNF-α with the supplementation of the probiotic *L. plantarum* IS-10506 and zinc because TGF-β1 is a mediator of anti-inflammation and TNF-α is as mediator of inflammation; therefore, a higher ratio describes a stronger anti-inflammation condition (Shiou *et al.*, 2013). The establishment of microbiota in the early post-natal period activates the innate and adaptive immune system and the uninterrupted microbial stimulus serves to mature the gut mucosal immune system. Early compromise of microbial stimulus may lead to reduced intestinal surface area, coordination and alteration in the mucosal intermediary metabolism, a sensitive mucosal barrier and a secretory mucosal IgA system. Correlating with this, the supplementation of probiotic can have a major function in improving the immune system in early life (Rather *et al.*, 2016).

Zinc supplementation increases body-weight gain

Zinc is an essential micronutrient that plays a vital role in child growth and development. Zinc is involved in multiple cellular and subcellular processes that are essential for cell proliferation and growth and brain development. Manifestations of zinc deficiency include poor growth, diarrhoea, rash and immune dysfunction (Shaikhkhalil *et al.*, 2014). The 90 days supplementation of probiotic and zinc significantly increased humoral immune response as well as zinc status (Surono *et al.*, 2014). Significant body-weight gained of children in zinc group compared with other groups (placebo, probiotic, and probiotic-zinc) is in line with results of Shaikhkhalil *et al.* (2014). Interestingly, combining zinc and probiotic, tended to result in less bodyweight gained as compared to zinc only. Standard deviation of bodyweight gain in the probiotic-zinc group is higher than the mean of body-weight gain, as a consequence of greater deviation of bodyweight gain, and the children were younger than in other groups (15 months of average age, while in the zinc group the average age was 18 months). Moreover, in the probiotic-zinc group, 64% children were below average bodyweight, while this was only 37% in the zinc group. Hence, we assume that the children in probiotic-zinc group may need a longer supplementation time to effectively increase the children's bodyweight gain.

5. Conclusions

Probiotic *L. plantarum* IS-10506 significantly increases the humoral immune response (sIgA). Zinc significantly increases TGF-β1 but not TNF-α titre and showed a positive correlation between the ratio of TGF-β1/TNF-α and IgA titre of children younger than two years after 90 days of supplementation.

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Conflict of interest

The corresponding author is an inventor of patent IDP0026922 (Indonesian patent granted On November 9, 2010 by Ministry of Justice Republic of Indonesia), on the '*L. plantarum* strain IS-10506 dan strain IS-20506, *Enterococcus faecium* IS-27526 asal Dadih Bersifat Probiotik' (*L. 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 the commercial product. Other authors declare to have no conflict of interest.

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