

## Lactobacillus plantarum IS-10506 supplementation reduced SCORAD in children with atopic dermatitis

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### Abstract

*Lactobacillus plantarum* IS-10506 is a novel probiotic isolated from dadih, an Indonesian traditional fermented buffalo milk. Its *in vitro* and *in vivo* probiotic properties have been assessed. Probiotic function has been shown *in vivo* by the suppression of allergic reactions in BALB/c mice through the action of T-regulatory cells cytokines by balancing Th1 and Th2 immune response. Atopic dermatitis (AD) is a chronic recurrent inflammatory skin disease characterised by the imbalance of Th1 and Th2. The aim of the study was to assess the probiotic function of *L. plantarum* IS-10506 in children with mild and moderate AD. A randomised double-blind placebo-controlled trial comparing microencapsulated *L. plantarum* IS-10506 ( $10^{10}$  cfu/day) and placebo (skim milk-Avicel) twice daily for 12 weeks was conducted in an outpatient clinic on children with mild and moderate AD. The trial included 22 AD children divided into intervention and control groups of n=12 and n=10 patients, respectively. Scoring Atopic Dermatitis Index (SCORAD) and serum immunoglobulin E (IgE), interleukin (IL)-4, interferon gamma (IFN- $\gamma$ ), forkhead box P3 (Foxp3+)/IL-10, and IL-17 levels were assessed. Demographic and baseline characteristics were not significantly different between the two groups. SCORAD and levels of IL-4, IFN- $\gamma$ , and IL-17 were significantly lower in the probiotic group than those in the placebo group, while the IgE levels were not significantly changed. The ratio of Foxp3+ to IL-10 was significantly higher in the probiotic group than that in placebo group. Supplementation with the probiotic *L. plantarum* IS-10506 offered a potential treatment for children with AD. Further long-term studies with a larger sample size are required to confirm the therapeutic efficacy of *L. plantarum* IS-10506 in AD.

**Keywords:** atopic dermatitis, children, *Lactobacillus plantarum* IS-10506, probiotic, SCORAD

### 1. Introduction

Atopic dermatitis (AD) is a chronic recurrent inflammatory skin disease. The symptoms include erythematous papules, intense pruritus, excoriation, and exudate formation. The prevalence of AD has increased over recent decades and the disease most often occurs in infants and children. From an immunological perspective, patients with AD are characterised by strong expression of the cytokine T-helper cell 2 (Th2). Skin lesions in AD show clear T cell infiltrates, which can induce the apoptosis of keratinocytes and affect the barrier function of the skin. This can lead

to further infection and the occurrence of complications (Rahman *et al.*, 2011). AD can also be caused by an imbalance of Th1 and Th2 (Boguniewicz, 2004; Flohr *et al.*, 2005). The role of Th2 in the cellular immune response against innocuous environmental antigens (allergens) in the immunopathogenesis of AD has been described: the expression of the cytokines, interleukin (IL)-4, IL-5, IL-9, and IL-13, at the beginning of the disease process may cause inflammation (Romagnani, 2004); and regulatory T cells (Treg) expressing forkhead box P3 (Foxp3+) and cells expressing Th17 play an important role in the process of Th1 and Th2 cell homeostasis.

Treg are a major regulator of the immune response in humans, and rodents such as mice, and severe deficiency in transcription factor Foxp3 leads to various autoimmune and atopic diseases. Overexpression of IL-10, either spontaneously or after administration of allergens, is also found in atopic disease. IL-10 is produced by various cells, including monocytes, activated B cells, macrophages, mast cells, keratinocytes, dendritic cells, and T cells. IL-10 can inhibit the synthesis of IL-2, IL-4, IL-6, IL-12, tumour necrosis factor  $\alpha$ , and interferon gamma (IFN- $\gamma$ ) (Gerasimov *et al.*, 2010).

Probiotics can modulate Toll-like receptors (TLR), and the introduction of proteoglycan protein in enterocytes can activate dendritic cells and the Th1 response, in which stimulation of Th1 will suppress the Th2 response. *Lactobacillus plantarum* IS-10506 is a novel probiotic isolated from dadih, an Indonesian traditional fermented buffalo milk (Suroño *et al.*, 2008a). Suppression of allergic reactions in BALB/c mice administrated with *L. plantarum* IS-10506 was observed through modulation of the immune response by balancing Th1 and Th2 immune response (Suroño *et al.*, 2008b).

The dysregulation of cellular immunity due to an imbalance in Th1 and Th2 cells is the basis of diseases such as AD, yet no therapies focus on this imbalance. Probiotics may be a promising treatment for this dysregulation as an alternative or adjuvant therapy, because they have immunomodulatory effects in both children and adults. This research is a randomised double-blind placebo-controlled clinical trial comparing a probiotic and placebo in children with mild and moderate AD.

The aim of this study was to investigate the probiotic function of *L. plantarum* IS-10506 in children with various degrees of AD severity. Its probiotic function was assessed by its ability to decrease the Scoring Atopic Dermatitis Index (SCORAD) and levels of serum immunoglobulin E (IgE), IL-4, and IL-17, and to increase levels of serum IFN- $\gamma$  and the Foxp3+ to IL-10 ratio.

## 2. Materials and methods

A randomised double-blind placebo controlled trial (IRB 430/Panke.KKE/X/2014) comparing the probiotic *L. plantarum* IS-10506 and placebo in paediatric patients with mild and moderate AD, as assessed by using SCORAD (Figure 1). The study population comprised new AD outpatients from the clinic at the Allergy Immunology Division of the Dermatology and Venereology Department of Faculty of Medicine, Universitas Airlangga / Dr Soetomo Teaching Hospital, Surabaya, Indonesia. The 22 AD subjects who met the inclusion criteria (see below) were divided into probiotic (n=12) and control (n=10) groups. Both groups received the standard treatment. The probiotic *L.*

*plantarum* IS-10506 was supplemented at a dose of  $10^{10}$  cfu/day for 12 weeks in microencapsulated form. *L. plantarum* IS-10506 of dadih origin was grown in 100 ml of De Man-Rogosa-Sharpe (MRS) broth (Oxoid, Basingstoke, UK) at 37 °C for 16 h, and then inoculated in 1000 ml of MRS broth, to be further inoculated in a fermentor SP30 L (BIOTRON, Oakland, CA, USA) containing 20 l MRS broth, incubated for 20 h at 37 °C. and 0.6% glucose feeding was conducted for 16 h. NaOH was used for maintaining pH at 6.0. The cells of *L. plantarum* IS-10506 were harvested by centrifugation at  $3,000\times g$  for 15 min at minus 4 °C using cold centrifuge (Thermo Fisher, Waltham, MA, USA), and the cell pellet was washed once with sterile aquadest, then resuspended in falcon tube (25 ml of cell pellet was added to 25 ml UHT milk) to form cell paste, with the initial viable counts was  $1.29\times 10^{12}$  cfu/ml.

The fluid bed dryer was sterilised at 120 °C to sterilise the instrument and filler materials prior to drying process. Then 200 ml cell paste of *L. plantarum* IS-10506 mixed with 600 ml UHT plain milk, and sprayed onto 2 kg of cellulose powder carrier material Flocel pH 101 (Gujarat, India) mixed with 2 kg of Skim Milk Powder (NZMP, New Zealand) previously loaded into a pilot scale fluid bed dryer (HongDau, Taiwan) of 4 kg maximum loading capacity, atomised by fluid nozzle in side-spray position using a peristaltic pump applying a spraying air pressure at 1.3 kPa with inlet air temperature was set to 45 °C which resulted in a maximal bed temperature of 37 °C and the spraying rate ranging 5-10 ml/ min was adjusted to avoid agglomeration of particles. Cells were encapsulated with 4.75% (w/v) sodium alginate and 5.5% (w/v) calcium chloride, and the viability of microencapsulated probiotic *L. plantarum* IS-10506 powder was  $1.2-1.3\times 10^{10}$  cfu/g.

The exclusion criteria were: the use of systemic corticosteroids or phototherapy in the previous month and systemic immunosuppressive drugs in the previous three months, probiotic use in the previous four weeks, use of topical medications such as corticosteroids or calcineurin inhibitors in the previous week, immunosuppressive conditions or other serious diseases, clinical skin disease, and other systemic diseases. The inclusion criteria were: paediatric patients aged 0-14 years, AD patients who met the Hanifin-Rajka diagnostic criteria, age-related total serum IgE levels of: 10-15 years, >200 IU/ml; 6-9 years, >90 IU/ml; 1-5 years, >60 IU/ml; <1 year, >1.5 IU/ml, patients in apparent good health, and willing to participate in the study and sign informed consent.

Consecutive randomisation was conducted on every subject who met the inclusion criteria. Microencapsulated probiotic *L. plantarum* IS-10506 at  $10^{10}$  cfu/day or placebo (skim milk-Avicel) was supplemented for 90 days. Total IgE from whole blood was assessed by an ELISA kit (Advia Centaur XPT®, Siemens, Wiesbaden, Germany) using reagent Advia

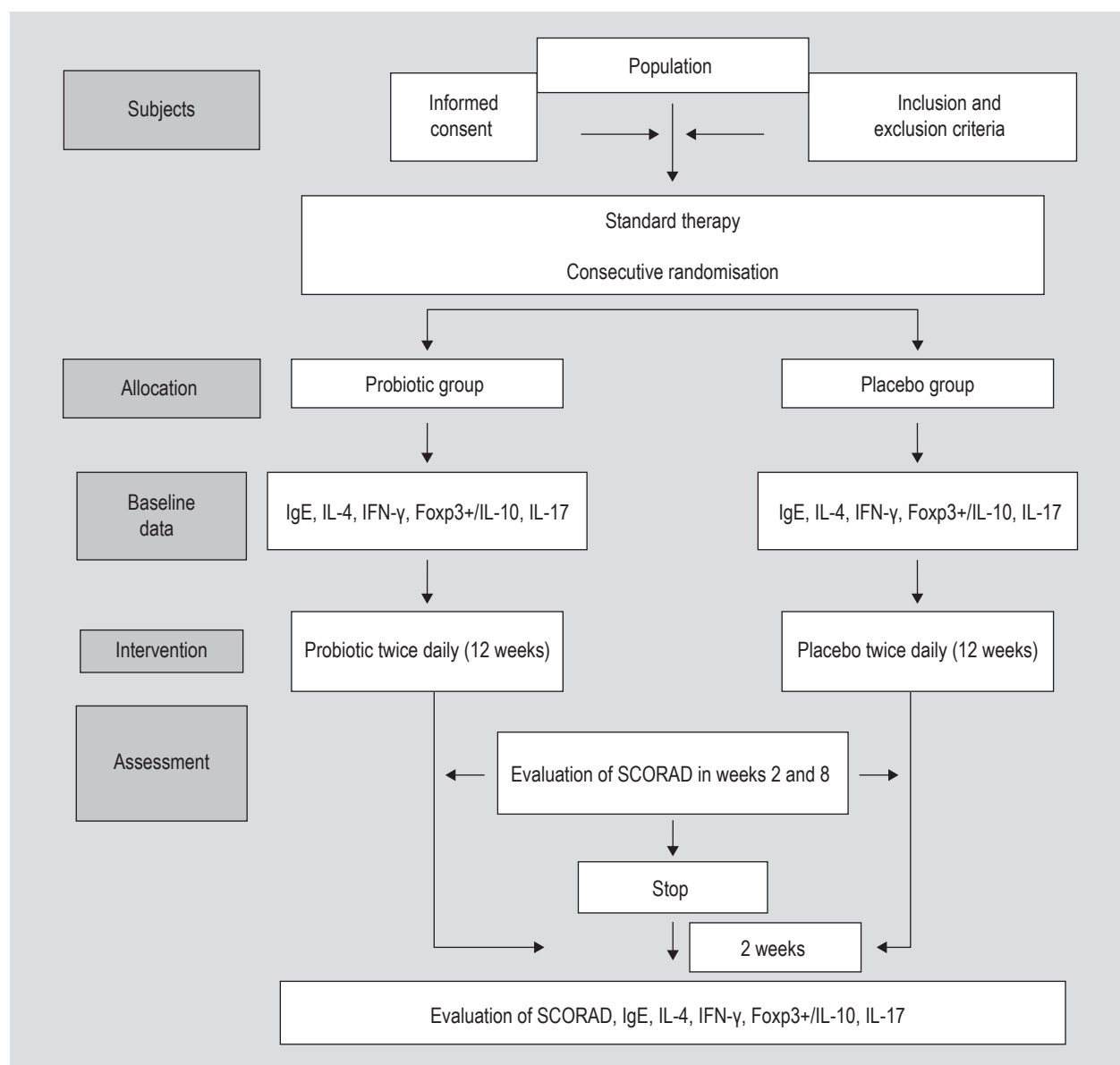


Figure 1. Research procedure.

Centaur IgE, and the T cell cytokines IL-4, IFN- $\gamma$ , ratio of Foxp3+ and IL-10 (Foxp3+/IL-10), and IL-17 were assessed by flow cytometry (Facs Calibur<sup>®</sup>, Singapore, Singapore). Purified cytokines (IL-4, IFN- $\gamma$ , IL-10, Foxp3+, and IL-17) were purchased from BD Plasminogen (San Jose, CA, USA). For flow cytometry reading, gating was performed to the lymphocyte population based on the size and granularity. We also performed gating to lymphocyte CD4+ resulting the CD4+ expression for IL-4, IFN- $\gamma$ , Foxp3+/IL-10, and IL-17. Baseline readings of these parameters were recorded before commencement of supplementation. The SCORAD index was recorded at 2 and 8 weeks into the study. SCORAD and other parameters were recorded 2 weeks after the end of the study in order to reduce the bias of *L. plantarum* colonisation in the gut (Han *et al.*, 2012).

### 3. Results

Twenty-two subjects who met the inclusion criteria (12 in the probiotic group and 10 in the placebo group) completed the study. No statistical differences were found between the two groups for age, sex, onset and duration of illness, SCORAD, and IgE level at baseline (Table 1).

#### Clinical severity

A decrease in SCORAD in both the placebo and probiotic groups was observed. However, the decrease in SCORAD in the probiotic group was significantly more than that in placebo group at the end of the study ( $P=0.000$ ) (Table 2).

**Table 1. Baseline characteristics.**

Variable <sup>1</sup>	Total (%)	
	Placebo n=10	Probiotic <sup>2</sup> n=12
Sex		
Boys, n (%)	7 (70.0)	5 (41.7)
Girls, n (%)	3 (30.0)	7 (58.3)
Age (years)	6.02±4.42	5.7±4.06
Onset (months, mean ± SD)	31.2±2.76	32.5±2.70
Duration of illness (week)		
<6 weeks, n (%)	6 (60.0)	8 (66.7)
6-24 weeks, n (%)	4 (40.0)	4 (33.3)
>24 weeks, n (%)	0	0
SCORAD	48.9±13.8	55.31±15.31
IgE (IU/ml)	1,792.22±1,209.47	1,009.1±902.25
IL-4 (IU/ml)	10.012±5.546	10.284±2.011
IFN-γ (IU/ml)	1.484±1.021	1.793±1.766
Foxp3+/IL-10 (IU/ml)	0.028±0.023	0.023±0.024
IL-17 (IU/ml)	0.382±0.343	0.412±0.462

<sup>1</sup> Foxp3+ = forkhead box P3; IFN-γ = interferon gamma; IgE = immunoglobulin E; IL = interleukin.  
<sup>2</sup> Microencapsulated *Lactobacillus plantarum* IS-10506.

### Immunoglobulin E level

There was no significant change in the total serum IgE level after 90 days of treatment in both groups, but the overall trend was a decrease. The mean total serum IgE level in the probiotic group tended to be lower than that in the placebo group (Table 2).

**Table 2. Level of SCORAD, IgE, IL-4, IFN-γ, Foxp3+/IL-10, and IL-17 in both groups after 12 weeks supplementation.**

Variable <sup>1</sup>	Placebo group (n=10)	Probiotic group (n=12)
SCORAD	22.040±8.817	18.533±14.200
IgE (IU/ml)	909.580±885.051	504.533±415.686
IL-4 (IU/ml)	5.815±6.633	4.277±4.892
IFN-γ (IU/ml)	0.684±1.006	0.528±0.634
Foxp3+/IL-10 (IU/ml)	0.014±0.018	0.050±0.135
IL-17 (IU/ml)	0.128±0.134	0.151±0.135

<sup>1</sup> Foxp3+ = forkhead box P3; IFN-γ = interferon gamma; IgE = immunoglobulin E; IL = interleukin.

### Th1 and Th2 activation of the adaptive immune response

Supplementation with the probiotic *L. plantarum* IS-10506 reduced both Th2 and Th1 cytokines. Th2 modulation of the adaptive immune response observed in this study occurred through the expression of IL-4 (Figures 2A,B and 3A,B) and modulation of the Th1 adaptive immune response occurred through the expression of IFN-γ (Figures 2C,D and 3C,D). IL-4 levels produced by CD4+ T lymphocytes significantly reduced in both the placebo group as well as the probiotic group, and the levels of IL-4 in CD4+ T lymphocytes in the probiotic group were significantly lower than those in the placebo group (Figure 2B and 3B). The IFN-γ produced by CD4+ T lymphocytes significantly reduced in the probiotic group, whereas in the placebo group, the reduction was not significant. Probiotic supplementation resulted in a significant decrease in more subjects (91.7%) than in those in the placebo group (80%), and levels of IFN-γ decreased more in subjects in the probiotic group (83.4%) than in those in the placebo group (70%) ( $P=0.006$ ) (Table 2).

### Regulatory T cell activation of the adaptive immune response

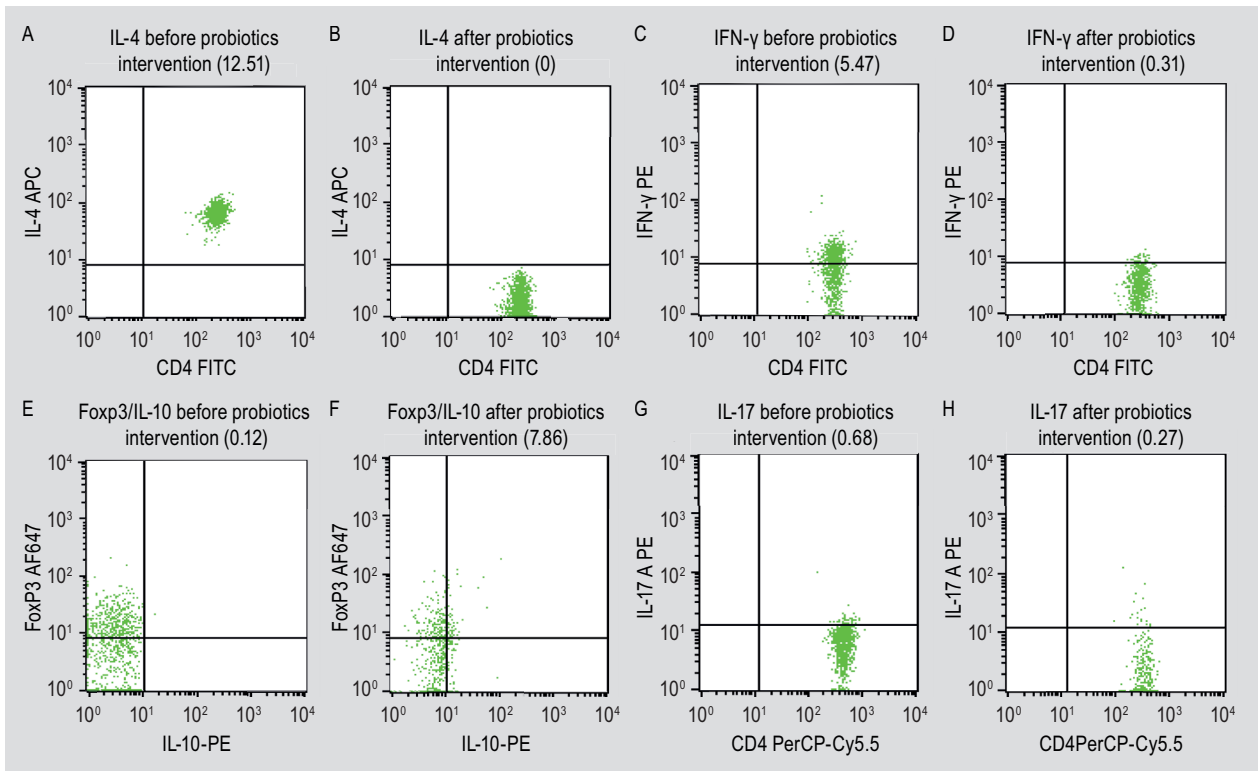
Treg activation of the adaptive immune response was observed in this study by analysing the ratio of the transcription factor Foxp3+ and IL-10. A significant increase in the probiotic group and a decreasing trend in the placebo group was observed. A significant increase of the Foxp3+/IL-10 ratio ( $P=0.001$ ) compared to that in the placebo group was observed after 90 days of supplementation (Figure 2F and 3F).

### Th17 activation of the adaptive immune response

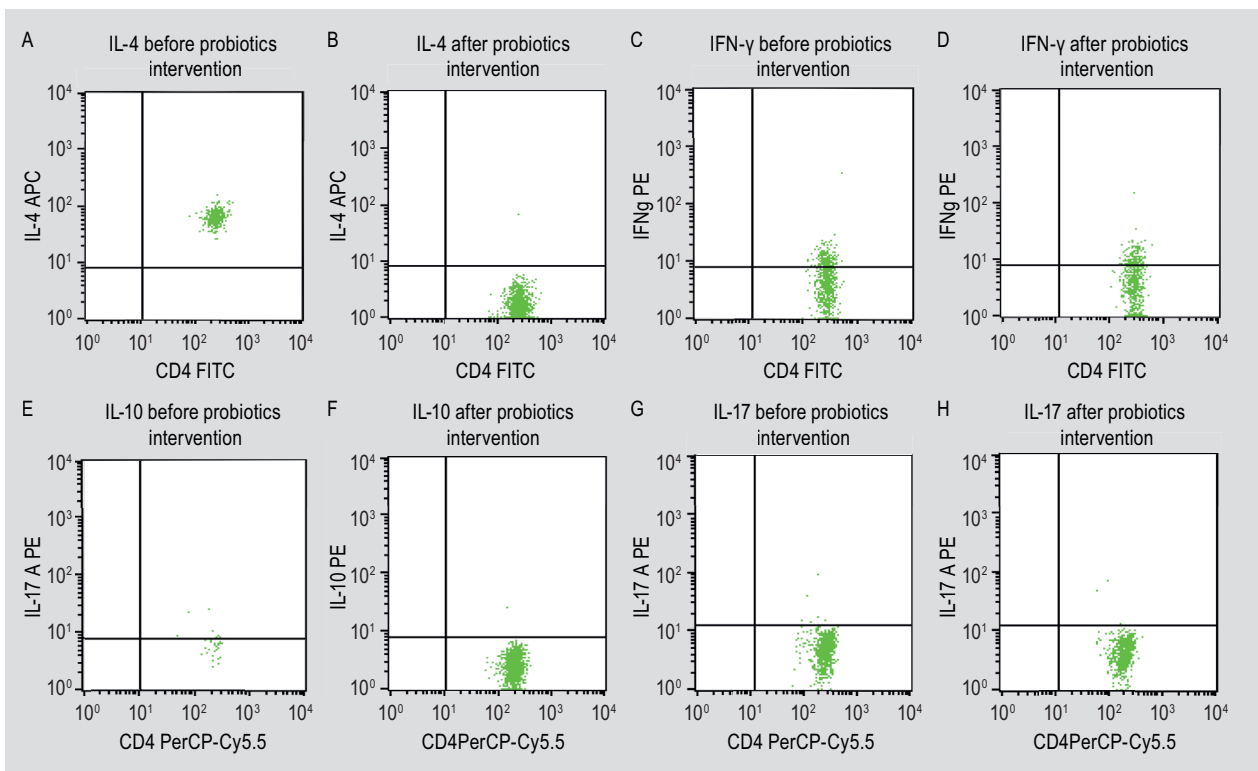
Th17 activation of the adaptive immune response was observed through the ratio of the IL-17 and CD4+ T lymphocytes. The ratio of IL-17/CD4+ T lymphocytes significantly reduced in both the probiotic and placebo groups ( $P=0.000$ ), and more decreased in the probiotic group was observed (Figure 2H and 3H).

## 4. Discussion

Homeostasis is required for life by all individuals. The maintenance of homeostasis requires permanent and effective surveillance of various defence mechanisms (Galdeano and Perdigon, 2006), such as the innate and adaptive immune mechanisms. AD is a homeostatic disorder caused by an imbalance of the Th1-Th2 response due to allergen exposure, which leads to mild to severe clinical symptoms. The decrease in clinical manifestation (SCORAD index) in the probiotic group was significantly more than in the placebo group. Administration of *L. plantarum* CJLP133 for 12 weeks reduced the SCORAD index significantly more than placebo ( $P=0.044$ ), and the



**Figure 2.** Profile of immune parameters probiotic group measured using flow cytometry, before and after treatment. (A) Interleukin (IL)-4, before; (B) IL-4, after; (C) interferon (IFN)- $\gamma$ , before; (D) IFN- $\gamma$ , after; (E) forkhead box P3 (Foxp3)/IL-10, before; (F) Foxp3+/IL-10, after; (G) IL-17, before; (H) IL-17, after.



**Figure 3.** Profile of immune parameters placebo group measured using flow cytometry. (A) Interleukin (IL)-4, before; (B) IL-4, after; (C) interferon (IFN)- $\gamma$ , before; (D) IFN- $\gamma$ , after; (E) forkhead box P3 (Foxp3)/IL-10, before; (F) Foxp3+/IL-10, after; (G) IL-17, before; (H) IL-17, after.



mean SCORAD index for probiotic and placebo was 9.1 and 1.8, respectively ( $P=0.004$ ) (Han *et al.*, 2012). In our research, both the probiotic and placebo groups showed a significant decrease in SCORAD ( $P=0.000$ ), and after 12 weeks of probiotic supplementation more subjects (100%) significantly ( $P=0.000$ ) experienced reduction in SCORAD index than the subjects in the placebo group did (90%) ( $P=0.000$ ). However, as the standard therapy of antibiotics, antihistamines, emollients, and topical corticosteroids were still provided, the improvement of clinical symptoms found in the placebo group of this study could also be due to these drugs treatment.

Our study showed probiotic therapy improved the clinical symptoms of AD. The symptom score declined significantly while the total IgE did not; this may be due to pathological process of allergic reaction in the target cells in which the histamine receptors H1, H2, and H3 mediate the production of mast cell degranulation. Allergic reactions occur due to the interaction between allergens, mast cells, and specific IgE allergens that produce mast cell degranulation (Prussin and Metcalfe, 2006).

In the respiratory tract, aeroallergens are captured by dendritic cells in the mucosa (mucosal DCs) located along the barrier of tight junctions. Similarly, in the gastrointestinal tract, food allergens are captured by dendritic cells of the intestinal mucosa (intestinal DCs), which are located between the basement membrane and the intestinal epithelium (Sung *et al.*, 2006; Takano *et al.*, 2005). The development of host tolerance to the allergen depends on the first response of the host immune system facing the allergen. When the mucosal DCs encounter an allergen containing a distress signal, the cells are activated. The danger signals can be signalled by the allergen itself or by microbial contaminants, such as proteolytic enzymes from mite allergens, pollution, or viral products (Hammad and Lambrecht, 2006).

The levels of IL-4 produced by CD4+ T lymphocytes significantly decreased in both the placebo and the probiotic groups. Levels of IL-4/CD4+ T lymphocytes decreased significantly more in the probiotic group than in the placebo group (Figure 1). Levels of IFN- $\gamma$ /CD4+ T lymphocytes significantly decreased in the probiotic group, and the placebo group showed a decreasing trend. Levels of IL-4 and IFN- $\gamma$  decreased more in probiotic group than the placebo group did ( $P=0.000$ , and  $P=0.006$ , respectively). These results are in agreement with the studies by Han *et al.* (2012), reported that IL-4 levels after 12 weeks' probiotic supplementation were significantly lower than placebo ( $P=0.049$ ); while, there was no significant change in the placebo group ( $P=0.209$ ). In another study Farid *et al.* (2011) also found that administration of synbiotics (*Lactobacillus rhamnosus*, *Streptococcus thermophilus*, *Bifidobacterium breve*, *Lactobacillus acidophilus*, *Bifidobacterium infantis*,

*Lactobacillus bulgaricus*, and fructo-oligosaccharides) twice daily for eight weeks showed no significant changes in the cytokines IL-4 and IFN- $\gamma$  ( $P=0.4$  and  $P=0.6$ ) in AD children aged 3-6 months. In this study, although the increased levels of IL-4 were not significant, they showed a greater downward trend in the synbiotic group than that in the placebo ( $3.528\pm 1.75$  to  $3.77\pm 2.40$  and  $3.267\pm 1.64$  to  $2.96\pm 0.86$ ). In a study by Han *et al.* (2012), the efficacy of a probiotic mix (*Lactobacillus casei*, *L. rhamnosus*, *L. plantarum*, and *Bifidobacterium lactis*) was compared to placebo in children aged 2-9 years with moderate to severe AD, and the results showed that levels of IL-4 were not significantly changed after a 6-week intervention. *In vitro* research by Tang *et al.* (1993) proved that children with severe AD produced significantly high levels of IL-4 and low levels of IFN- $\gamma$  compared to non-atopic controls. The ratio of IL-4 to IFN- $\gamma$  increased significantly, indicating an imbalance between the two cytokines. In addition, there was a relationship between IgE levels and the IL-4/IFN- $\gamma$  ratio, indicating that this cytokine imbalance was a regulatory factor of *in vivo* IgE levels. Unlike in children with severe AD, in moderate AD, IL-4 did not increase and IFN- $\gamma$  did not decrease. These findings indicated that the production of IL-4 and IFN- $\gamma$  were generally associated with the severity of AD. In children with severe AD (total serum IgE >600 kIU/l) with eczema and severe acute exacerbations, elevated levels of IL-4 and decreased IFN- $\gamma$  were found, whereas levels of IL-4 and IFN- $\gamma$  were normally found in children with moderate AD (<600 kIU/l) and mild eczema. There was evidence that the increase in IL-4 and decrease in IFN- $\gamma$  was not only limited to AD. Rousset *et al.* (1991) showed that the production of IL-4 and IFN- $\gamma$  were not normally found in subject with atopy or allergies, but only in two subjects with eczema. Tang *et al.* (1993) reported an increase in the number of cells with IL-4 mRNA in bronchoalveolar fluid of patients with asthma; and children with asthma showed an increase in IL-4 in circulation. One report also mentioned that through H4 receptor subtypes, histamine plays a role in the immune regulation complex in the inflammatory response process of acute and chronic allergies. Through the H1 receptor, histamine increased the capacity of antigen presenting cells (APC) to capture antigens, which increased the release of histamine and other mediators from mast cells and basophils, down regulated humoral immunity, increased the proliferation of Th1, the production of IFN- $\gamma$ , and the expression of adhesion molecules, and chemotaxis of eosinophils and neutrophils (Simons and Simmons, 2011).

Endaryanto (2006) reported that *L. plantarum* IS-10506 regulated Th1, Th2, and Treg through balancing TLR2 and TLR4 in the decrease of allergic reactions in BALB/c mice. This did not occur through decreased IL-4 and total IgE levels, but through an increase in Th1 cytokines, Treg, and synthesis of specific IgA preventing mast cell degranulation through a reduction in specific IgE, without decreasing Th2.

In agreement with our study, Niers *et al.* (2005) showed that the effect of Th2 suppression by probiotic bacteria, including *L. plantarum*, might be due to immunoregulatory functions and was not associated with an increase in IFN- $\gamma$  rather than due to increase production of IL-10 and IL-6 in monocytes by *L. plantarum* and *Bifidobacterium adolescentis*. In addition, probiotics work through many pathways and each strain has a specific function or effect.

An important finding of this study was the decrease in clinical symptoms by the probiotic through suppressing the Th2 adaptive immune response, but not increasing the Th1 adaptive immune response; the mechanism of homeostasis was due to increasing the immune response of Treg and decreasing Th17. When processing harmful organisms and food allergens, Treg cells will be enabled by the mucosal immune system as inductor tolerance (Sampson, 2005).

Limitation of this study are small sample size and using whole blood to assess the cytokines. also antihistamines were given here as the standard therapy and affected the humoral immunity, they will therefore affect the results. The sample size of our pilot study is small, therefore the results were not representative of the whole population. Assessment of the cytokines by flow cytometry using whole blood without stimulation may obtain less lymphocytes in comparison to the PBMC cultures.

## 5. Conclusions

Probiotic *L. plantarum* IS-10506 showed ability to reduce clinical symptoms in AD children, as shown by a decrease in SCORAD and levels of serum IgE, IL-4, and IL-17, also an increase in the levels of Foxp3+ to IL-10 ratio. An important finding of this study was the decrease in clinical symptoms by the probiotic through down regulating Th2 adaptive immune response, but not up regulating Th1 adaptive immune response. Probiotics *L. plantarum* IS-10506 is a potential treatment for preventing recurrence or progression to chronic AD in children who are unable to eliminate allergenic ingredients and the emphasis on alternative therapies through the induction of immunological tolerance. This finding may lead to the use of the probiotic *L. plantarum* IS-10506 for AD adults. Further studies with a larger sample size and longer observation period are needed to strengthen evidence for the beneficial role of the probiotic *L. plantarum* IS-10506 in children with AD.

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