

A randomized trial of *Lactobacillus rhamnosus* IDCC 3201 tyndallizate (RHT3201) for treating atopic dermatitis

Kyunguk Jeong¹  | Mincheol Kim² | Se Ah Jeon¹ | Young-Hoo Kim² | Sooyoung Lee¹ 

¹Department of Pediatrics, Ajou University School of Medicine, Suwon, Korea

²ILDONG Pharmaceutical Co., Ltd., Seoul, Korea

Correspondence

Sooyoung Lee, Department of Pediatrics, Ajou University School of Medicine, Worldcup-ro 164, Yeongtong-gu, Suwon 16499, Korea.
Email: jsjs87@ajou.ac.kr

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Abstract

Background: Probiotic therapies, mainly live bacteria, have been proven to be effective in treating atopic dermatitis (AD) with some controversies. Killed probiotics or postbiotics would have immunomodulatory effect in allergic diseases including AD. This study was performed to evaluate the therapeutic effect and safety of tyndallized *Lactobacillus rhamnosus* (IDCC 3201, isolated from the feces of a Korean breast-fed infant, repeated heat-treated and incubated, RHT3201) in children with AD.

Methods: In a randomized, double-blind, placebo-controlled study, RHT3201 at a dose of 1.0×10^{10} CPU/d or placebo was given in children (aged 1–12 years) with moderate AD for 12 weeks. SCORing of AD (SCORAD) scores, allergic inflammatory markers, and safety parameters were evaluated.

Results: For evaluating the therapeutic effects of RHT3201, 33 subjects in each group were analyzed. The change of SCORAD total score at 12 weeks (primary outcome) from baseline was significantly greater in the RHT3201 group (-13.89 ± 10.05) compared to the control group (-8.37 ± 9.95). Levels of eosinophil cationic protein (ECP) and interleukin (IL)-31 showed tendency to decrease in the RHT3201 group and significant decreases in subgroup analysis in AD for ≥ 50 months. For safety analysis, a total of 100 subjects (50 in the treated group and 50 in the control group) were evaluated, and there were no significant differences in safety parameters between two groups.

Conclusion: In children with moderate AD, oral administration of RHT3201 showed the therapeutic effect on AD, the effects in part correlated with decrement of ECP and IL-31, and the effect was more remarkable in subgroup analysis.

KEYWORDS

atopic dermatitis, children, *Lactobacillus rhamnosus*, treatment

1 | INTRODUCTION

Atopic dermatitis (AD) is characterized by intense itching, eczematous skin lesions with typical locations, and chronic and relapsing features. Children with AD are typically treated with topical corticosteroids (TCS), antihistamines, systemic steroids, and antibiotics. However, long-term use of these medications may induce several adverse effects.¹⁻³ Previous meta-analyses have suggested that exposure to environmental microbes or harmonized intestinal microbes can modulate the development of allergic diseases including AD.^{4,5} Recently, microbes such as probiotic bacteria have been highlighted and considered candidates for immunomodulating pharmabiotics to prevent and treat allergic diseases; they have been mainly studied in AD.⁵⁻⁹

The term “probiotics” was originally defined as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” and are distinct from commensal microorganisms by definition.¹⁰ From studies on the therapeutic effects of probiotics, most of the materials used are live bacteria, and of these, *Lactobacillus* and *Bifidobacterium* are the most frequently investigated.⁶⁻⁸ While there are many studies on the immunomodulatory properties of probiotic microorganisms beyond their viability, the concept of inactivated, ghost probiotics named “parabiotics” or non-viable bacterial products, metabolites, or “postbiotics” has been proposed.¹¹⁻¹⁴ Here, we investigated the therapeutic effect of tyndallized *Lactobacillus rhamnosus* IDCC 3201 (*Lactobacillus rhamnosus* IDCC 3201 tyndallizate, RHT3201) on AD in children. RHT3201 was produced using a modified tyndallization method by repeated heat and pressure treatment of industrially grown *L. rhamnosus* IDCC 3201, which was isolated from a Korean breastmilk-fed infant; this product contains cell fractions and culture supernatants.¹⁵ Previously, RHT3201 had shown therapeutic effects on AD in a murine model and its mechanism was proposed.¹⁶ We conducted a 12-week, double-blind, randomized, placebo-controlled trial using RHT3201 and investigated its therapeutic effect on AD in children compared to a placebo group, along with several biomarkers in the immunomodulatory mechanism of AD.

2 | SUBJECTS AND METHODS

2.1 | Study population and design

One hundred children aged 1-12 years who were diagnosed with moderate AD, defined as SCORing of Atopic Dermatitis (SCORAD) score between 25 and 50, were included in the study. The following subjects were excluded: (a) subjects with other skin diseases, (b) subjects with immunosuppression, autoimmune diseases, malignancies, and other systemic diseases; (c) subjects taking systemic antibiotics, corticosteroids, and immunosuppressants for AD within 4 weeks of screening; (d) subjects taking medications such as antihistamines, oriental medicine, and other supplements for AD within 2 weeks of

Key Message

- Probiotics (mainly live form of bacteria) have been considered to have the preventive and therapeutic effect on atopic dermatitis (AD), and several immunomodulatory mechanisms were proposed. However, there is still lack of strong evidence to advocate the actual use of probiotics for treatment of AD.
- RHT3201 (*Lactobacillus rhamnosus* IDCC 3201 tyndallizate, the killed bacteria, and its culture supernatant condensate) showed the therapeutic effect in children with moderate AD. Eosinophil cationic protein and interleukin-31 were proposed as the immunologic markers related to improvement of AD, especially in AD with long duration.

screening; and (e) subjects receiving phototherapy within 4 weeks of screening.

A randomized, double-blind, placebo-controlled parallel study was conducted at the Department of Pediatrics, Ajou University, from October 27, 2014, to May 12, 2016; clinical evaluation was performed by the same physician during the entire study. This study was approved by the institutional review board at Ajou University Hospital in Suwon, Korea (AJIRB-MED-FOD-14); written informed consent was obtained from each patient or their parents (or legal guardian) before enrollment. The randomized subjects were provided with experimental treatment, RHT3201, prepared by ILDONG Pharmaceutical Co. Ltd. in the form of heat-killed bacteria (1.0×10^{10} CFU/d) or the placebo.¹⁵ Sample size determination and randomization process are summarized in Supplementary Table 1. The protocol for the use of TCS and emollients during the study period is presented in Supplementary Table 2.

2.2 | Clinical evaluation and laboratory tests

The subjects were evaluated for demographic characteristics, past history, physical examination, anthropometric measurements, vital signs, blood tests, and severity of AD at 0, 6, and 12 weeks of study. The SCORAD index was evaluated based on objective and subjective SCORAD as reported previously.¹⁷ Eosinophil count, eosinophil cationic protein (ECP), interleukin (IL)-4, IL-10, IL-31, C-reactive protein (CRP), tumor necrosis factor- α (TNF- α), and chemokine levels in blood were assessed at the baseline and at weeks 6 and 12. The primary outcome was the difference of SCORAD total score improvement at week 12, and the secondary outcomes were SCORAD total scores at week 6, SCORAD objective and subjective scores, respectively, at week 12, TCS usage, and immunologic markers listed above. For safety evaluation, full sets of physical examination and tests for vital signs, body weight, and laboratory parameters (complete blood

TABLE 1 Demographic characteristics of the subjects before treatment

	Per-protocol set			Subjects with AD for more than 50-mo duration of per-protocol set		
	RHT3201	Placebo	P value	RHT3201	Placebo	P value
	N = 33	N = 33		N = 17	N = 12	
Gender N (%)						
M	18 (54.55)	12 (36.36)	.1380 [†]	12 (70.59)	5 (41.67)	.1479 [†]
F	15 (45.45)	21 (63.64)		5 (29.41)	7 (58.33)	
Age (y)						
Mean ± SD	5.67 ± 3.30	5.33 ± 2.53	.6471 [†]	8.18 ± 2.24	7.50 ± 1.68	.3850*
Min, max	1.00, 11.00	1.00, 10.00		5.00, 11.00	5.00, 10.00	
Body weight (kg)						
Mean ± SD	22.27 ± 10.61	21.72 ± 7.52	.8083*	29.50 ± 9.72	28.06 ± 6.45	.6580*
Min, max	9.40, 46.70	10.40, 37.50		16.70, 46.70	18.30, 37.50	
Family history of allergies N (%)						
Y	27 (81.82)	24 (72.73)	.3782 [†]	13 (76.47)	10 (83.33)	1.0000 [†]
N	6 (18.18)	9 (27.27)		4 (23.53)	2 (16.67)	
Family history N (%)						
If yes to family history of allergies						
Father						
Y	8 (29.63)	11 (45.83)	.2322 [†]	3 (23.08)	5 (50.00)	.2213 [†]
N	19 (70.37)	13 (54.17)		10 (76.92)	5 (50.00)	
Mother						
Y	16 (59.26)	11 (45.83)	.3377 [†]	8 (61.54)	5 (50.00)	.6850 [†]
N	11 (40.74)	13 (54.17)		5 (38.46)	5 (50.00)	
Brother, Sister						
Y	11 (40.74)	10 (41.67)	.9465 [†]	6 (46.15)	4 (40.00)	1.0000 [†]
N	16 (59.26)	14 (58.33)		7 (53.85)	6 (60.00)	
Years of atopic dermatitis (mo)						
Mean ± SD	58.18 ± 39.75	48.12 ± 31.16	.2568*	9.71 ± 25.41	83.33 ± 19.48	.4063*
Min, max	6.00, 131.00	6.00, 119.00		54.00, 131.00	60.00, 119.00	
Breastfeeding status N (%)						
Past	32 (96.97)	33 (100.00)	1.0000 [†]	17 (100.00)	12 (100.00)	-
Present	1 (3.03)	0 (0.00)		0 (0.00)	0 (0.00)	
Breastfeeding						
Breastfeeding only	14 (42.42)	13 (39.39)	1.0000 [†]	7 (41.18)	2 (16.67)	.4364 [†]
Breastfeed and formula feeding	16 (48.48)	16 (48.48)		8 (47.06)	8 (66.67)	
Formula feeding only	3 (9.09)	4 (12.12)		2 (11.76)	2 (16.67)	
Baby food start (months after birth)						
Mean ± SD	6.97 ± 2.60	6.85 ± 1.95	.8313*	7.35 ± 3.14	7.92 ± 2.43	.6070*
Min, max	4.00, 18.00	5.00, 12.00		4.00, 18.00	5.00, 12.00	

[†]P-value by two-sample t test, *P-value by chi-square test, [†]P-value by Fisher's exact test.[‡]The dose of RHT3201 was 1.0 × 10¹⁰ CPU/d.

TABLE 2 SCORAD total score and SCORAD objective score after treatment with RHT3201 or placebo

			RHT3201 Mean ± SD	Placebo Mean ± SD	P-value*
SCORAD total score	Total subjects		N = 33	N = 33	
		Baseline	38.15 ± 7.09	36.49 ± 7.20	.3514
		Week 6	28.70 ± 12.12	29.43 ± 12.75	
		Change (weeks 6-0)	-9.44 ± 9.80	-7.06 ± 8.27	.2906
		Week 12	24.25 ± 12.75	28.13 ± 13.44	
		Change (weeks 12-0)	-13.89 ± 10.05	-8.37 ± 9.95	.0283
	Subjects with atopic dermatitis for ≥ 50-mo duration		N = 17	N = 12	
		Baseline	38.19 ± 6.88	39.99 ± 6.38	.4817
		Week 6	28.58 ± 14.15	34.80 ± 12.56	
		Change (weeks 6-0)	-9.62 ± 12.52	-5.19 ± 8.76	.3013
		Week 12	22.86 ± 11.16	32.76 ± 13.63	
		Change (weeks 12-0)	-15.34 ± 9.30	-7.23 ± 10.54	.0376
SCORAD objective score	Total subjects	Objective total score	N = 33	N = 33	
		Extent of disease			
		Intensity of disease			
	Subjects with atopic dermatitis for ≥ 50-mo duration	Objective total score	N = 17	N = 12	
		Extent of disease			
		Intensity of disease			

*Compared between groups; P-value by two-sample t test

†The dose of RHT3201 was 1.0×10^{10} CPU/d.

cell count, blood chemistry test, and urinalyses) were performed at weeks 0 and 12.

2.3 | Statistical analyses

The efficacy analysis was performed using a per-protocol (PP) set and full analysis (FA) set, respectively, and safety analysis was primarily performed using the safety set. In case of any missing data, the most recently obtained data from the missing dates were analyzed using the last observation carried forward (LOCF) method. Paired *t* test was used to compare changes before and after ingestion within each group; the two-sample *t* test or Wilcoxon rank-sum test was used for comparison between two groups. Chi-square test or Fisher's exact test was used to test independence and probability. A significance level of 0.05 was set for two-sided tests. Additionally, subgroup analyses were performed for subjects with AD for ≥ 50 months. The characteristics of subjects before treatment initiation were considered as baseline values. All statistical analyses were performed using SAS® (version 9.4, SAS Institute, Cary, North Carolina, USA).

3 | RESULTS

3.1 | Demographic characteristics

In total, 104 subjects eligible for the study were screened: 100 (50 in the RHT3201 group and 50 in the placebo group) were randomly assigned; of these, 90 subjects (45 in the RHT3201 group and 45 in the placebo group) completed the study (Figure 1). In total, 66 subjects (33 in the RHT3201 group and 33 in the placebo group) who completed the trial without violating any selection criteria were finally included in the PP set analysis. At the baseline of this study, no statistically significant differences were noted between the two groups (Table 1).

3.2 | Clinical effect and use of TCS

In PP analysis, the change of SCORAD total score at week 12 (primary outcome) was -13.89 ± 10.05 in the RHT3201 group ($P < .0001$) and -8.37 ± 9.95 in the placebo group ($P < .0001$) and this difference was statistically significant between the two groups ($P = .0283$, Figure 2A). The SCORAD total score at week 12 in the subjects with AD for ≥ 50 months was reduced more profoundly by 15.34 ± 9.30 ($P < .0001$) in the RHT3201 group and by 7.23 ± 10.54 ($P = .0367$) in the placebo group (between groups, $P = .0376$, Figure 2B).

The SCORAD objective score at week 12 was significantly decreased in both groups, and the degree of change was more profound in the RHT3201 group (Figure 3A). The reduction in disease extent was not statistically different in both groups (Figure 3B), but the intensity of the disease at week 12 was significantly reduced in the RHT3201-treated group (Figure 3C). In subgroup analysis at week 12 among

subjects with AD for ≥ 50 months, the difference of objective SCORAD score was statistically different between groups (Figure 3D). The difference in the reduction of extent of disease was significantly greater in the RHT3201-treated group than in the placebo group (Figure 3E), but the difference in the reduction in disease intensity was not statistically different in both groups (Figure 3F). The entire results of SCORAD total and objective scores in PP set analysis are shown in Table 2. The change of SCORAD total and objective scores did not show significant differences between the groups in FA set analysis (Table S3).

The proportion of subjects and the total amount of TCS used were not statistically different in both groups; 81.82% in the RHT3201 group and 84.85% in the placebo group used TCS; 7.09 ± 9.12 g of TCS was used in the RHT3201 group, whereas in the placebo group, 6.47 ± 7.13 g of TCS was used. The amount of TCS and emollients used in both groups and the change of SCORAD total scores after adjustment with TCS and emollients are presented in Tables S4 and S5, respectively.

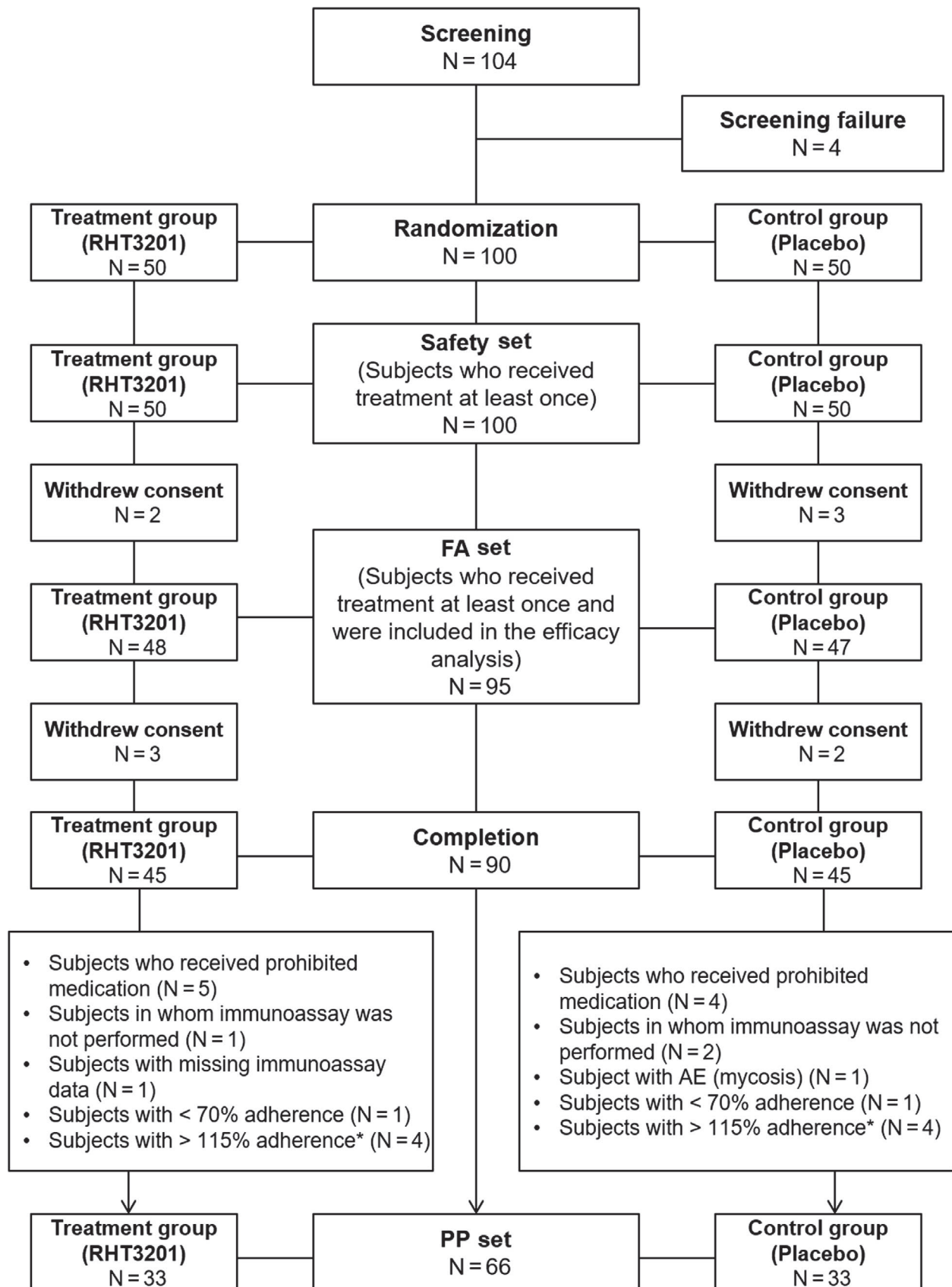
3.3 | Laboratory tests

The mean level of ECP at week 12 was increased slightly from the baseline, but was not statistically significant in both groups ($P = .9582$ in the RHT3201 group and $P = .1220$ in the placebo group) (Figure 4A). Contrastingly, in terms of the proportion of subjects with improvement of the ECP level at week 12, there were statistical differences between the RHT3201 and placebo groups (51.52% vs 24.24%, $P = .0224$). The changes in eosinophil count and IL-31 levels at week 12 showed a decreasing tendency in the RHT3201 group, but these decreases were not statistically significant (Figure 4B,C in the entire PP set). There was no statistical difference between the two groups in terms of altered IL-4, IL-10, CRP, TNF- α , and TARC/CCL17 levels.

By subgroup analysis at week 12 in subjects with AD for ≥ 50 months, the changes in ECP levels, eosinophil count, and IL-31 levels were significantly different between the two groups. The mean ECP level was decreased by 16.76 ± 33.77 mg/dL in the RHT3201 group and was increased by 12.42 ± 21.16 mg/dL in the placebo group ($P = .0084$, Figure 4D); the mean eosinophil count at week 12 was reduced by 83.53 ± 195.22 cells/ μ L in the RHT3201 group, whereas it was increased by 62.50 ± 155.40 cells/ μ L in the placebo group ($P = .0486$, Figure 4E). Furthermore, the mean IL-31 levels were decreased by 599.86 ± 985.95 pg/mL in the RHT3201 group and were increased by 330.28 ± 1038.98 pg/mL in the placebo group; this change was statistically significant between the two groups ($P = .0431$, Figure 4F).

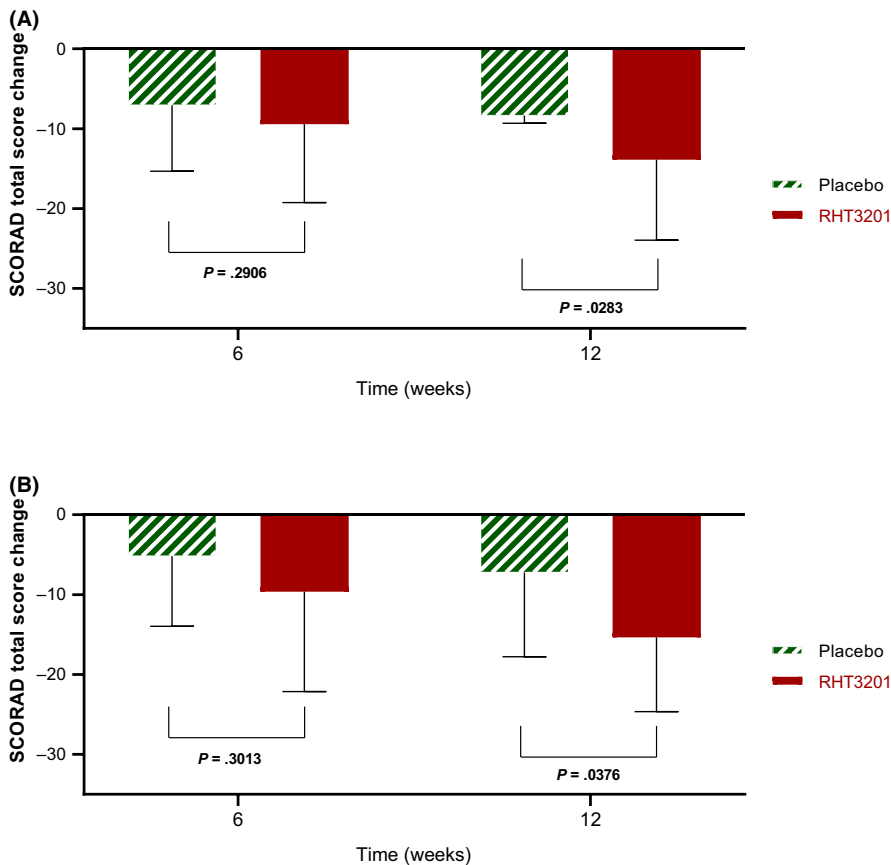
3.4 | Adherence and safety analyses

The adherence rate was $90.59 \pm 7.99\%$ in the RHT3201 group and $91.89 \pm 7.72\%$ in the placebo group ($P = .5070$). Safety analysis was performed in all randomized subjects who received at least one treatment (50 in each group). In the RHT3201 group, 27 subjects



* Adherence > 115% in our manuscript means the subjects who took more than 115% dose of the investigational product as 120% of IP was provided to each subject considering the potential loss of IP and the possible delay of the next visit.

FIGURE 1 Flowchart of the study population



Compared between groups; p-value by Two-sample t-test

FIGURE 2 Change of SCORAD total scores from weeks 0 to 12 in total subjects (A) and in subjects with atopic dermatitis for ≥ 50 -mo duration (B)

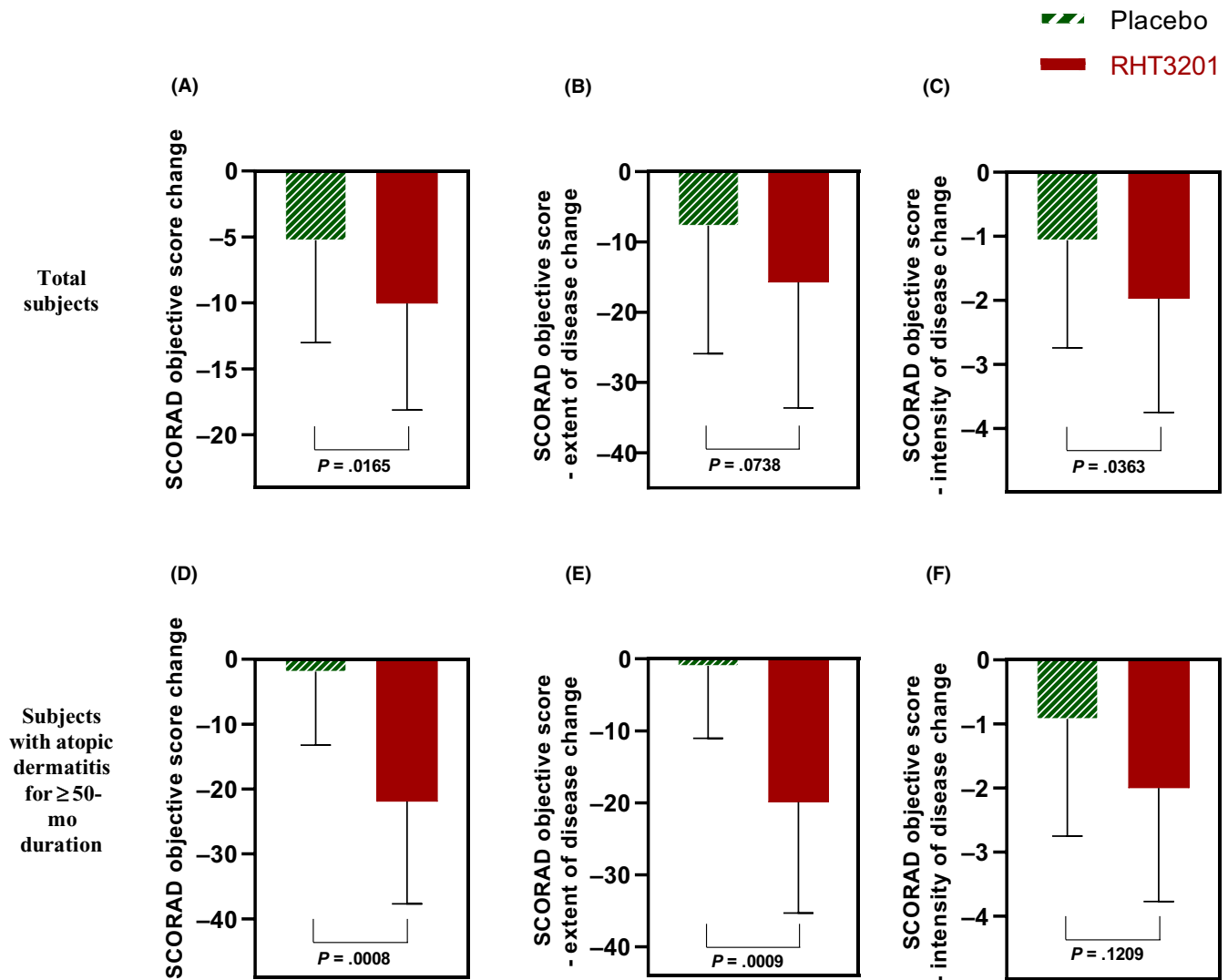
reported 38 episodes of adverse events, and, in the placebo group, 29 subjects reported 39 episodes of adverse events, with no statistical difference between the two groups. All of them were temporary common conditions in children or were caused by concurrent allergic diseases, which were “definitely not related” to the study treatments. The overall summary and the details for each adverse event are presented in Tables S7 and S8.

4 | DISCUSSION

In this study, we conducted a double-blind, randomized, placebo-controlled trial for evaluating the therapeutic effect of *Lactobacillus rhamnosus* IDCC 3201 tyndallizate (RHT3201) on AD in children aged 1–12 years. The primary outcome was the change in SCORAD total score from the baseline to week 12, and other clinical and immunologic parameters were compared between two groups. The change in SCORAD total score was significantly reduced in the RHT3201-treated group compared to the placebo group at week 12; thus, the primary outcome was fulfilled. We also found that the reduction in SCORAD total score and objective score was significantly greater in the RHT3201-treated group in subjects with AD duration of ≥ 50 months. In immunologic analysis, there was a decreasing tendency of serum ECP and IL-31 concentrations at week 12 in the RHT3201-treated group compared to the placebo group; these

findings were statistically significant in subgroup analysis with AD duration of ≥ 50 months. In our study, due to the ethical concerns for clinical trials in young children, minimal use of TCS and emollients was permitted; the amount of TCS and emollient used was not significantly different in both groups, and there was no safety issue related to the RHT3201 treatment.

In recent meta-analytic studies and systematic reviews published between 2016 and 2019, it has been evident that probiotics contribute to regulating allergic reactions in various manners and ultimately have preventive and/or therapeutic effect on AD in children.^{5–9} In most of the studies analyzed, the primary outcome was the difference of an improvement in SCORAD score between the treated and control groups. The duration of the dietary intervention was 8–12 weeks in most studies. In a meta-analysis with 13 selected clinical studies, the therapeutic efficacy was higher in children of 2 years or more compared to infants; improvement of SCORAD score was observed in the Asian population, whereas no effect was observed in Europeans.⁷ Among 26 systemic reviews regarding the therapeutic effect of probiotics on AD in human, 19 studies showed improvement in AD after probiotics treatment; a therapeutic effect was observed only in subgroup analysis in several studies.⁸ Collectively, these conceded studies could partly conclude that probiotics, mostly *Lactobacillus* and *Bifidobacterium*, have a therapeutic effect on AD when the administration and the effective duration are suggested for at least 8 weeks.^{6–8,18–23}



Compared between groups; p-value by Two-sample t-test

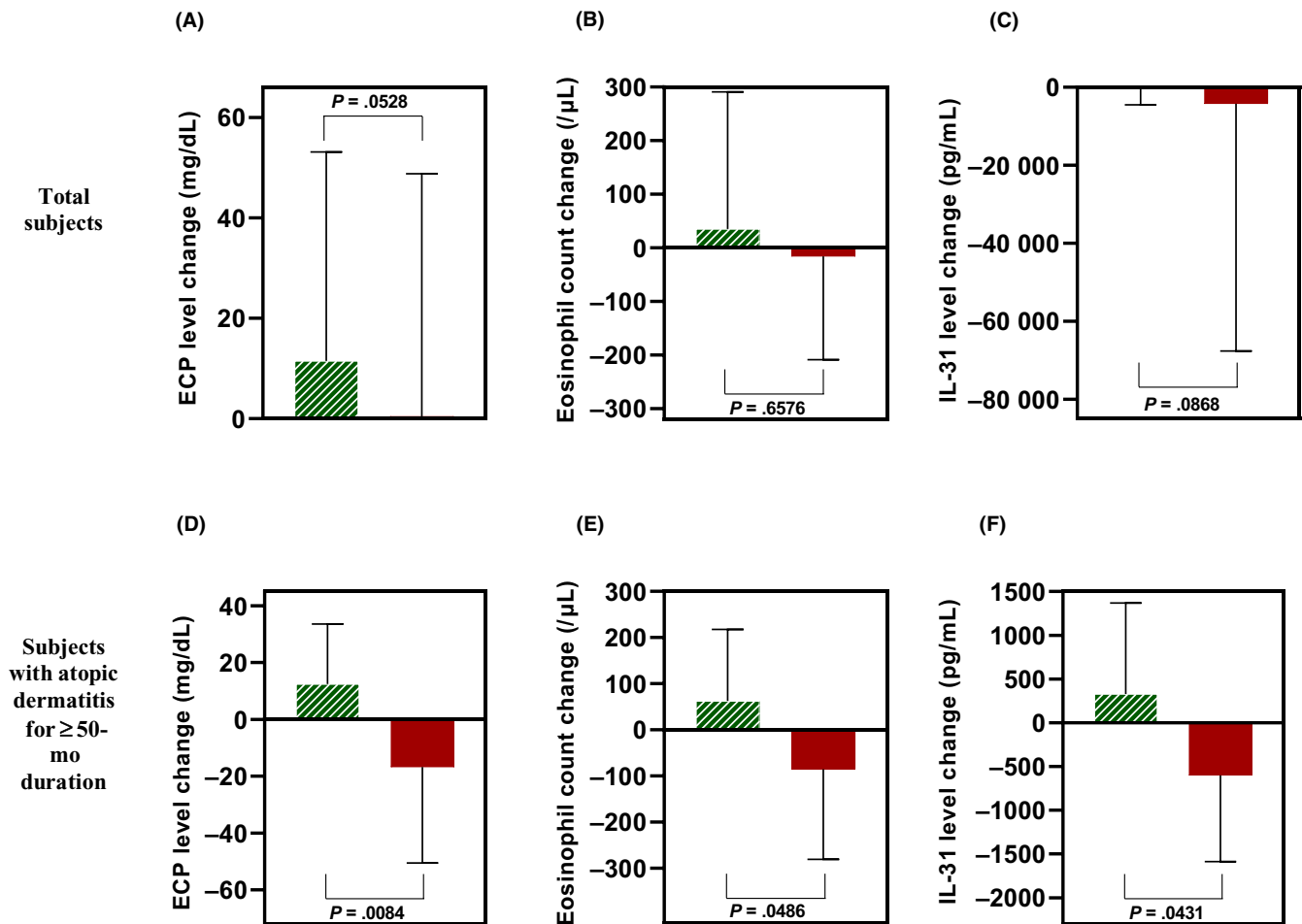
FIGURE 3 Change of SCORAD objective scores (objective total score, extent of disease score, and intensity of disease score) from weeks 0 to 12 in total subjects (A, B, C) and in subjects with atopic dermatitis for ≥ 50 -mo duration (D, E, F)

In previous clinical trials, various immunologic markers were proposed as mechanisms related to the therapeutic effect of probiotics on AD, although the results were not consistent.^{6-9,18-25} With the improvement of AD symptoms, reduced levels of eosinophils and/or IL-4,^{18,23,24} decreases or increases in regulatory cytokines^{23,24} and serum chemokine levels,²⁵ and controversial findings in IFN- γ levels^{18,24} were found. In the present study, we found that the proportion of subjects with decreased ECP was significantly higher in the RHT3201 group, and the changes in eosinophil count and IL-31 level at week 12 showed a decreasing tendency in the RHT3201 group. In the subgroup analysis of subjects with AD for ≥ 50 months, the changes in ECP levels, eosinophil counts, and IL-31 levels were significantly different between the two groups. Thus, we propose that the therapeutic effects of RHT3201 on childhood AD are related to the decreased production

or activity of eosinophils and IL-31. IL-31 has been identified as the cytokine that is preferentially produced by Th2 cells; its emerging role in pruritus in AD has been studied in both animal model and human studies²⁶⁻²⁹; thus, we cautiously propose that IL-31 could have a mechanistic role. Some immune parameters increased in the placebo group, possibly due to unknown irritating factors in the clinical study, unlike animal studies with constant control of the environment.

In most previous studies, live forms of *Lactobacillus* and *Bifidobacterium* are the most investigated organisms,^{5-9,18-25} while there have been limited studies of heat-killed probiotics in adults.^{23,30} Distinctively in the present study, we used RHT3201, which was tyndallized killed *Lactobacillus rhamnosus* (strain obtained from a Korean breastmilk-fed infant) for investigating the therapeutic effect on AD in children. Previously, RHT3201 showed

Placebo
RHT3201



Compared between groups; p-value by Wilcoxon rank-sum test

FIGURE 4 Group comparison of immune parameter change between RHT3201 and placebo from weeks 0 to 12 in total subjects (A, B, C) and in subjects with atopic dermatitis for ≥ 50 -mo duration (D, E, F)

an AD treating effect in the mouse model and has been discussed as one of the promising postbiotics in clinical use¹⁴⁻¹⁶; thus, we re-confirmed that RHT3201 is one of the candidates for treating AD in children.

This study has several limitations. First, the protocol could not completely stop the use of emollients and TCS; second, we could not measure IFN- γ due to the insufficiency of blood sample amounts. However, to overcome these weak points, we performed statistical analysis with adjustment for the amount of emollient and TCS used to minimize these influences. Instead of IFN- γ , we measured the IL-31 levels and found that the changes in IL-31 after treatment with RHT3201 are unique to this study, compared to previous clinical studies on AD.

Thus, the results of this randomized, double-blinded, placebo-controlled trial show that RHT3201 has therapeutic effect on

AD in children. The improvement in clinical symptoms was in part related to decreased ECP, eosinophil count, and IL-31; these positive findings are more profound in subgroup children with AD for ≥ 50 months. No statistically significant findings and differences in various safety parameters were found between both groups.

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CONFLICT OF INTEREST

Mincheol Kim and Young-Hoo Kim are employed by ILDONG Pharmaceutical Corporation. Other authors declare that there are no potential conflicts of interest with respect to the authorship and/or publication of this article.

AUTHOR CONTRIBUTIONS

Sooyoung Lee has conceptualized and designed the study, conducted the study, interpreted the results, and managed whole process. Kyunguk Jeong involved in the design and data collection, and mainly drafted the initial manuscript, and revised manuscript. Mincheol Kim and Young-Hoo Kim contributed to interpretation of the results, and statistical analyses. Sooyoung Lee and Mincheol Kim reviewed and revised the manuscript. Se Ah Jeon performed the data collection and data management. All authors approved the final manuscript as submitted and agreed to be accountable for all aspects of the work.

ORCID

Kyunguk Jeong  <https://orcid.org/0000-0002-1261-4507>

Sooyoung Lee  <https://orcid.org/0000-0003-1734-4101>

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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SUPPORTING INFORMATION

SUPPLEMENTARY TABLES

Supplementary Table 1. Summary of sample size determination and randomization

Sample size determination		- Referring to the existing clinical studies conducted in a design similar to this study, the appropriate number of subjects required for each group was 38. - Considering the dropout rate of 24%, the minimum number of subjects for randomization was 100 in total.
Randomization	Method used for allocation sequence generation	- At visit 2 (randomization visit, week 0), all subjects who met the inclusion criteria were assigned to each group by the allocation code of the block randomization method. - The ratio of the number of subjects in each group was 1:1.
	Type of randomization	The same block size (block size 4) was applied for balanced randomization among the groups.
	Implementation of random allocation sequence	- The randomization table was applied by sequentially applying the permutation of random numbers generated by the randomization program of SAS®, starting with the subject number 1. - The randomization table, reproducible by the SAS® randomization program, was generated by independent statistical experts prior to the start of the study. - The sponsor's investigational product (IP) manager attached the label according to the randomization table when packaging the IP, and supplied it to Ajou University Hospital before the start of the study. - At screening, the principal investigator (PI) assigned the screening numbers to the subjects who signed the consent form. - At visit 2, the randomization numbers were assigned to the subjects who met the inclusion criteria.
	Blinding	- The assignment of unique codes for each subject was managed in a sealed state by the PI, and was not disclosed until the end of the study. - In case of loss or damage of IP, the extra for each specific code was used to maintain the blindness.

Supplementary Table 2. The protocol for the use of topical corticosteroids and emollients

Types of TCS	< 2 years of age: Hydrocortisone (Class VII) > 2 years of age: 0.25% prednicarbate (Class V)
TCS prescription	TCS was provided only when our investigator determined the rescue medication was necessary, or the subject determined the rescue treatment was urgently needed according to the prior education by our investigator for acute flare of atopic dermatitis. Subjects were instructed to use the minimum amount of TCS for the relief of acute flare.
Checkup of TCS usage	Instead of the “total days” of TCS use, the total dose of TCS used during the test period was measured in this study, by weighing the remaining dose of TCS (in grams) at every visit
Types of emollient	AtobARRIER lotion (Aestura, Korea)
Checkup of emollient usage	Instead of the “total days” of emollient use, the total dose of emollient used during the test period was measured in this study, by weighing the remaining dose of emollient (in grams) at every visit

Supplementary Table 3. SCORAD total score and SCORAD objective score after treatment with RHT3201 or placebo (FA Set)

		Full analysis Set				
		RHT3201	Placebo	p-value*		
		Mean ± SD	Mean ± SD			
.SCORAD total score	Total subjects	N = 48	N = 47			
		Baseline	38.08 ± 7.10	36.57 ± 7.13	0.3014	
		Week 6	29.85 ± 11.55	29.15 ± 12.48		
		Change (weeks 6 - 0)	-8.23 ± 9.84	-7.42 ± 8.23	0.6630	
		Week 12	27.20 ± 13.18	27.36 ± 12.48		
	Change (weeks 12 - 0)	-10.89 ± 11.93	-9.21 ± 9.56	0.4515		
	Subjects with atopic dermatitis for ≥ 50 months duration	N = 24	N = 16			
		Baseline	38.04 ± 7.20	39.91 ± 6.85	0.4174	
		Week 6	30.28 ± 13.22	34.42 ± 12.08		
		Change (weeks 6 - 0)	-7.75 ± 11.87	-5.49 ± 8.51	0.5145	
Week 12		25.65 ± 12.65	31.24 ± 13.47			
Change (weeks 12 - 0)	-12.38 ± 11.50	-8.66 ± 11.33	0.3196			
SCORAD objective score	Total subjects	Objective total score	N = 48	N = 47		
			Baseline	28.79 ± 5.83	26.67 ± 6.65	0.1017
			Week 6	22.27 ± 9.13	21.36 ± 9.77	
			Change (weeks 6 - 0)	-6.52 ± 6.46	-5.31 ± 6.88	0.3784
			Week 12	20.53 ± 10.25	20.25 ± 10.66	
		Change (weeks 12 - 0)	-8.26 ± 8.21	-6.42 ± 7.94	0.2696	
		Extent of disease	Baseline	42.24 ± 14.65	38.04 ± 13.10	0.1447
			Week 6	31.86 ± 17.64	30.48 ± 18.36	
			Change (weeks 6 - 0)	-10.38 ± 16.97	-7.56 ± 14.60	0.3894
			Week 12	29.74 ± 19.82	28.66 ± 20.87	
	Change (weeks 12 - 0)		-12.50 ± 20.01	-9.38 ± 18.34	0.4310	
	Intensity of disease	Baseline	5.81 ± 1.41	5.45 ± 1.50	0.2238	
		Week 6	4.54 ± 1.90	4.36 ± 1.96		
		Change (weeks 6 - 0)	-1.27 ± 1.53	-1.09 ± 1.52	0.5532	
		Week 12	4.17 ± 2.02	4.15 ± 2.06		
	Change (weeks 12 - 0)	-1.65 ± 1.69	-1.30 ± 1.63	0.3102		
	Subjects with atopic dermatitis for ≥ 50 months duration	Objective total score	N = 24	N = 16		
			Baseline	29.29 ± 6.05	30.22 ± 7.30	0.6631
Week 6			23.28 ± 10.58	26.48 ± 10.00		
Change (weeks 6 - 0)			-6 ± 7.81	-3.74 ± 6.85	0.3515	
Week 12			20.11 ± 9.68	25.62 ± 11.57		
Change (weeks 12 - 0)			-9.18 ± 7.77	-4.6 ± 8.77	0.0913	
Extent of disease			Baseline	43.63 ± 14.58	40.63 ± 15.79	0.5410
			Week 6	31.83 ± 16.70	40.53 ± 18.95	
			Change (weeks 6 - 0)	-11.79 ± 15.02	-0.09 ± 11.00	0.0111
			Week 12	27.65 ± 17.22	37.31 ± 20.28	
		Change (weeks 12 - 0)	-15.98 ± 17.38	-3.31 ± 14.07	0.0200	
Intensity of disease		Baseline	5.88 ± 1.42	6.31 ± 1.45	0.3502	
		Week 6	4.83 ± 2.26	5.25 ± 1.98		
		Change (weeks 6 - 0)	-1.04 ± 1.73	-1.06 ± 1.57	0.9694	
		Week 12	4.17 ± 2.04	5.19 ± 2.26		
Change (weeks 12 - 0)		-1.71 ± 1.71	-1.13 ± 1.86	0.3130		

* p-value by Two sample t-test

‡ The dose of RHT3201 was 1.0×10^{10} CPU/day

Supplementary Table 4. The amount of topical corticosteroids and emollients used (PP set)

			RHT3201 N = 33	Placebo N = 33	p-value*
Topical steroid	Baseline–12 weeks (g)	Mean ± SD	7.09 ± 9.12	6.47 ± 7.13	0.7577
		Min, Max	0.00, 31.60	0.00, 33.30	
	Baseline–6 weeks (g)	Mean ± SD	3.38 ± 4.73	3.62 ± 4.85	0.8377
		Min, Max	0.00, 18.70	0.00, 25.20	
	6 weeks–12 weeks (g)	Mean ± SD	3.72 ± 6.03	2.85 ± 3.10	0.4663
		Min, Max	0.00, 24.10	0.00, 11.90	
Emollients	Baseline–12 weeks (g)	Mean ± SD	648.70 ± 332.18	757.91 ± 360.06	0.2049
		Min, Max	101.00, 1467.00	102.00, 1492.00	
	Baseline–6 weeks (g)	Mean ± SD	353.61 ± 240.94	414.58 ± 194.83	0.2626
		Min, Max	0.00, 958.00	33.00, 749.00	
	6 weeks–12 weeks (g)	Mean ± SD	295.09 ± 160.48	343.33 ± 210.11	0.2985
		Min, Max	39.00, 743.00	45.00, 800.00	

* p-value by Two sample t-test

Supplementary Table 5. The change of SCORAD total scores after adjustment with the total doses of topical corticosteroids and emollients used (PP set)

Variable	Group	N	LS Mean	SE	P-value* for ANCOVA	Difference With Placebo	95% CI for LS mean
SCORAD total score (V4-baseline)	RHT3201	33	-13.91	1.74	0.0293	-5.56	(-10.54, -0.58)
	Placebo	33	-8.35	1.74			

LS Mean: Least Square mean

*P-value for ANCOVA adjusted by the total doses of topical steroids and emollients used (H0: LS mean(RHT3201)-LS mean(Placebo)=0)

Supplementary Table 6. Measurement results of immune-parameters after treatment with RHT3201 or placebo

		Per-protocol set			Full analysis Set			
		RHT3201	Placebo	p	RHT3201	Placebo	p	
		Mean ± SD	Mean ± SD	value**	Mean ± SD	Mean ± SD	value**	
		N = 33	N = 33		N = 48	N = 47		
Total subjects	ECP (mg/dL)	Baseline	51.63 ± 55.49	51.46 ± 49.31	0.6079	47.40±48.42	49.86±49.08	0.6959
		Week 6	48.86 ± 33.99	60.30 ± 50.51		48.60±37.90	55.97±47.84	
		Change (weeks 6 - 0)	-2.77±49.11	8.84 ± 41.07	0.3902	1.20±48.14	6.11±43.60	0.6418
		Week 12	52.08 ± 45.22	62.98 ± 47.80		47.32±40.96	62.46±50.98	
		Change (weeks 12 - 0)	0.44±48.34	11.51 ± 41.64	0.0528	-0.07±42.32	12.60±48.67	0.0230
	Eosinophil (µL)	Baseline	488.48 ± 406.97	386.06 ± 219.96	0.0695	484.38±377.44	400.85±297.98	0.3194
		Week 6	435.76 ± 293.80	445.45 ± 261.12		453.75±278.51	427.66±305.53	
		Change (weeks 6 - 0)	-52.73 ± 243.61	59.39 ± 220.67	0.0695	-30.63±318.40	26.81±230.82	0.4083
		Week 12	472.73 ± 391.53	421.21 ± 283.68		458.33±343.22	417.02±295.12	
	Change (weeks 12 - 0)	15.76 ± 192.86	35.1 5± 255.80	0.6576	-26.04±231.22	16.17±299.36	0.5570	
	IL-31* (pg/ml)	Baseline	11623.45 ± 52658.03	1897.48 ± 4122.20	0.4197	8623.57±43769.42	1860.10±3682.45	0.7536
		Week 6	1644.15 ± 2394.44	1538.32 ± 2955.48		3073.02±11864.02	1664.37±2897.95	
		Change (weeks 6 - 0)	-9979.30 ± 52396.57	-359.16 ± 2868.77	0.8904	-5550.55±44611.96	-195.74±2444.31	0.6527
		Week 12	7447.95 ± 36256.53	1566.32 ± 3006.14		15899.49±78428.17	1678.79±2890.73	
	Change (weeks 12 - 0)	-4175.50 ± 63437.23	-331.15 ± 4128.24	0.0868	7275.92±90124.75	-181.31±3479.96	0.3823	
	IL-4 (pg/ml)	Baseline	0.15 ± 0.05	0.16 ± 0.06	0.5160	0.15±0.05	0.16±0.06	0.3802
		Week 6	0.15 ± 0.05	0.16 ± 0.07		0.15±0.05	0.16±0.06	
		Change (weeks 6 - 0)	-0.00 ± 0.05	-0.00 ± 0.06	0.7578	-0.00±0.05	-0.00±0.05	0.9457
		Week 12	0.15 ± 0.06	0.15 ± 0.08		0.14±0.06	0.15±0.08	
		Change (weeks 12 - 0)	0.00 ± 0.06	-0.01 ± 0.08	0.1399	-0.01±0.06	-0.01±0.08	0.2365
	IL-10 (pg/ml)	Baseline	1.19 ±2.67	1.12 ± 2.19	0.1683	0.97±2.24	0.90±1.86	0.4494
		Week 6	1.06 ±1.34	0.54 ± 0.38		0.98±1.23	0.57±0.46	
		Change (weeks 6 - 0)	-0.13 ± 2.87	-0.59 ± 2.15	0.0266	0.01±2.45	-0.33±1.85	0.0538
		Week 12	1.18 ± 1.42	1.16 ± 2.23		1.02±1.22	0.99±1.89	
	Change (weeks 12 - 0)	-0.01 ± 2.58	0.04 ± 2.79	0.2619	0.05±2.13	0.09±2.33	0.3236	
	CRP (ug/mL)	Baseline	0.06 ± 0.12	0.09 ± 0.28	0.6686	0.06±0.11	0.08±0.24	0.4035
		Week 6	0.15 ± 0.47	0.06 ± 0.06		0.19±0.48	0.10±0.22	
		Change (weeks 6 - 0)	0.09 ± 0.49	-0.03 ± 0.30	0.7766	0.14±0.49	0.02±0.33	0.9018
		Week 12	0.06 ± 0.07	0.09 ± 0.12		0.06±0.07	0.08±0.11	
	Change (weeks 12 - 0)	-0.00 ± 0.12	0.00 ± 0.29	0.1002	-0.00±0.10	0.01±0.25	0.0471	
	TNF-α (pg/mL)	Baseline	2.63 ± 3.18	2.99 ± 3.65	0.3974	2.51±2.74	2.81±3.26	0.6636
		Week 6	2.03 ± 0.83	5.27 ± 10.61		2.59±2.77	4.34±8.94	
		Change (weeks 6 - 0)	-0.59 ± 3.30	2.29 ± 11.42	0.0692	0.08±3.93	1.52±9.64	0.1746
		Week 12	4.18 ± 4.97	5.81 ± 9.19		3.81±4.58	7.12±13.05	
	Change (weeks 12 - 0)	1.55 ± 6.16	2.82 ± 10.15	0.5245	1.31±5.44	4.31±13.68	0.8234	
	TAR C/CC L17 (pg/mL)	Baseline	939.14 ± 581.52	847.75 ± 590.87	0.6042	969.65±590.25	833.84±514.02	0.4748
		Week 6	769.05 ± 451.74	893.03 ± 526.28		863.18±513.52	916.57±557.51	
		Change (weeks 6 - 0)	-170.09 ± 451.30	45.28 ± 527.09	0.0918	-106.48±478.53	82.73±516.30	0.0678
		Week 12	815.84 ± 510.63	883.42 ± 724.47		877.00±538.11	852.26±650.93	
	Change (weeks 12 - 0)	-123.30 ± 407.20	35.67 ± 730.90	0.6698	-92.65±542.39	18.42±648.37	0.4842	
Subjects with atopic dermatitis for 50 months duration		N = 17	N = 12		N = 24	N = 16		
ECP (mg/dL)	Baseline	53.87 ± 62.85	37.39 ± 30.54	0.8420	50.42 ± 54.80	45.21 ± 49.14	0.8359	
	Week 6	45.08 ± 32.09	55.63 ± 52.34		45.40 ± 39.39	59.26 ± 53.26		
	Change (weeks 6 - 0)	-8.79 ± 47.88	18.24 ± 32.54	0.2406	-5.02 ± 52.58	14.04 ±51.36	0.2878	
	Week 12	37.11 ± 35.19	49.81 ± 41.56		38.70 ± 33.43	57.77 ± 48.38		
	Change (weeks 12 - 0)	-16.76 ± 33.77	12.42 ± 21.16	0.0084	-11.71 ± 34.19	12.56 ± 54.52	0.0244	
Eosinophil	Baseline	512.94 ± 427.31	395.83 ± 215.81	0.9285	550.83 ± 408.73	496.88 ± 396.85	0.9001	
	Week 6	447.06 ± 346.62	491.67 ± 271.22		470.83 ± 335.55	531.25 ± 355.38		

(µL)	Change (weeks 6 - 0)	-65.88 ± 187.19	95.83 ± 105.44	0.0102	-80.00 ± 345.20	34.38 ± 177.69	0.1186
	Week 12	429.41 ± 375.44	458.33 ± 284.31		454.17 ± 333.60	581.75 ± 322.94	
	Change (weeks 12 - 0)	-83.53 ± 195.22	62.50 ± 155.40	0.0486	-96.67 ± 247.96	21.88 ± 386.85	0.0593
IL-31* (pg/ml)	Baseline	1392.27 ± 1587.87	935.67 ± 1463.38		2044.36 ± 4080.88	896.83 ± 1357.07	0.1703
	Week 6	1119.46 ± 1341.50	1188.81 ± 1908.07		4454.26 ± 16624.27	1160.02 ± 1841.76	
	Change (weeks 6 - 0)	-272.81 ± 799.68	253.14 ± 622.18	0.0599	2409.90 ± 12772.30	263.18 ± 623.21	0.1989
	Week 12	792.41 ± 1155.25	1265.95 ± 2457.83		21886.13 ± 103389.64	1260.16 ± 2245.22	
	Change (weeks 12 - 0)	-599.86 ± 985.95	330.28 ± 1038.98	0.0431	19841.77 ± 103318.29	363.32 ± 935.99	0.0969

* One of placebo group was excluded due to lack of baseline testing.

** Compared between groups; p-value by Wilcoxon rank sum test

Supplementary Table 7. Summary of adverse events

		RHT3201 (Number of cases)	Placebo (Number of cases)	P-value*
Grading scale for adverse events	Mild	34	35	0.1277
	Moderate	1	4	
	Severe	3	0	
Serious adverse event		0	0	-
Dropout due to adverse event		1	0	1.0000
Relation with the study treatments	Definitely not related	38	39	-
Total adverse event		38	39	0.6870

*p-value by Fisher's exact test

Supplementary Table 8. The details of individual adverse event defined as definitely not related to the study treatments

Adverse event	RHT3201 (Number of cases)	Placebo (Number of cases)	Reason for “definitely not related to the study treatments”
Acute otitis media	0	2	Cured after acute treatment and no additional symptoms occurred.
Abdominal pain	1	0	Cured by administration of a digestant and no additional symptoms occurred.
Diarrhea	2	0	Improved by administration of an antidiarrheal and no additional symptoms occurred.
Anaphylactic reaction	1	0	Occurred acutely after buckwheat exposure in a subject allergic to buckwheat
Fungal skin infection	1	1	Cured by using antifungal drugs and no additional symptoms occurred.
Hand-foot-and-mouth disease	0	1	Cured after conservative care and no additional symptoms occurred.
Hordeolum	1	1	Cured after treatment and no additional symptoms occurred.
Skin papilloma	0	1	A temporary infectious disease that was determined to have no causal relationship with the test material.
Viral rash	5	4	A common comorbidity in patients with atopic dermatitis and repeated symptoms of viral rash were already present before the clinical trial.
Contusion	0	1	A condition caused by physical injury and therefore unassociated with the test material.
Lip injury	0	1	A condition caused by physical injury and therefore unassociated with the test material.
Wound	0	1	A condition caused by physical injury and therefore unassociated with the test material.
Tibial torsion	1	0	A condition caused by physical injury and therefore unassociated with the test material.
Asthma	1	1	A common concurrent allergic disease in patients with atopic dermatitis. The intermittent symptoms were already present before the clinical trial.
Pneumonia	1	0	An infectious respiratory disease that was cured after conventional treatment. No additional symptoms occurred.
Rhinitis allergic	2	2	A common concurrent allergic disease in patients with atopic dermatitis. No additional symptoms occurred.
Upper respiratory tract infection	22	20	Cured by conservative care for cold and no additional symptoms occurred.
Impetigo	0	1	Cured after antibiotic treatment and no additional symptoms occurred.
Laceration	0	1	A condition caused by physical injury and therefore unassociated with the test material.
Urticaria	0	1	Cured by administration of temporary antihistamine and no additional symptoms occurred.