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Title Page

Oral administration of *Lactobacillus casei* variety *rhamnosus* partially alleviates TMA-induced atopic dermatitis in mice through improving intestinal microbiota

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Running Title: Probiotic LCR35 for atopic dermatitis

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Abstract

Aims: The purpose of this study was to investigate the effect of *Lactobacillus casei* variety *rhamnosus* (LCR35) on AD-like symptoms in mice.

Methods and Results: AD-like skin lesions in BALB/C mice was induced by sensitization to and repeated topical challenges with trimellitic anhydride (TMA) for 10 days. LCR35 was orally administered to the mice once daily throughout the study. In the TMA-induced AD model, orally administered LCR35 suppressed significantly irritant-related scratching behavior and skin dehydration as well as apparent severity of AD. LCR35 also significantly decreased serum levels of IgE and IL-4, but not IFN- γ , implying the restoration of TMA-induced disruption of Th1/Th2 balance. Quantitative real-time PCR targeting hypervariable regions of 16S rDNA gene of fecal microbiota indicated that the LCR35 treatment increased population of *Bifidobacterium*, *Lactobacilli*, *Enterococcus* and *Bacteroides fragilis* group, but decreased those of *Clostridium coccoides* group.

Conclusions: LCR35 has the ability to suppress the development of AD in mice, possibly through the modulation of Th1/Th2 balance and gut microbiota.

Significance and Impact of the Study: LCR35 has a strong potential as a probiotic for preventing AD.

Key Words: *Lactobacillus casei* variety *rhamnosus*, probiotics, atopic dermatitis, microbiota, immunomodulation, Th1/Th2 balance

Introduction

Atopic dermatitis (AD) is a common chronic relapsing inflammatory skin disease accompanied by severe itching and multiple lesions with erythema, edema, erosion, dry skin, and susceptibility to cutaneous infection. Although the exact pathogenesis of AD is not yet completely understood, it is known to be associated with strong polarization to T-helper (Th) 2 type responses, thereby resulting in the elevated serum immunoglobulin E

(IgE) levels (Grewe *et al.* 1998; Leung and Bieber 2003). Depending on the type of cytokines produced, Th cells are classified into two major subtypes, Th1 and Th2 cells. Th1 cells secrete interferon (IFN)- γ and interleukin (IL)-2 responsible for cell-mediated immunity toward humoral immunity, whereas Th2 cells produce mainly IL-4 associated with B cell proliferation and IgE production (Paul and Seder 1994). Cytokine secretion profiles correlate well with the activation and distinctive functions of these cells. Therefore, an imbalance in the Th1/Th2 cytokines with a shift toward a predominantly Th2 cytokine is considered to be associated with AD pathogenesis.

Furthermore, the hyperreactivity to environmental triggers and barrier dysfunction of the atopic skin play a central role in the exacerbation of clinical symptoms (Leung *et al.* 2004). Itch is one of the most serious clinical symptoms of AD. Itch-associated scratching destroys the skin tissue and increase the inflammation, which in turn results in more itching further (Wahlgren 1999). It is therefore important to reduced itching to prevent aggravation of the skin lesion in AD and to improve the quality of life of the patients with AD.

Recently, abnormal gut microbiota has been proposed as a factor in the development of allergic diseases. Many epidemiological evidences and experimental researches have demonstrated differences in the fecal microbiota between allergic and nonallergic infants, indicating differences in the composition of the gut microbiota that may disturb the development of a normal Th1-/Th2-balance in allergic children (Bottcher *et al.* 2000; Watanabe *et al.* 2003). In addition, it was reported that failure of germ-free mice to establish oral tolerance was restored normally when germ-free mice were associated with *Bifidobacterium infantis* (Sudo *et al.* 1997). Therefore, the importance of administration of probiotics for the primary prevention and even treatment of atopic disease by means of normalization of intestinal microbiota has been emphasized.

Probiotics are live microorganisms that provide health benefits when consumed. It has been suggested that administration of probiotics may have preventive and/or therapeutic potential in AD (Matricardi and Bonini 2000; Kalliomaki *et al.* 2001). Probiotics have been shown to improve intestinal permeability, to stimulate and/or modulate the host immune system through the gut immune system and to normalize the composition intestinal microbiota by increasing bifidobacteria and decreasing the numbers of clostridia (Kalliomaki *et al.* 2001; Isolauri *et al.* 2001). However, evidence regarding their utility is still limited at this time.

The purpose of this study was to investigate whether oral administration of LCR35 has the benefit effects on a TMA-induced atopic dermatitis in mice showing significant increases in ear thickness, lymph node weight, IgE production, and skin inflammation and, if so, whether it is relevant to the change in the composition of the intestinal microbiota.

Materials and Methods

Animals

Male BALB/c mice (6 weeks old, 18–22 g body weight) were purchased from Samtaco Co. (Seoul, Korea). The animals were group housed at $23 \pm 2^\circ\text{C}$ and 40–60% humidity with a 12 h light/dark cycle (08:00–20:00 h light, 20:00–08:00 h dark) with access to a mouse pellet diet and water *ad libitum*. All procedures with animals were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of Kyung Hee University.

TMA-induced atopic dermatitis

Experimental protocols were illustrated in Fig. 1. The sensitization and challenge protocol was carried out according to the procedure described by Schneider et al. (Schneider *et al.* 2009). Briefly, mice were sensitized with 50 μl of 5% TMA by topical application on the shaved abdominal skin on day 0, received 10 μl of 5% TMA on the both ears on day 5, and repeatedly challenged with 10 μl of 2% TMA on the both ears once a day on days 6–14. TMA was dissolved in a mixture of acetone and isopropylmyristate (4:1, v/v) immediately before application. Mice with no induction of AD-like skin lesion were treated with vehicle alone (a mixture of acetone and isopropylmyristate) on the ear skin.

Experimental groups and LCR35 administration

Lyophilized *Lactobacillus casei* variety *rhamnosus* (LCR35; 1×10^9 CFU g^{-1}) was used in this study. LCR35 was obtained from Han Wha Pharma Co., Ltd. (Chuncheon, Korea). For administration, LCR35 lyophilized to dry powder form was suspended in saline and used immediately for administration.

Animals were randomly divided into 4 groups of 10 mice each; NOR, TMA, LCR-25, and LCR-250. With the exception of the mice in NOR group, all of mice were treated with TMA to induce AD-like skin lesion. The LCR-25 and LCR-250 groups received 2.5×10^7 and 2.5×10^8 CFU kg^{-1} body weight per day of LCR35, respectively. LCR35 was orally administered once a day for 15 consecutive days from the day of the first TMA sensitization. On the each day of TMA treatment, the administration of LCR35 was performed 30 min before the TMA application. The NOR and TMA groups received a comparable volume of saline.

Evaluation of skin lesion severity and lymph node weight

The relative AD severity was estimated macroscopically by the extent of erythema, edema, erosion and scaling (Hanifin *et al.* 2001). As a parameter of cutaneous inflammation, ear thickness was also measured 24 h after the last challenge using a dial micrometer (Mitsutoyo Co., Tokyo, Japan). Water content of the ear skin was assessed by measuring electrical capacitance using a Corneometer CM825 (Courage Khazaka, Cologne, Germany), with values reported in arbitrary units (AU). The values obtained were determined as the average of five measured values at the same skin site. These measurements were performed at a temperature of $23 \pm 1^{\circ}\text{C}$ and under $60 \pm 10\%$ humidity. Weights of lymph nodes collected from the sacrificed mice were also measured using an electronic balance (Mettler Toledo, Greifensee, Switzerland).

Observation of scratching behavior

Scratching behavior was observed after the last TMA challenge on day 15. For measurement of scratching behavior, mice were individually placed in an acrylic cage divided into compartments ($8 \times 8 \times 15$ cm) to acclimate for 60 min. And then, the mice were administered LCR35 or vehicle and quickly returned to the cage. Immediately after TMA challenge at 30 min after oral administration of LCR35, each mouse was put back into its cage, and its behavior was recorded using a digital video camera for 30 min in an unwatched condition. The number of scratching episodes was counted manually from video recordings. The mice generally scratched several times with their hind paws for 1 s, and a series of these movements was counted as one episode of scratching.

Measurement of serum levels of total IgE, histamine, IFN- γ and IL-4

Blood was collected by cardiac puncture and serum was harvested from each blood samples and stored at -80°C until analyzed. Serum levels of total IgE, histamine, IFN- γ and IL-4 were measured by ELISA method using commercial quantitative kit (BD Biosciences Pharmingen, San Diego, CA, USA for IFN- γ ; R&D Systems Inc., MN, USA for IL-4; Labor Diagnostika Nord GmbH & Co., KG, Nordhorn, Germany for histamine; Bethyl Laboratories Inc., TX, USA for total IgE) according to the manufacturer's instructions.

RT-PCR

The expression of proinflammatory cytokine mRNA was evaluated by reverse transcriptase polymerase chain reaction (RT-PCR). In brief, total RNA was extracted from excised skin lesion of each mouse using TRIzol

reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instruction. cDNA was synthesized from 2 µg total RNA using reverse transcriptase (Takara Bio, Otsu, Japan) with random hexamers (COSMO Genetech, Seoul, Korea), and then amplified at 58 °C for 32 cycles for TNF- α and at 58 °C for 30 cycles for IL-4 by PCR using Taq DNA polymerase (Takara, Kyoto, Japan) on a thermal cycler (MJ Research, MA, USA). The following sequences of primers were used: TNF- α forward, 5'-GCAGAAGAGGCACTCCCCCA-3' and reverse, 5'-GATCCATGCCGTTGCCAGG-3' (product size, 328 bp); IL-4 forward, 5'-TCAACCCCCAGCTAGTTGTC-3' and reverse, 5'-TGTTCTTCGTTGCTGTGAGG-3' (product size, 177 bp); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward, 5'-AACTTTGGCATTGTGGAAGG-3' and reverse, 5'-ACACATTGGGGGTAGGAACA-3' (product size, 223 bp). Primer sequences were designed using Primer 3 software (The Whitehead Institute for Biomedical Research, <http://frodo.wi.mit.edu/primer3>). The PCR products were electrophoresed on 1.2% agarose gel containing GelRed stain (Biotium Inc., Hayward, CA). The bands were photographed under UV light and evaluated with the image-analysis software Image Master Total Lab (Amersham Pharmacia Biotech, NJ, USA). Data were normalized against GAPDH expression in the corresponding samples.

Fecal microbiota analysis by real-time PCR

The bacterial DNA was extracted from fecal samples using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. The relative amounts of target bacteria were analyzed using SYBR Green I-based real-time PCR with FastStart Essential DNA Green Master (Roche Applied Science, Mannheim, Germany) and LightCycler Nano System (Roche). The 16S rDNA-targeted primers used are shown in Table 1. Amplifications were performed in a 20 µl final volume containing 20 ng of fecal DNA under the following temperature profiles: 1 cycle at 95 °C for 300 s, followed by 45 cycles of denaturation at 95 °C for 20 s, annealing at the appropriate target-specific temperature for 20 s, and extension at 72 °C for 20 s. To determine the specificity of amplification, analysis of product melting curve was performed after the last cycle of each amplification. The threshold PCR cycle number (Ct) values were determined using the LightCycler Relative Quantification software. The fold value in target bacteria was calculated for each sample using the $2^{-\Delta\Delta C_t}$ method (Tuomisto *et al.* 2013).

Statistical analysis

Data are presented as means $\pm$ SEM. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test with GraphPad Prism 5.01 for Windows (GraphPad Software, San Diego, USA). $p < 0.05$ was considered to be statistically significant.

Results

Oral administration of LCR35 reduces inflammatory responses in AD mice induced by repeated application of TMA

Repeated topical exposure to TMA is an established mouse model of AD (Schneider *et al.* 2009). Typical photographs of each group on the next day after the last challenge are shown in Fig. 2A. Repeated topical application of TMA to mice developed AD-like skin lesions, whereas vehicle application in NOR group did not induce any change in normal ear appearance. Erythema and edema were observed from day 7-8 on TMA-treated ears and became progressively worse with time (data not shown). At day 15, these ears showed erythema, edema, erosion and dryness (Fig. 2A, compare NOR and TMA). However, despite ongoing challenges with TMA, oral administration of LCR35 during the entire period of TMA application dose-dependently reduced development of the skin lesions (Fig. 2A, see LCR-25 and LCR250). Edema expressing the severity of inflammatory process was next determined by measuring ear thickness. Ear thickness was also significantly increased with repeated topical application of TMA. However, LCR35-treated mice showed a dose-dependent reduction (up to 12.3 %) in TMA-induced ear thickening (Fig. 2B). Ear thickness in the NOR group did not change during the experimental periods. Weight of draining lymph node was measured to examine whether topical exposure to TMA provokes immune activation in mice. TMA treatment resulted in elevation of lymph node weight in mice compared with vehicle treatment. Oral administration of LCR35 significantly inhibited an increase in lymph node weight to a maximum decreased of 28.3 % at a dose of 2.5×10^8 CFU kg⁻¹ (Fig. 2C).

Oral administration of LCR35 inhibits itching response in AD mice induced by repeated application of TMA

Itching is the most severe problem in AD. Because the frequent rubbing and scratching reflects itching, we analyzed the degree of itching by the frequency of scratching behavior to assess the effect of LCR35 on scratching. Significantly frequent scratching behavior was elicited by repeated challenge with TMA. The frequencies of scratching episodes in mice corresponded to the severity of skin lesions caused by repeated

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applications of TMA. The LCR35 at dose of 2.5×10^8 CFU kg⁻¹ inhibited the scratching behavior episode by 30 % compared with TMA group (Fig. 3A). Skin dryness is an important non-allergic factor involved in the development of itching associated with AD. The dermal water content at day 15 is shown in Fig. 3B. As expected, skin hydration declined significantly in TMA-treated mice. The water content of the skin was 9.6 ± 0.7 AU in the TMA-treated mice, compared with 26.0 ± 1.4 AU in the NOR group. LCR35 significantly suppressed skin dehydration induced by TMA in a dose-dependent manner. The level of histamine, one of itching-related factors, was also significantly increased in serum of TMA-treated mice (Fig. 3 C). LCR-25 and LCR-250 groups exhibited a dose-dependent reduction of 19.4 % and 25.9 % in histamine level compared with TMA group; the difference is statistically significant in LCR-250 group but not in LCR-25 (Fig. 3 C).

Oral administration of LCR35 suppresses the total IgE and cytokines in serum and the balance of Th1/Th2 in AD mice induced by repeated application of TMA

An increase in serum total IgE levels, a major feature of atopic disease, reflects a Th1 to Th2 switch (Elghazali *et al.* 1997). As shown in Fig. 4A, serum IgE level was significantly increased after repeated exposure of mice skin to TMA, but oral administration of LCR35 dose-dependently inhibited this increase of total IgE level in serum.

IFN- γ and IL-4 are representative of Th1 and Th2 cytokines, respectively. The TMA group exhibited higher levels of INF- γ produced by Th1 cells than the NOR group. LCR35 slightly lowered the levels in TMA-treated mice, but it was not statistically significant (Fig. 4B). IL-4 levels produced by Th2 cells were also much greater in the TMA group than the NOR group, whereas only LCR35 at a dose of 2.5×10^8 CFU kg⁻¹ significantly suppressed the increase in TMA-treated mice (Fig. 4C). The ratio of IFN- γ to IL-4, an index for Th1/Th2 balance, was significantly decreased in TMA group than normal control, whereas administration of LCR35 to TMA-treated mice led to a dose-dependent increase in the ratio, but only LCR-250 statistically significantly increased the ratio (Fig. 4D).

Oral administration of LCR35 inhibits cutaneous cytokine production in AD mice induced by repeated application of TMA

To determine whether LCR35 is able to suppress cutaneous TNF- α and IL-4 expression in AD mice induced by repeated application of TMA, mRNA expression of TNF- α and IL-4 were also measured in the inflamed dorsal skin lesions using RT-PCR analysis (Fig. 5). The expression of both TNF- α and IL-4 increased remarkably in

the ear skin tissues of TMA-treated mice than those of the normal controls. LCR35, however, significantly inhibited the expressions of these cytokines in a dose-dependent manner by upto 36.8 %, and 31.5 %, respectively.

LCR35 modifies relative abundance of the gut microbiota

The relative abundance of each bacterial group in fecal microbiota was assessed by quantitative real-time PCR. TMA treatment changed significantly the population levels of dominant groups in gut microbiota. The major changes were a lower abundance of *Bifidobacterium* and *Lactobacilli* and a higher proportion of *Clostridium coccoides* group in TMA-treated mice compared with the NOR group (Fig. 6). But, there were no differences in the relative abundance of *Clostridium leptum* group and *Enterobacterium* spp. in AD mice induced by repeated application of TMA. LCR administration for 2 weeks resulted in a significant increase in the relative quantities of *Lactobacilli*, and *Bacteroides fragilis* group and a marked decrease in those of *Clostridium coccoides* group, compared with TMA group, while there was no significant differences in *Clostridium leptum* group and *Enterobacteria*. The relative level of *Bifidobacterium* was clearly higher in the LCR-250 group, but the difference was not statistically significant.

As shown the above, the relative abundance of *Lactobacilli* significantly increased after administering LCR35 for 2 weeks compared with TMA group (Fig. 7). Importantly, reduced quantity of *L. rhamnosus* caused by TMA treatment was greatly increased by LCR35 administration and even much higher than those of the normal mice, confirming the presence of LCR35 in the intestine. Furthermore, *L. johnsonii* also increased significantly after administering LCR35 for 2 weeks compared with TMA group.

Discussion

The representative symptoms of AD are cutaneous inflammation and skin barrier dysfunction, which result in dry itchy skin. Itch-evoked scratching results in skin barrier defects and inflammation exacerbation, both of which eventually increase itching (Wahlgren 1999). Although the molecular mechanisms underlying the AD pathogenesis still remain unclear, accumulating data suggests that that gut microbiota interact dynamically with immune system, playing a crucial role in the immune response (Iweala and Nagler 2006) and subsequently contributing to the pathogenesis of immune disease such as AD in humans (Round and Mazmanian 2009) and even in animal models (Mader *et al.* 2010). Changes in gut microbiota were also observed in TMA-induced AD

model used in this study; a lower abundance of *Bifidobacterium* and *Lactobacilli* and a higher proportion of *Clostridium coccoides* group in TMA-treated mice compared with the NOR group. This result is in correspondence with another study which state that oxazolone-induced skin inflammation itself likely induces a pathophysiologic response that alters the composition of the gastrointestinal microbiota (Lundberg *et al.* 2012). It would appear from these that there is a close link between the development of hapten-induced AD-like dermatitis and the change in gut microbiota.

These results support the hypothesis that modulation of the gut microbiota may prevent or even treat allergic diseases. Hence, probiotics likely to modulate the gut microbiota have emerged recently as potential therapeutic alternatives in the prevention and/or treatment of immune diseases such as AD. Indeed, the amelioration of AD progression after treatment with probiotics such as *Bifidobacterium* and *Lactobacillus* has been demonstrated in both human and animal model studies (Wakabayashi *et al.* 2008; Inoue *et al.* 2009). Since there are, however, some controversial results (Brouwer *et al.* 2006; Gruber *et al.* 2007), the influences of probiotics on prevention and/or management of AD requires further investigation.

LCR35 is a representative probiotic strain that is commercially available as health functional food for more than 60 years. In several human studies, the oral administration of LCR35 improves gastrointestinal diseases, including irritable bowel syndrome (Dapoigny *et al.* 2012) and chronic constipation (Bu *et al.* 2007). However, while the effects of many other probiotics on AD has been well researched, there has been little research done on the effects of LCR35 on AD. LCR35 has been demonstrated in this study to significantly inhibit skin inflammation and scratching behavior induced by TMA treatment in mice, suggesting that LCR35 may exert anti-allergic effect by reducing scratching behavior and suppressing the aggravation of skin lesions. These results were similar to previous report showed the beneficial effect of LCR35 on the manifestations of AD in children (Passeron *et al.* 2006).

Although not fully understood, it is widely accepted that the Th1/Th2 imbalance and consequent increase in IgE synthesis plays an important role in the pathogenesis of AD. The beneficial effects of LCR35 on TMA-induced AD-like symptoms observed in this study may have been attributed to the adjustment of the Th1/Th2 imbalance and the decrease in plasma IgE levels. Unlike LCR35, some probiotic bacteria strains having a positive effect on allergic diseases, such as allergic rhinitis and AD improve the subjective symptoms without changes in the immunological parameters including Th1/Th2 imbalance and IgE levels (Ishida *et al.* 2005; Sawada *et al.* 2007). This difference is due to specific properties and various ability of each probiotic strain. Probiotic effects on certain diseases or conditions, therefore, cannot be generalized and the characteristic

of each probiotic strain needs to be investigated with regard to specific disease states. This study shows that LCR35 can influence non-gut inflammation, AD, that is not gastrointestinal disease such as constipation and diarrhea. The findings of this study are concordance with those reported in other recent studies (Kim *et al.* 2012; Kim *et al.* 2013) and provide additional support for the preventive effects of LCR35 against allergic disease.

Although it is widely accepted that probiotics could modulate the balance of the gut microbiota and introduce beneficial functions to gut microbial communities, the relationship between probiotic strains and commensal gut bacteria remains uncertain as well. Administration of LCR35 affected the gut microbiota of TMA-treated mice. *Lactobacilli* and *Bacteroides fragilis* group were significantly increased far above the level of NOR group, while the *Clostridium coccoides* group was significantly decreased. *Bifidobacteria* and *Enterococcus* which were decreased by TMA treatment showed a tendency to increase by LCR35 intake, but no statistical significance was achieved. However, since our data provide a potential of LCR35 to change only some microbiota, it remains unclear how other microbiota are affected by administration of LCR35. One of common mechanisms of probiotic action is reducing pH of the colon, resulting in survival of commensal organisms that prefer acidic conditions and the reduction of potentially harmful bacteria. As, therefore, a reduced pH of the colon is expected due to a survival of LCR35 in gut, it seems natural that fecal *Lactobacilli*, *Bifidobacteria* and *Enterococcus* increased after LCR35 administration, whereas the *C. coccoides* group decreased. The increase of *B. fragilis* after LCR35 intake, meanwhile, is very interesting.

The *B. fragilis* group has been suggested to be associated with the modulation of host immunity. Previous studies showed that *B. fragilis* has anti-inflammatory effects, making it effective in treating symptoms of inflammatory bowel disease and multiple sclerosis in mice (Mazmanian *et al.* 2008; Ochoa-Reparaz *et al.* 2010). In addition to this effect, another study reported that there was a delay in fecal colonization of *B. fragilis* in cesarean delivered infants known to be at higher risk of developing AD (Gronlund *et al.* 1999). Polysaccharide A (PSA), the capsular polysaccharide produced by *B. fragilis*, plays an essential role in the mechanisms of *B. fragilis*-mediated immune modulation. For example, CD4⁺ T cells from germ-free mice produced more of the Th2 cytokine IL-4 than cells from conventionally colonized mice, but purified PSA as well as monocolonization with *B. fragilis* decreased production of IL-4 and restored production of IFN- γ to levels in conventionally colonized mice; PSA directs the development of CD4⁺ T cells and eventually corrects immunologic defects such as impaired systemic CD4⁺ T cell maturation, aberrant Th1/Th2 lineage differentiation, and defective lymphoid-organ development (Mazmanian *et al.* 2005). Therefore, the beneficial effects of LCR35 in developing TMA-induced AD-like symptoms may be mediated by the relatively increased

B. fragilis group after LCR35 administration.

In summary, this study provides evidence that LCR35 is efficient on AD-like symptoms via the adjustment of the Th1/Th2 balance and, at least in part, the improvement of its intestinal microbial balance in repeated TMA-treated mice, suggesting that LCR35 could be considered as a potential novel therapeutic agent for the prevention and/or treatment of AD.

Conflict of Interest

The authors state no conflict of interest.

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Target		Sequence (5' to 3')	Annealing temperature (°C)	References
<i>Bifidobacterium</i> spp.	forward	GATTCTGGCTCAGGATGAACGC	50	(Gueimond <i>et al.</i> 2004)
	reverse	CTGATAGGACGCGACCCCAT		
<i>Lactobacillus</i> spp.	forward	GCAGCAGTAGGGAATCTT	55	(Castillo <i>et al.</i> 2006)
	reverse	GCATTYCACCGCTACACATG		
<i>Enterococcus</i> spp.	forward	CCCTTATTGTTAGTTGCCATCATT	50	(Rinttila <i>et al.</i> 2004)
	reverse	ACTCGTTGTACTTCCCATTGT		
<i>Clostridium coccooides</i> group	forward	AAATGACGGTACCTGACTAA	50	(Gomez-Hurtado <i>et al.</i> 2011)
	reverse	CTTTGAGTTTCAATCTTGCGAA		
<i>Clostridium leptum</i> subgroup	forward	GCACAAGCAGTGGAGT	61	(Gomez-Hurtado <i>et al.</i> 2011)
	reverse	CTTCCTCCGTTTTGTCAA		
<i>Bacteroides fragilis</i> group	forward	ATAGCCTTTCGAAAGRAAGAT	58	(Gomez-Hurtado <i>et al.</i> 2011)
	reverse	CCAGTATCAACTGCAATTTTA		
<i>Enterobacterium</i> spp.	forward	CATTGACGTTACCCGCAGAAGAAGC	50	(Gomez-Hurtado <i>et al.</i> 2011)
	reverse	CTCTACGAGACTCAAGCTTGC		
<i>Lactobacillus rhamnosus</i>	forward	GTGCTTGCATCTTGATTTAATTTT	50	(Furet <i>et al.</i> 2004)
	reverse	TGCGGTTCTTGATCTATGCG		
<i>Lactobacillus acidophilus</i>	forward	AGAGGTAGTAAGTGGCCTTTA	58	(Malinen <i>et al.</i> 2003)
	reverse	GCGGAAACCTCCCAACA		
<i>Lactobacillus johnsonii</i>	forward	CACTAGACGCATGTCTAGAG	60	(Furet <i>et al.</i> 2004)
	reverse	AGTCTCTCAACTCGGCTATG		
Total bacteria	forward	TGGCTCAGGACGAACGCTGGCGGC	66	(Gomez-Hurtado <i>et al.</i> 2011)
	reverse	CCTACTGCTGCCTCCCGTAGGAGT		

TABLE 1. Primer sequences for real-time PCR analysis of gut microbiota

Figure legends

Fig. 1. Experimental schedule for induction of AD-like skin lesions and oral administration of LCR35 in mice.

Mice were sensitized on the abdomen with 5 % TMA 5 days before the first challenge, received 5 % TMA and repeatedly challenged on the ear with 2 % TMA every day until day 14. LCR35 was orally administered once a day for 14 days starting from the day of the sensitization with TMA, as described in the materials and methods. () Sensitization with 5% TMA on dorsal skin; () Second sensitization with 5% TMA to both ears; () Challenge with 2% TMA to both ears; (★) Scratching behavior test; (↑) Ear thickness, skin water content, and sampling.

Fig. 2. Effect of LCR35 on inflammation parameters in AD mice induced by repeated application of TMA.

Clinical features of skin (A), ear thickness (B), and weight of draining (auricular) lymph node weight (C) were observed as inflammation parameters. On a day after the last challenge, immediately after photographs of the representative site were taken, ear thickness and lymph node weight were measured, as described in the materials and methods. NOR, normal control; TMA, TMA-induced AD; LCR-25, 2.5×10^7 CFU kg⁻¹ LCR35 administered; LCR-250, 2.5×10^8 CFU kg⁻¹ LCR35 administered. Data represent mean $\pm$ SEM of ten mice. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.005$ vs. TMA; # $P < 0.05$ vs. LCR-25.

Fig. 3. Effect of LCR35 on itching responses induced by repeated TMA challenge in mice.

Irritant-induced scratching behavior (A), skin water content (B) and serum histamine (C) were measured. Scratching behavior was measured for 30 min after the last application of TMA, and the number of scratching episodes with hind paws was recorded by digital video and counted twice. Skin hydration was measured on the next day of the last challenge by determining epidermal water content using electrical capacitance measurement. Serum was also collected at day 15 and serum histamine levels were analyzed by ELISA. NOR, normal control; TMA, TMA-induced AD; LCR-25, 2.5×10^7 CFU kg⁻¹ LCR35 administered; LCR-250, 2.5×10^8 CFU kg⁻¹ LCR35 administered. Each value represents the mean $\pm$ SEM of ten mice. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.005$ vs. TMA; # $P < 0.05$ vs. LCR-25.

Fig. 4. Effect of LCR35 on serum levels of IgE, IFN- γ and IL-4 and Th1/Th2 balance in AD mice induced by repeated application of TMA.

After the completion of all treatments, blood samples were collected, and the

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serum was separated. The concentration of IgE (A), IFN- γ (B) and IL-4 (C) was quantified by ELISA. Th1/Th2 balance (D) was calculated from the ratio of IFN- γ concentration to IL-4. NOR, normal control; TMA, TMA-induced AD; LCR-25, 2.5×10^7 CFU kg⁻¹ LCR35 administered; LCR-250, 2.5×10^8 CFU kg⁻¹ LCR35 administered. Each value represents the mean $\pm$ SEM of ten mice. * $P < 0.05$, ** $P < 0.001$ and *** $P < 0.005$ vs. TMA; ### $P < 0.005$ vs. LCR-25.

Fig. 5. Effects of LCR35 on the mRNA expression of TNF- α and IL-4 in the lesional ear induced by repeated TMA challenge. The mRNA levels of TNF- α and IL-4 in the ear homogenates at 24 h after the final TMA challenge were measured by RT-PCR. NOR, normal control; TMA, TMA-induced AD; LCR-25, 2.5×10^7 CFU kg⁻¹ LCR35 administered; LCR-250, 2.5×10^8 CFU kg⁻¹ LCR35 administered. Each value represents the mean $\pm$ SEM of ten mice. * $P < 0.05$, ** $P < 0.001$ and *** $P < 0.005$ vs. TMA; # $P < 0.05$ vs. LCR-25.

Fig. 6. Effect of LCR35 on changes in fecal microbiota induced by repeated TMA challenge in mice. Relative abundance of each microbiota in fecal samples were determined by qPCR and quantitated as fold changes by the $2^{-\Delta\Delta C_t}$ method. Values are represented as the mean $\pm$ SEM from ten samples for each group. * $P < 0.05$, ** $P < 0.001$ and *** $P < 0.005$ vs. TMA; ### $P < 0.001$ vs. LCR-25.

Fig. 7. Effect of LCR35 on levels of the *Lactobacillus* group and some species in AD mice induced by repeated application of TMA. The levels of *Lactobacillus* group, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Lactobacillus johnsonii* were assessed by qPCR and quantitated as fold changes by the $2^{-\Delta\Delta C_t}$ method. Values are represented as the mean $\pm$ SEM from ten samples for each group. * $P < 0.05$ vs. TMA.

Fig. 1.

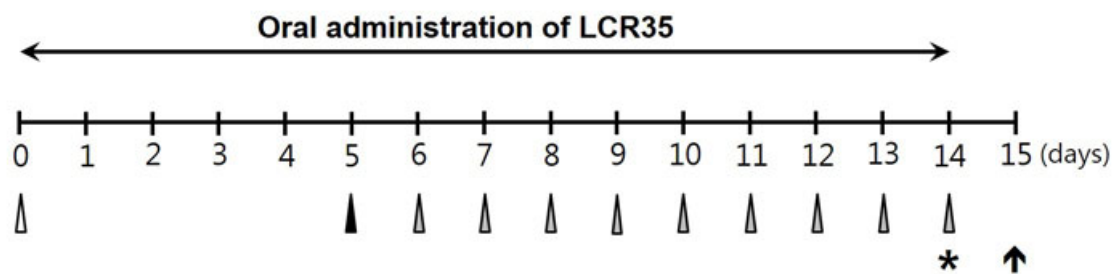


Fig. 2.

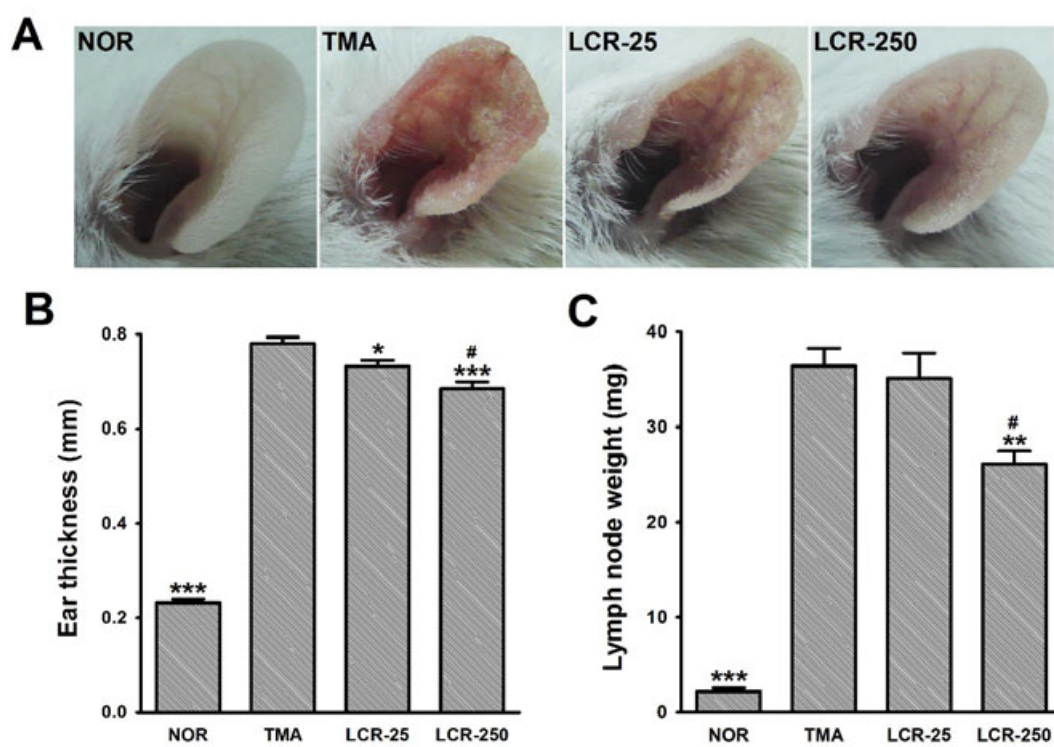


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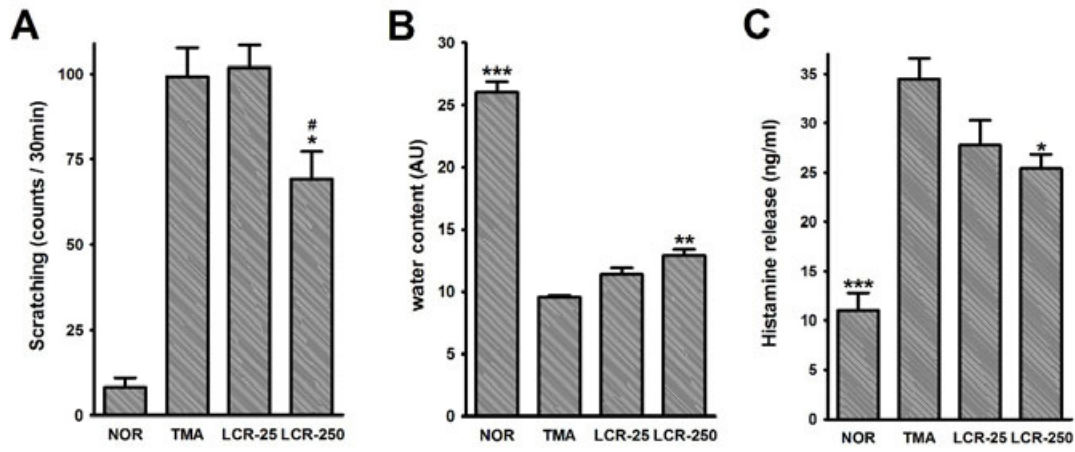


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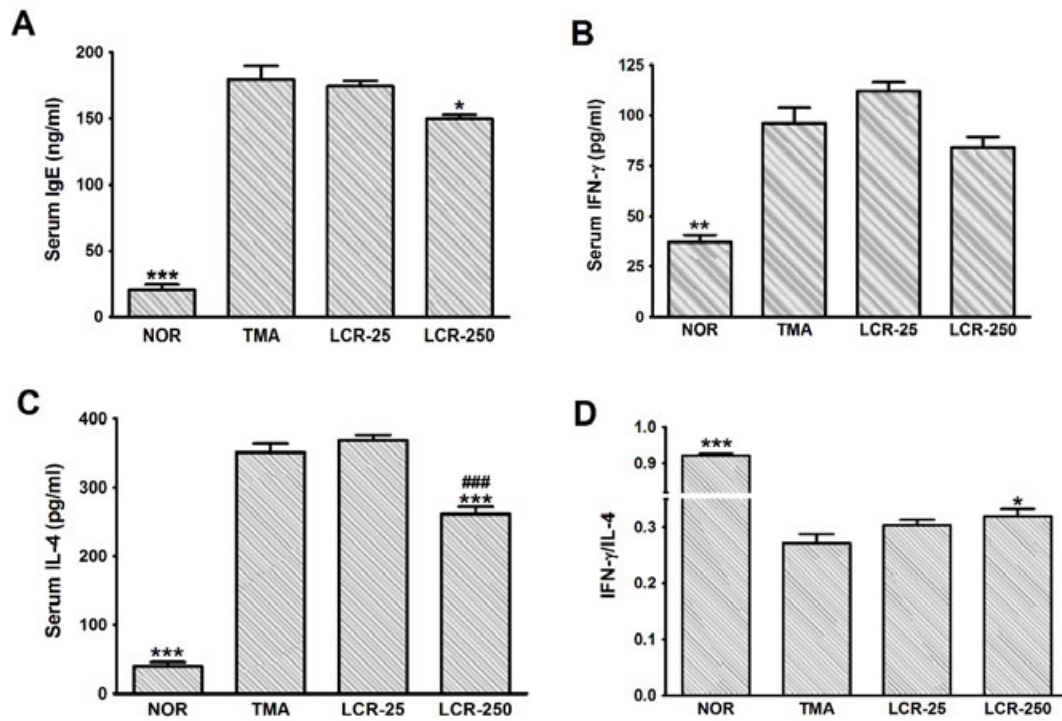


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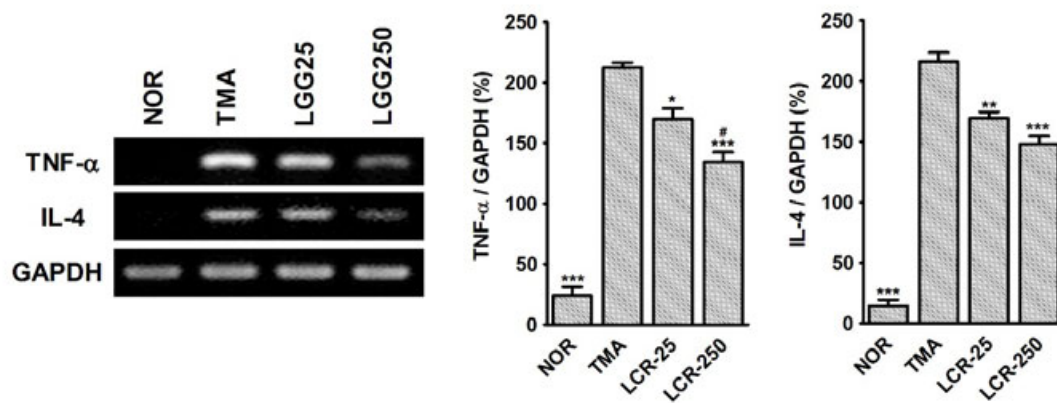


Fig. 6.

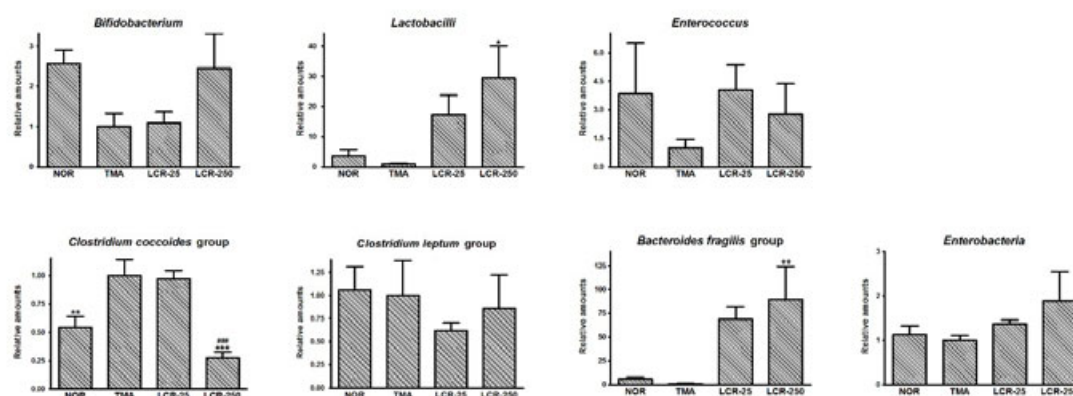


Fig. 7.

