Oral Intake of *Lactobacillus helveticus* NS8 Alleviates Ovalbumin-Induced Atopic Dermatitis in SKH-1 Hairless Mice

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Abstract Atopic dermatitis (AD) is the most common chronic inflammatory skin disease and is driven by strong type 2 immune responses. *Lactobacillus helveticus* NS8 (NS8), a probiotic strain isolated from Mongolian koumiss, has anti-inflammatory activities. Here, we evaluated the therapeutic potential of NS8 on AD-like skin lesions by using SKH-1 hairless mice that underwent three cycles of epicutaneous sensitization (EC) with ovalbumin (OVA). NS8 (5×10^8 CFU/day) was orally administered to mice from 2 weeks before the first sensitization until the end of the study. NS8 attenuated the symptoms and pathological changes in the skin of AD mice. For example, NS8 reduced epidermal and dermal thickening and significantly restrained the infiltration of mast cells, eosinophils, and CD4⁺ T cells into the dermis. By analysing the Th1/Th2 cytokines produced in skin lesions, we found that NS8

significantly suppressed the expression of IL-4, IL-5, and IL-13 ($P < 0.05$), while it had no discernible effect on the expression of IFN- γ . Systemically, NS-8 reduced the total IgE and OVA-specific IgE levels in serum ($P < 0.05$). Our study demonstrates that oral administration of *L. helveticus* NS8 effectively alleviates AD severity in mice by suppressing the Th2 immune response. NS8 may be a promising candidate for prophylactic and therapeutic treatments of allergic diseases, such as AD.

Keywords Atopic dermatitis · Probiotic · *Lactobacillus helveticus* · Th1/Th2 balance · IgE

Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease with a complex aetiology that usually starts in early infancy and is associated with an increased tendency to develop asthma and allergic rhinitis later in life, but AD is also found in a substantial number of adults [1]. Genetic susceptibility and environmental changes are thought to contribute to the increasing prevalence of AD [2]. Accumulating evidence has suggested that impaired skin barriers, a robust Th2-skewed response, defects in innate immunity, and an altered microbiome are critical biological features of AD [3]. For patients with AD, topical corticosteroids (TCS) and topical calcineurin inhibitors (TCIs) are mainstays of anti-inflammatory treatments. However, skin local or systematic side effects from these pharmacological topical therapies have been reported [4].

Epidemiological and experimental evidence has supported the theory of the “hygiene hypothesis”: early interrupted microbial exposure could favour less mature Th2-mediated immune systems and therefore lead to the

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development of atopic sensitization [5, 6]. Probiotics are increasingly thought to have many health-promoting functions [7, 8]. Many studies have explored the potential of probiotics in the prevention of AD and have proposed that these bacterial products might induce a Th1 immune response instead of a Th2 immune response and inhibit the production of the allergy-associated IgE antibody [9, 10]. In 2015, the World Allergy Organization (WAO) recommended the use of probiotics by pregnant and lactating women and their breastfed infants to prevent the development of AD [11]. Even so, evidence of the effectiveness of probiotics on AD treatment remains inconclusive. The specific probiotic strain used, time of administration, duration of exposure, dosage, and other factors could influence the intervention effect. Additionally, using probiotic combinations or mixing probiotics with prebiotics or a hydrolysed whey formula leads to inconsistent results between studies [12]. The discovery of a specific probiotic strain with preventive or therapeutic effects on AD is still needed.

Lactobacillus helveticus NS8, isolated from Mongolian koumiss, has shown anti-inflammatory activities, including elevating the production of interleukin (IL)-10 in monocytes [13]. In this study, by establishing a hairless mouse model with epicutaneous sensitization (EC) with ovalbumin (OVA), we investigated whether NS8 treatment could alleviate OVA-induced AD severity and the pathologic parameters as well as modulate the Th1/Th2 balance in skin lesions. Therefore, we set out to determine the efficacy of probiotics for the treatment of AD.

Materials and Methods

Preparation of Lactobacilli Suspension

Lactobacillus helveticus NS8, isolated from koumiss, was stored at -80°C in de Man, Rogosa and Sharpe (MRS) medium supplemented with 15% (v/v) glycerol [13]. For preparation of NS8 suspensions, bacterial cells were revitalized and then cultured in fresh MRS broth at 37°C for 48 h. Cell pellets were obtained by centrifugation at $3000\times g$ for 10 min at room temperature and resuspended in sterile saline. The concentration of lactobacilli was adjusted to approximately 5×10^9 CFU/mL.

OVA-Induced AD Mouse Models and Administration of NS8

Female SKH-1 hairless mice (6 weeks of age, ten per group) were obtained from Laboratory Animal Center of Hangzhou Normal University. Mice were fed a standard diet and tap water and kept in a specific-pathogen-free

environment at 23°C with a 12-h light/12-h dark cycle. The protocol of OVA-induced skin sensitization was performed following the method of Kim et al. [14] with slight modification. Briefly, mice were epicutaneously immunized with OVA patches for a total of three 1-week cycles at 2-week intervals. OVA ($100\text{ }\mu\text{g}$ in $100\text{ }\mu\text{L}$ of PBS, grade V, Sigma, St Louis, MO, USA) was contained in a $1 \times 1\text{ cm}$ patch of sterile gauze, which was secured to the dorsum skin with Tegaderm™ dressing (3M, St Paul, MN, USA). The OVA patches were replaced twice per week. The negative control group received patches containing $100\text{ }\mu\text{L}$ of PBS by the same procedure. Mice from the treatment group were daily intragastrically administered $100\text{ }\mu\text{L}$ of a NS8 suspension (5×10^8 CFU) from 2 weeks before the first sensitization until the endpoint of the study (Fig. 1a). The Animal Care and Ethics Committee at Hangzhou Normal University approved all of the animal experiments in our study.

AD Severity Scoring

The severity of dermatitis was assessed macroscopically according to the symptoms of itching, erythema, edema, excoriation, and dryness in dorsal lesions as described by Taniguchi et al. [15]. Individually, AD was graded as 0 (no symptoms), 1 (mild), 2 (moderate), and 3 (severe). Scoring was performed by two different people who did not know the grouping.

Histological Analyses

Histological analysis was performed on haematoxylin/eosin (H&E)-stained skin samples fixed in 4% paraformaldehyde and embedded in paraffin. Mast cells in the skin were stained by using a toluidine blue staining kit and following the protocol supplied by the manufacturer (Biogenex, Fremont, CA, USA). Slide images were captured by a Panoramic Scanner and analysed using a Panoramic Viewer (PerkinElmer, Waltham, MA, USA). The thickness of the epidermis and dermis and numbers of eosinophils and mast cells were quantified by selecting three different microscopic high-power fields (HPF, $400\times$) per sample. Two slides per mouse were examined.

Immunofluorescence Assay

To detect CD4-positive T-cells by immunofluorescence in OVA-sensitized skin, skin sections were incubated overnight with a primary anti-CD4 antibody (1:1000, Abcam, Cambridge, MA, USA). The next day, the slides were incubated for 1 h with an anti-rabbit secondary antibody conjugated with Alexa Fluor® 647 (Abcam). The cells were counterstained with DAPI (Sigma) before the cell

Fig. 1 Oral administration of NS8 alleviates AD symptoms in OVA-sensitized SKH-1 mice. **a** Scheme of the AD mouse model. NS8 was orally administered to SKH-1 mice 2 weeks before the first

sensitization until the endpoint. Mice were epicutaneously sensitized with OVA patches for three rounds, as shown. **b** Scoring of AD symptoms. * $P < 0.05$

morphology was captured by a fluorescence microscope (Zeiss, Oberkochen, Germany).

Quantitative Real-Time PCR

Total RNA of skin homogenates was extracted with TRIzol reagent (Invitrogen, USA). Reverse transcription was performed with a cDNA Reverse Transcription Kit (TaKaRa, China) according to the manufacturer's instructions. Quantitative real-time PCR was carried out in an Applied Biosystems 7300 machine (Life Technologies, USA). The reaction mixture was performed with SYBR® *Premix Ex Taq*TM (TaKaRa, Dalian, China) according to the manufacturer's protocols. The primer sequences were as follows: *Il4*, forward 5'gggtcacaacccagctagt3' and reverse 5' gcc-gatgatctctctcaagtgt3'; *Il5*, forward 5'ctctgttgacaagcaatgacag3' and reverse 5'tcttcagtattgtctagccctg3'; *Il13*, forward 5'cctggctcttgcctgcct3' and reverse 5'ggctctgtgtgatgtgtc-ca3'; *Ifng*, forward 5'acagcaaggcgaaaaggatg3' and reverse 5'tggtggaccactcggatga3'; and *Gapdh*, forward 5'aggtcggt-gtgaacggatttg3' and reverse 5'ggggtcgttgatggcaaca3'. For the relative comparison of the mRNA expression levels, the data were analysed with the $\Delta\Delta C_t$ method with normalization to GAPDH.

Quantitation of Immunoglobulin by ELISA

Blood was collected from each animal by cardiac puncture, and the serum supernatant was separated from clotted blood. For the measurement of total IgE and specific OVA-IgE in serum, a mouse anti-IgE ELISA kit and mouse anti-OVA IgE ELISA kit were used, respectively, according to the manufacturer's protocols (Cayman, Ann Arbor, MI, USA).

Statistical Analysis

Comparisons between groups were conducted using one-way ANOVA. Data are expressed as the mean $\pm$ SEM. A P value of < 0.05 was considered significant. Each experiment was repeated at least three times.

Results and Discussion

NS8 Alleviates AD-Like Skin Symptoms

Macroscopical assessment of skin lesions was first performed to evaluate the effects of *L. helveticus* NS8 against atopic dermatitis. Compared with mice treated with OVA only, oral administration of NS8 effectively reduced the severity of OVA-induced AD. For example, these mice showed less scratching behaviour and less erythema, which manifested as a lower AD score ($P < 0.05$, Fig. 1b). Microscopically, SKH-1 hairless mice sensitized with OVA had obvious epidermal and dermal thickening and excessive infiltration of inflammatory cells into the dermis, whereas the NS8 treatment significantly reduced the epidermal thickness from $61.3 \pm 6.1 \mu\text{m}$ to $25.8 \pm 1.1 \mu\text{m}$ ($P < 0.001$), dermal thickness from $250.3 \pm 52.4 \mu\text{m}$ to $150.8 \pm 33.2 \mu\text{m}$ ($P < 0.01$), and numbers of eosinophils and mast cells in the dermis ($P < 0.01$, Fig. 2). AD was also characterized by dermal infiltration of CD4^+ T cells in response to the allergenic stimulation [16]. Thus, activation of CD4^+ T cells in skin lesions was immunohistochemically detected. Dermal infiltration of CD4^+ T cells in OVA-sensitized skin was observably aggravated compared with normal mice, but it was significantly suppressed by the NS8 treatment (Fig. 3).

Fig. 2 NS8 attenuates OVA-sensitized skin histological lesions. **a** Representative photomicrographs of H&E-stained (upper panels) and toluidine blue-stained skin sections (lower panels). Scale bars: 100 μ m. **b** Epidermal thickness, dermal thickness, and numbers per high-power field (HPF, $\times 400$) of eosinophils and mast cells in mice skin. Data are mean $\pm$ SEM ($n = 10$ per group). ** $P < 0.01$, *** $P < 0.001$ (colour figure online)

NS8 Downregulates Th2 Cytokines

In recent years, some strains of probiotics have been reported to alleviate the severity of AD-like lesions in murine models [17, 18]. Although the models differ in the sensitization antigens and administration routes, the anti-allergic effects of probiotics mostly resulted from suppressing the excessive Th2 polarization, one of the hallmark immune responses of AD. Th2 cytokines (IL-4, IL-5, IL-13, and IL-31) can downregulate terminal differentiation genes and tight junction products, contributing to the skin barrier defect and subsequent *Staphylococcus aureus*

infection in patients with AD [19]. Therefore, we measured mRNA expression of the Th2 cytokines IL-4, IL-5 and IL-13 and Th1 cytokine IFN- γ in skin lesions by quantitative real-time PCR. Compared with the blank group, the Th2 cytokines IL-4, IL-5, and IL-13 but not the Th1 cytokine IFN- γ were increased in the AD group. NS8 treatment significantly suppressed the Th2 cytokines levels in the skin ($P < 0.05$), while it had no impact on the expression of IFN- γ (Fig. 4). These results suggest that NS8 lactobacilli could alleviate the enhanced Th2 response to OVA sensitization.

Fig. 3 NS8 suppresses infiltration of CD4⁺ cells in dermis. CD4⁺ T cells in skin specimens were identified by an immunofluorescence assay. Pictures are representative of three experiments

Fig. 4 NS8 suppresses the expression of Th2 cytokines. IL-4, IL-5, IL-13, and IFN-γ mRNAs were detected in skin lesions by real-time PCR. The values are expressed as the mean $\pm$ SEM (n = 6–7 per group). *P < 0.05

AD shows a complex interplay between the intrinsic immune system and skin barrier. Here, we demonstrated that *L. helveticus* NS8 suppressed the Th2-prone immune

response. However, different probiotic species vary in their ability to stimulate immune cytokines. Some strains can elicit Th1 cytokine production, while others can stimulate a

Fig. 5 NS8 reduces IgE production in OVA-sensitized mice serum. Serum levels of total IgE and OVA-specific IgE were determined by ELISA. Each value represents the mean $\pm$ SEM ($n = 8$). $*P < 0.05$

Th2 response or even a mixed Th1/Th2 response [20]. On the other hand, probiotics have strain-specific effects on the gut microbiota that are closely associated with the host immune system, which may explain why certain strains have better effects in inhibiting allergic responses [21]. Profiling specific probiotic strains' immune-stimulating effects is necessary for probiotic use in the management of atopy.

NS8 Attenuates Systemic Immune Responses

IgE antibodies are key mediators of allergic diseases, and their production is highly dependent on CD4⁺ T cells and Th2 cytokines [22]. To further investigate the effect of NS8 on the systemic immune response of AD, the total IgE and OVA-specific IgE antibodies in serum were measured by ELISA. Both IgE antibodies were upregulated in the AD group compared with the blank group, whereas the serum total IgE and OVA-IgE antibodies were significantly reduced by NS8 treatment ($P < 0.05$, Fig. 5). These results suggest that NS8 effectively suppressed the severe allergic response both locally and systemically. In our previous study, NS8 suppressed the level of IL-12 and increased the production of IL-10 in peripheral blood mononuclear cells (PBMCs) [13]. It seems that NS8 improves the host immune homeostasis and therefore benefits the allergic status of the body, including the skin. The biological pathways and mechanisms that are triggered by NS8 in the treatment of AD require further investigation.

Live bacteria present preventive and therapeutic effects on AD lesions, and heat-killed probiotic cells were also reported to alleviate allergic responses in animal models [23]. For example, oral administration of heat-killed *Lactobacillus brevis* ameliorated the symptoms of dust mite-induced AD in NC/Nga mice by enhancing Th1-prone immunity [24]. Investigations on *Lactobacillus rhamnosus* GG revealed that intracellular or cell wall components of

LGG, such as lipoteichoic acid, exopolysaccharides, and hydrolytic peptidoglycan, potentially cause immunomodulatory effects [25]. The use of non-viable probiotics will make such products safer and more convenient for local application on the skin. Blanchet-Rethore et al. [26] conducted a clinical trial by applying a lotion containing heat-treated *Lactobacillus johnsonii* and found that this treatment controlled *S. aureus* colonization in skin lesions of patients with AD. In our previous study, topical use of a NS8-femented supernatant improved the skin barrier function by reducing transepidermal water loss in mice [27]. Our future studies will try to identify the active components of NS8 that can be used to treat skin allergic diseases or confer protective effects to the skin.

Conclusion

Our study demonstrates that oral administration of *L. helveticus* NS8 significantly attenuated the symptoms of AD, such as restrained scratching behaviour, decreased epidermal thickening, and suppressed infiltration of mast cells and eosinophils, by downregulating the Th2 cytokines in skin lesions. Furthermore, the serum levels of the IgE and OVA-IgE antibodies were significantly reduced by the NS8 treatment, which were closely correlated with the modulatory effects of NS8 on the systematic immune response. These findings suggest that NS8 might exert a preventive or attenuating effect on allergic diseases, including AD.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no competing interests.

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