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Effect of *Enterococcus faecalis* EF-2001 on experimentally induced atopic eczema in mice

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Abstract Here, the effects of heat-killed *Enterococcus faecalis* EF-2001 (EF-2001) on atopic eczema (AE) were assessed. An AE model was established *in vivo* by repetitious topical exposure to 1-chloro-2,4-dinitrobenzene (CDNB) and dermatophagoidesfarinae extract (DFE) via application on each ear. Mice were administered EF-2001 orally for 4 weeks, dermal and epidermal ear thickness, mast cell infiltration of the ear tissue, and serum IgE and IgG2a levels were evaluated. Moreover, pathogenic cytokines levels of the ears, splenocytes, and cervical lymph nodes were determined. EF-2001 reduced AE symptoms grounded in the ear thickness, histopathological analysis, and serum IgE levels. Furthermore, EF-2001 attenuated mast cell infiltration in the ears and CDNB/DFE-induced various pathogenic cytokines levels of the ears, splenocytes and cervical lymph nodes. Thus, our data suggested that EF-2001 may have potential medicinal applications in the treatment of AE through its immunomodulatory properties.

Keywords: atopic eczema, heat-killed *Enterococcus faecalis* EF-2001, immunomodulatory properties, pathogenic cytokines

Introduction

The first colonizer in the human gastrointestinal (GI) tract, *Enterococcus faecalis*, is a facultative anaerobic gram-positive bacterium that has been shown to have immunostimulatory and immunoregulatory activities (1,2). Lots of probiotic bacteria have been investigated to act as agents for the prevention and treatment of atopic eczema (AE) by influencing the human immune system (3,4). Although therapeutic use of probiotics has been applied for more than a century, the safety of probiotics has not been definitively clarified; thus, many recent studies have focused on the application of nonviable microbial cell extracts or microorganisms (5). For example, heat-killed *E. faecalis*, derived from healthy human feces, has been investigated to improve immune system and antitumor activity (6,7). However, the effects of heat-killed *E. faecalis* on AE are not completely understood.

AE is a very common, refractory inflammatory skin disease (8) involving abnormalities in the balance between pro-inflammatory and regulatory cytokines (9). In the early stage of atopic skin inflammation, the infiltration mostly consists of interleukin (IL)-4-inducing Th2 cells, but the infiltration with a Th1 phenotype is generally observed in the chronic atopic lesions (10). Activation of each of the two T-helper type (Th) 2 (IL-4) and Th22 (IL-22) is a feature of AE, with some contribution of Th1 (interferon [INF]-γ) and Th17 (IL-17) elements. However, the molecular basis of the immunopathogenesis of AE remains elusive.

Therefore, the goal of this investigation was to determine the immunomodulatory effects of nonviable probiotic heat-killed *E. faecalis* EF-2001 (EF-2001) on AE to make clear the complicated pathogenic mechanisms of AE *in vivo* using a mouse model.

Materials and Methods

Animals Eight-week-old female BALB/c mice were obtained from Samtako Co., Ltd. (Osan, Korea) and dwelled under specific pathogen-free conditions (SPC). All experiments were approved by the Institutional Animal Care and Use Committee of Konkuk University (KU14011).

EF-2001 EF-2001 which was originally isolated from healthy human feces is a commercially available probiotic from Nihon Berumu Co., Ltd. (Tokyo, Japan). It was supplied as a heat-killed, dried powder. One gram of dried EF-2001 was consistent with 7.5×10^{12} colony-forming units (CFU) prior to being heat-killed.

Induction of AE lesions in the ear AE was induced in mice by repeated local exposure to 1-chloro-2,4-dinitrobenzene (CDNB) and dermatophagoidesfarinae extract (DFE) on the ears, as previously described (11). For induction of AE, the mice were randomly divided into four groups (control, AE only, EF-2001 only, and AE+EF-2001), and the both of earlobes surfaces were stripped with surgical tape (21N; Nichiban Co., Ltd., Tokyo, Japan) five times. After stripping, each ear was spread with 20 μL of 1% CDNB, followed by application of 20 μL of DFE (10 mg/mL) 4 days later. DFE or CDNB treatment was applied once a week alternately for 4 weeks. Animals received EF-2001 (17 mg/kg orally administered) throughout the 4 weeks of AE induction.

Earthickness was measured 24 h after CDNB or DFE application with a dial thickness gauge (A-1; Kori Seiki MFG, Co., Ltd., Tokyo, Japan). On days 14 and 28, blood samples were collected by orbital puncture. Plasma samples were prepared from the blood samples and stored at −70°C for next analysis. After blood collection, the ears were removed for histopathological analysis. On days 14 and 28 after the first induction, the serum levels of immunoglobulin (Ig)E and IgG2a were determined by using an IgE enzyme-linked immunoassay kit (Bethyl Laboratories Inc., Montgomery, TX, USA), according to the manufacturer's instructions.

Histological observations Excised ears were fixed with 4% of paraformaldehyde for 16 h and embedded in paraffin. The 6 μm of thin sections were stained with hematoxylin and eosin (H&E, baton Rouge, LA, USA). The thickness of the dermis and epidermis was determined under a microscope. For the investigation of mast cell infiltration, the skin sections of the ears were stained with toluidine blue, and the number of mast cells was measured in five randomly chosen fields of view.

Quantitative real-time polymerase chain reaction (qPCR) qPCR (Thermal Cycler Dice TP850; TaKaRa Bio Inc., Kusatsu, Japan) was performed by the manufacturer's protocol. Total RNA was separated from the ear tissues, splenocytes and cervical lymph nodes of each group. The qPCR conditions were similar to previously described experiments (11). Briefly, 2 μL of 100 ng cDNA, 1 μL of 0.4 μM sense and antisense primer solution, 12.5 μL of SYBR® Premix Ex Taq (TaKaRa Bio Inc.), and 9.5 μL of dH_2O were mixed to make a final 25 μL reaction mixture into the each reaction tube. The primers used for qPCR were as follows: mouse tumor necrosis factor-α (TNF-α) forward primer: AAGCCTGTAGCCCACGTCGTA, reverse primer: GGCA CCACTAGTTGGTTGTCTTTG; mouse IFN-γ forward primer: TCAAGTGG CATAGATGTGGAAGAA, reverse primer: TGGCTCTGCAGGATTTTCATG; mouse IL-4 forward primer: ACAGGAGAAGGGACGCCAT, reverse primer: GAAGCCGTACAGACGAGCTCA; mouse IL-17 forward primer: CCTACCAGACCAAGGTCAAC, reverse primer: AGGGGGTAATAAAGGG ATTG; mouse IL-22 forward primer: TCCCTCTGTCATCTGGGAAG, reverse primer: CTCGACCCTGAAAGTGAAGG; and mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward primer: GCACAGTC AAGGCCGAGAAT, reverse primer: GCCTTCTCCATGGTGGTGAA. The cycling conditions were as follows: 95°C for 10 s, followed by 40 cycles of 95°C for 5 s, 60°C for 30 s, 95°C for 15 s, 60°C for 30 s, and 95°C for 15 s. In each sample, the normalized mRNA expression value of the specific genes relative to the GAPDH were determined by the formula: relative mRNA expression=2−(specific gene ΔCt−GAPDH ΔCt), where Ct is the cycle threshold value.

Statistical analysis Statistical analysis of data was performed by the SAS statistical software (SAS Institute Inc., Cary, NC, USA). Multiple group data were analyzed with one-way analysis of variance (ANOVA). Post hoc multiple comparisons were made with the Dunnett's multiple range tests. All data were presented as the mean±standard deviation (SD) of comparative fold-changes. Data are representative of at least three independent experiments. Differences with p values of less than 0.05 were considered to be statistically significant.

Results and Discussion

Effect of EF-2001 on the thickness of ear tissue and histopathological observation Probiotic lactic acid bacteria including *E. faecalis*, produce acid end-products that dramatically affect epithelial cell functionality and viability. In some cases, epithelial cells are extremely sensitive to these acid end-products. Heat-killing probiotic bacteria have been used to minimize the effects of acids (12). Recently, beneficial immunomodulatory effects of nonviable lactobacilli have been investigated in experimental humans (13) and animals (14,15). However, the beneficial effects of nonviable probiotics on AE have not been well studied yet. In this study, to investigate of the immunomodulatory properties of nonviable lactobacilli, we determined the effects of EF-2001 on AE mice model (11). As shown in Fig. 1A, repetitious topical exposure of CDNB/DFE significantly augmented ear thickness in AE mice model. Moreover, EF-2001 decreased CDNB/DFE-induced ear thickening. CDNB/DFE also caused notable AE lesions, including edema, hemorrhage, scaling, and excoriation, which were reduced by EF-2001 treatment (Fig. 1A).

To confirm the effects of EF-2001 on swollen skin and mast cell infiltration, ears tissues were stained and made observation under an optical microscope. Repetitious topical exposure of CDNB/DFE induced marked inflammatory changes, including thickening of the dermis and epidermis, fibrosis in the dermis, and amassment of inflammatory cell such as eosinophils, neutrophils, and lymphocytes in the ear tissues of AE mice (Fig. 1B, 1C, and 1E). On the contrary,

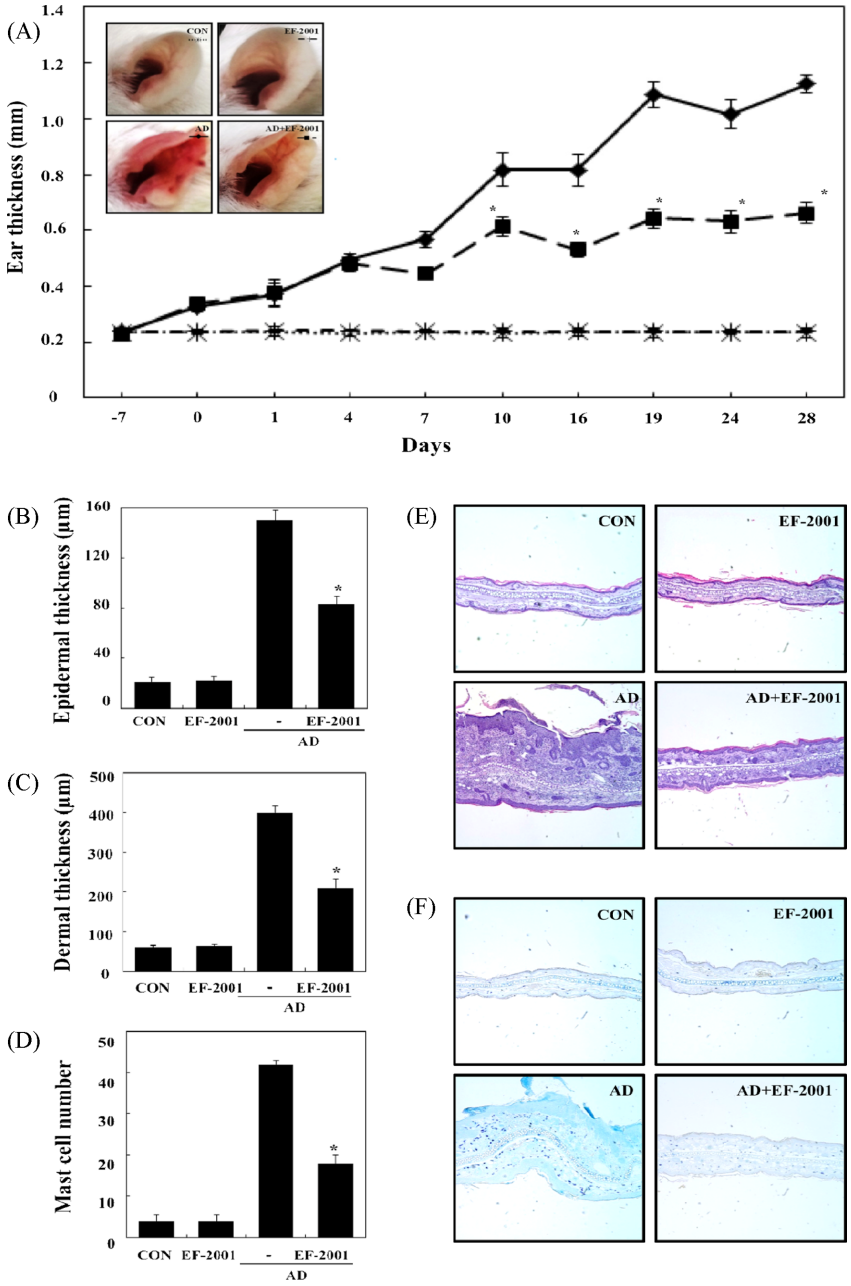

Fig. 1. Histopathological analysis to assess the effect of EF-2001 on the ear thickness and mast cell infiltration. (A) The ear thickness was measured 24 h after 1-chloro-2,4-dinitrobenzene (CDNB) or Dermatophagoidesfarinae extract (DFE) application with a dial thickness gauge. Photographs of the ears of mice from each group on day 28. Epidermal (B) and dermal (C) thickness was measured from hematoxylin and eosin-stained microphotographs. (D) The number of infiltrated mast cells was determined on the basis of toluidine blue staining. Representative photomicrographs of ear sections stained with hematoxylin (E) or toluidine blue (F). Data are presented as the mean±SD of six determinations. *Significant difference from the CDNB/DFE-treated value at $p<0.05$. The pictures shown are representative of each group (n=3-6). Original magnification was 200X. CON, control; EF-2001, heat-killed *Enterococcus faecalis* EF-2001; AE, atopic eczema.

CDNB/DFE-induced thickening of the dermis and epidermis were diminished by EF-2001 treatment (Fig. 1B, 1C, and 1 E).

Mast cells stimulate some remarkable signaling molecules, among which histamine shows markedly strong pro-inflammatory activities (16). Thus, to further investigate the above-described changes, we determined the properties of EF-2001 on the mast cells infiltration in the ears. In addition, EF-2001 treatment attenuated the mast cells infiltration induced by CDNB/DFE (Fig. 1D and 1F). These data regarding the reduced the mast cells infiltration of the skin lesions in AE mice suggested that EF-2001 may directly target inactivation of mast cells in AE. Thus, our results showed that EF-2001 alleviated the histological and typical changes of AE, such as severe ear thickening, ulcers, epidermal hyperplasia, epidermal thickening, and mast cells infiltration.

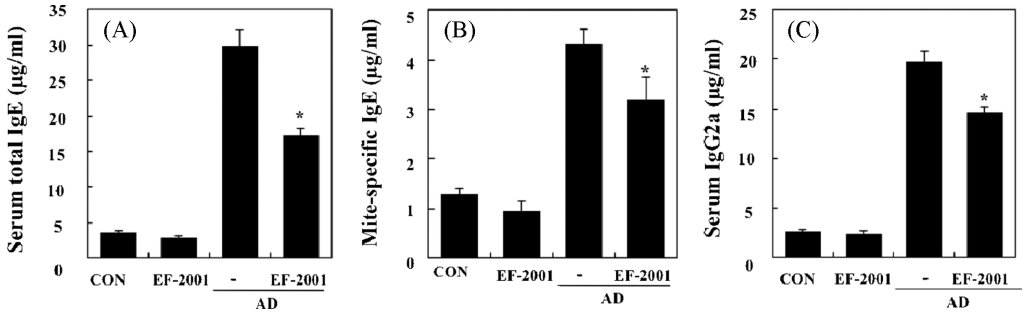

Fig. 2. Effect of heat-killed *Enterococcus faecalis* EF-2001 (EF-2001) on serum immunoglobulin levels. The blood samples for the control (CON), EF-2001, 1-chloro-2,4-dinitrobenzene/dermatophagoidesfarinae extract (CDNB/DFE), and EF-2001 plus DFE/DNCB groups were collected by orbital puncture at day 28, and plasma levels were quantified by enzyme-linked immunosorbent assay. The serum levels for IgE (A), mite-specific IgE (B), and IgG2a (C) are shown. Data are presented as mean±SD of triplicate determinations. *Significant difference from the CDNB/DFE-treated value at $p<0.05$. AE: atopic eczema induced by DFE and CDNB treatment

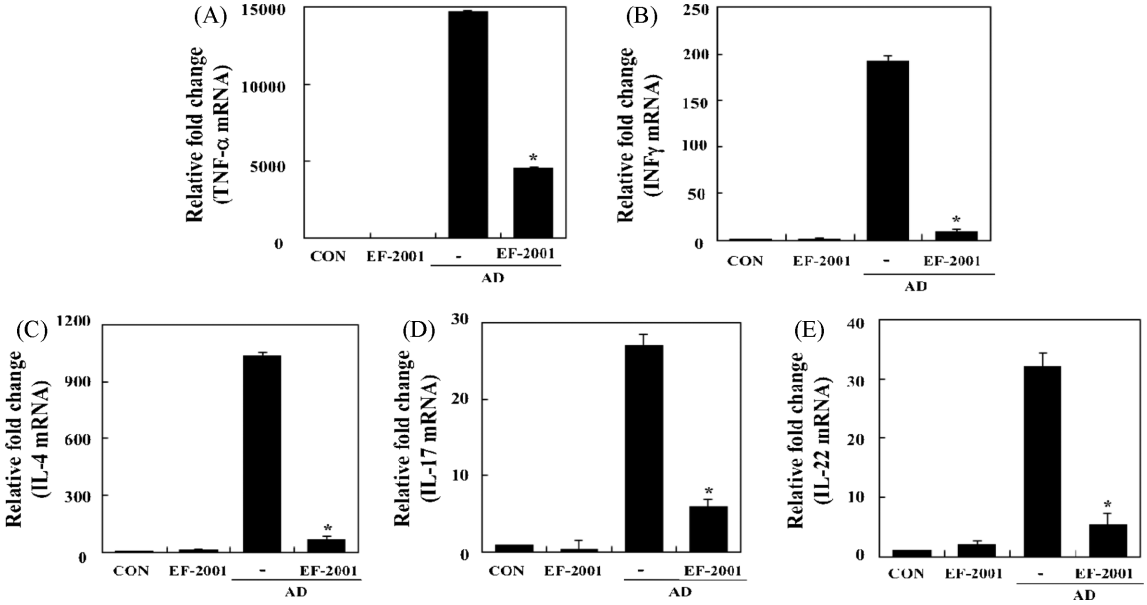

Fig. 3. Effect of heat-killed *Enterococcus faecalis* EF-2001 (EF-2001) on the expression of various pathogenic factors in the ear. The ears were excised on day 28 and total RNA was isolated. Quantitative real-time polymerase chain reaction was performed as described in the Methods. The relative fold change in mRNA for TNF-α (A), INF-γ (B), IL-4 (C), IL-17 (D), and IL-22 (E) are shown. Data are presented as mean±SD of triplicate determinations. *Significant differences from the CDNB/DFE-treated value at $p<0.05$. DFE, Dermatophagoidesfarinae extract; CDNB, 1-chloro-2,4-dinitrobenzene; AE, atopic eczema induced by DFE and CDNB treatment.

Inhibitory activity of EF-2001 on serum Ig concentrations Hyperproduction of IgE is related with the Th2 cellular immune response and is a key feature of AE. On the contrary, IgG2a production is related with the Th1 cellular response (17). To confirm whether EF-2001 exhibits its effects mainly through the Th1 or Th2 immune response, we determined the serum concentrations of IgE (total and DFE-specific) and IgG2a. Repetitious topical exposure of CDNB/DFE induced significant ascent in total IgE (Fig. 2A), DFE-specific IgE (Fig. 2B), and IgG2a (Fig. 2C). In contrast, EF-2001 diminished significantly the CDNB/DFE-caused serum total IgE, DFE-specific IgE, and IgG2a concentrations.

Inhibitory activity of EF-2001 on the cytokines expression Next, to elucidate the mechanisms through which EF-2001 alleviated the AE symptom, we determined the AE-derived inflammatory cytokines levels of the ear tissue, splenocytes and cervical lymph nodes using qPCR. All of the inflammatory cytokines from the ear tissue, splenocytes, and cervical lymph nodes of AE mice were elevated, and EF-2001 attenuated the levels of inflammatory cytokines (TNF-α, Fig. 3A, 4A, and 5A), Th1 (IFN-γ, Fig. 3B, 4B, and 5B), Th2 (IL-4, Fig. 3C, 4C, and 5C), Th17 (IL-17, Fig. 3D, 4D, and 5D), and Th22 (IL-22, Fig. 3E, 4E, and 5E) from the ears tissues (Fig. 3), splenocytes (Fig. 4), and cervical lymph nodes (Fig. 5). Th2 cytokines are known to be highly expressed during the acute stage of AE, whereas Th1 cytokines are predominantly responsive during the chronic stage (17). The present results suggested that EF-2001 inhibited the expression levels of both Th1 and Th2 cytokines from the ears, splenocytes, and cervical lymph nodes in the context of AE.

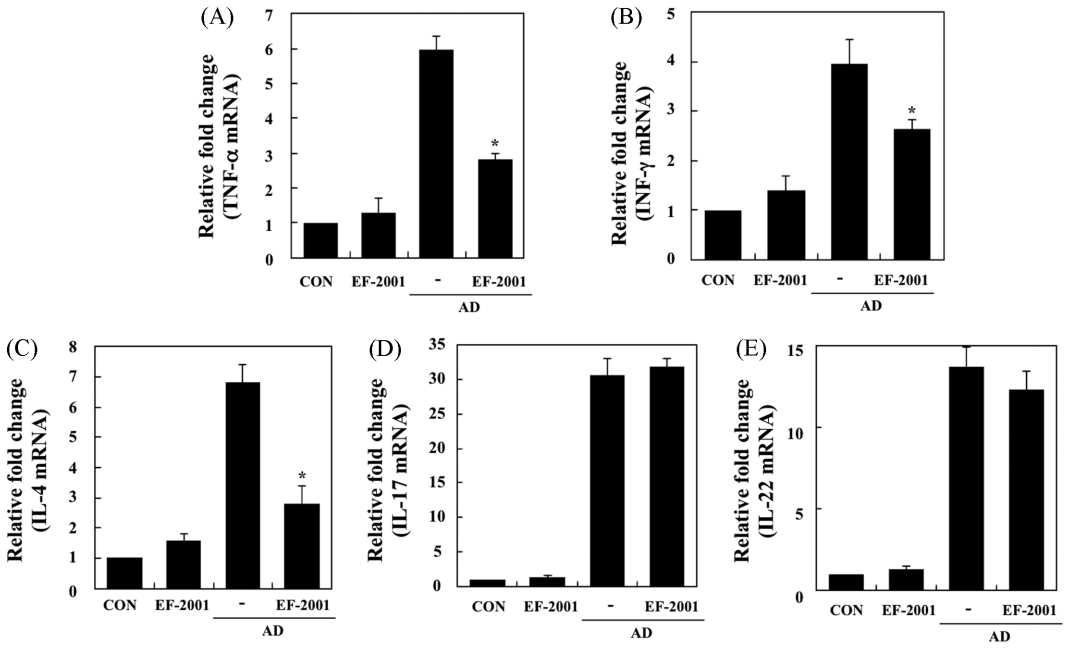

Fig. 4. Effect of heat-killed *Enterococcus faecalis* EF-2001 (EF-2001) on the expression of various pathogenic factors in the cervical lymph nodes. The ears were excised on day 28 and total RNA was isolated. Quantitative real-time polymerase chain reaction was performed as described in the Methods. The relative fold change in mRNA for TNF-α (A), INF-γ (B), IL-4 (C), IL-17 (D), and IL-22 (E) are shown. Data are presented as the mean±SD of triplicate determinations. *Significant differences from the CDNB/DFE-treated value at $p<0.05$. DFE, dermatophagoidesfarinae extract; CDNB, 1-chloro-2,4-dinitrobenzene; AE, atopic eczema induced by DFE and CDNB treatment.

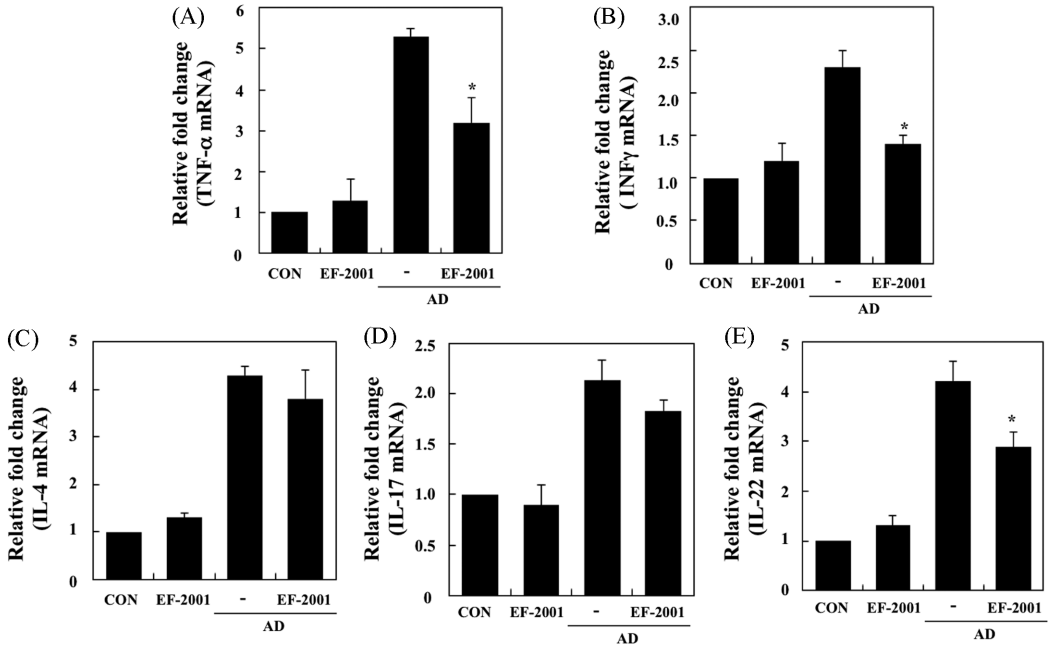

Fig. 5. Effect of heat-killed *Enterococcus faecalis* EF-2001 (EF-2001) on the expression of various pathogenic factors in the splenocytes. The ears were excised on day 28 and total RNA was isolated. Quantitative real-time polymerase chain reaction was performed as described in the Methods. The relative fold change in mRNA for TNF-α (A), INF-γ (B), IL-4 (C), IL-17 (D), and IL-22 (E) are shown. Data are presented as the mean±SD of triplicate determinations. *Significant differences from the CDNB/DFE-treated value at $p<0.05$. DFE, dermatophagoidesfarinae extract; CDNB, 1-chloro-2,4-dinitrobenzene; AE, atopic eczema induced by DFE and CDNB treatment.

In patients with AE, elevation of total IgE and DFE-specific IgE which is specific to environmental allergens are normally found (18). Historically, AE has been thought to be caused by a Th1/Th2 imbalance. Th1-induceed inflammation works to fight infections via its key cytokine, IFN-γ, while Th2-related cytokines including IL-4 and IL-5 are connected with allergic responses and arbitrate IgE class

conversion, among other functions (19,20). In this study, EF-2001 diminished IL-4, which plays a key role in Ig isotype switching. These data suggested that EF-2001 could inhibit the ascension of serum Ig concentrations by reducing the Th2 response, particularly IL-4 expression (Fig. 3C, 4C, and 5C). AE is associated with Th2 expansion in the skin (21). Recently, Th17 and Th22 were recognized as distinct T-cell subunits related in the various pathogenic conditions, such as allergic skin diseases (22,23). A role for IL-17 in allergic skin diseases accord with the phenomenon that IL-17-deficient mice exhibit delayed-type and impaired contact hypersensitivity responses upon challenge and sensitization with the corresponding allergen (24). In AE patients, the number of IL-17+CD4+T cells from peripheral blood relates with severe disease (25). In addition, IL-17+cells have been often shown to infiltrate acute AE lesions (25,26). In the skin, IL-22 stimulates epidermal hyperplasia and keratinocyte proliferation, and the frequency of IL-22-expressing T cells in AE skin is linked with disease severity (27,28). In our AE model, EF-2001 markedly inhibited serum levels of IgE and IgG2a, and the expression levels of pro-inflammatory, Th1, Th2, Th17, and Th22 cytokines from the ear tissue, and splenocytes, cervical lymph nodes. These data implied that EF-2001 could inhibit Th1, Th2, Th17, and Th22 responses in the AE skin injuries of the ear tissue. Previous studies have investigated that nonviable heat-killed lactic acid bacteria inhibited pro-inflammatory, Th1, Th2, Th17, and Th22 cytokines from splenocytes (29). Similarly, we imply that EF-2001 can suppress significantly the inflammatory response by inhibiting Th1, Th2, Th17, and Th22 in the splenocytes cervical and lymph nodes.

In conclusion, we found that EF-2001 attenuated the AE symptoms which were induced by CDNB/DFE in a murine model by reducing histopathological observation, Ig expression, and pathogenic cytokine production. Our data suggested that heat-killed EF-2001 may represent a promising therapeutic candidate for AE as a food supplement or pharmacological agent.

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Disclosure The authors declare no conflict of interest.

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