Kazachstania turicensis CAU Y1706 ameliorates atopic dermatitis by regulation of the gut–skin axis

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ABSTRACT

Atopic dermatitis (AD) causes chronic inflammatory skin disease that results in a considerable economic expense and social burden. Certain *Lactobacillus* strains ameliorate AD, but the effects of probiotic yeast on AD have not been investigated to date. In this study, we isolated *Kazachstania turicensis* CAU Y1706, commonly known as a kefir yeast, and evaluated its mitigating effects using an ovalbumin-sensitized AD mouse model. Overall, *K. turicensis* CAU Y1706 was generally effective against AD. Oral administration of *K. turicensis* CAU Y1706 suppressed T helper type 2 immune response factors by regulatory T cells and upregulation of T helper type 1 cytokine levels. *Kazachstania turicensis* CAU Y1706 also reduced IgE levels as well as the number of eosinophil and mast cells. Furthermore, feces from *K. turicensis* CAU Y1706-treated mice had more butyrate-producing bacteria, such as *Lactobacillus*, *Bacteroides*, *Ruminococcus*, and *Akkermansia*, although the level of *Fecalibacterium* was significantly reduced. Therefore, *K. turicensis* CAU Y1706 modulates immune responses as well as gut microbiota, thus indicating that it has potential for application as a supplement for alleviation of AD.

Key words: *Kazachstania turicensis*, atopic dermatitis, kimchi, microbiota, immune response

INTRODUCTION

Atopic dermatitis (AD) is a chronic, itchy, inflammatory skin disease caused by a variety of genetic, environmental, and immunological factors, as well as by exposure to microorganisms or allergens (Williams and Flohr, 2006). Traditionally, corticosteroids and calcineurin inhibitors are used as basic AD treatments

to reduce inflammation (Draeos, 2008). However, these approaches can occasionally trigger side effects, and they treat the symptoms rather than the underlying cause of AD (Cury Martins et al., 2015). Therefore, treatments that target AD without triggering systemic side effects are needed. To achieve this, recent studies of probiotics and gut microbiota, which have important nutrient and immune functions, have focused on the prevention or treatment of AD (Noverr and Huffnagle, 2005).

Lactic acid bacteria (LAB) include species of the genera *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, and *Vagococcus*. These bacteria are usually found in dairy products and produce lactic acid as the major metabolic end product of carbohydrate fermentation (Stiles and Holzapfel, 1997). Recently, some LAB have been shown to have beneficial effects on AD, such as *Lactobacillus fermentum* VRI-003 (Weston et al., 2005), *Lactobacillus rhamnosus* GG (Fölster-Holst et al., 2006), *Lactobacillus salivarius* LS01 (Drago et al., 2011), *Lactobacillus acidophilus* L-92 (Yamamoto et al., 2016), and *Lactococcus chungangensis* CAU 28 (Choi et al., 2016).

Lactic acid bacteria play an important role in gut microbial translocation and provide health benefits by balancing T helper type 1 (Th1) and T helper type 2 (Th2) immune responses (Nonaka et al., 2008).

However, few reports have been done to date on the effects of probiotic yeasts on AD. Kefir is a well-known probiotic dairy product produced by symbiotic fermentation of milk by LAB and yeasts (Fiorda et al., 2017). A member of kefir yeasts, *Kazachstania turicensis*, previously known as *Saccharomyces turicensis*, has been isolated from a variety of kefir grains (Garofalo et al., 2015) and listed in the fungal diversity of microorganisms with beneficial use (Bourdichon et al., 2012).

In this study, we evaluated the mitigating effect of *K. turicensis* CAU Y1706 isolated from kimchi, which is a Korean traditional fermented vegetable, modulation of immune responses, and gut microbiota changes in an ovalbumin (OVA)-induced AD mouse model.

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MATERIALS AND METHODS

Isolation and Identification of *K. turicensis* CAU Y1706

Kimchi samples were obtained from local markets in Seoul, Republic of Korea. The samples were chopped into pieces and blended with 10 mL of peptone water (0.85% mass/vol). After serial dilution, the samples were spread on de Man, Rogosa, and Sharpe (BD BBL, Sparks, MD) agar plates and incubated at 30°C for 2 to 3 d in an MIR-254-PK aerobic incubator (Panasonic, Osaka, Japan). White colonies were picked and streaked several times to obtain pure cultures on Tryptic Soy Agar (TSA; BD BBL) plates at 30°C for 24 h. The yeast-like isolates were identified by rRNA gene (18S, 26S, and 5.8S-ITS) sequence analysis (Nisiotou and Nychas, 2008) and sequenced using a 3730 automatic DNA sequencer (Applied Biosystems, Foster City, CA). The sequences obtained were analyzed using the NCBI BLAST program (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The morphology of the cells was observed both by a DM 1000 light (Leica, Wetzlar, Germany) and a Sigma field-emission scanning electron microscope (Carl Zeiss, Dresden, Germany) using cells growing exponentially in TSA at 30°C. The biochemical characteristics were determined using the API 20C AUX and API 50CH according to the manufacturer's instructions (bioMérieux, Marcy-l'Étoile, France). *Kazachstania turicensis* CAU Y1706 was one of the isolates and was used for evaluation of AD studies.

Ethics and Experimental Design

Animal studies were performed in accordance with the Korean Food and Drug Administration guidelines (KFDA, 2011). Blood and tissue samples were collected from mice under the animal ethics guidelines approved by the Chung-Ang University Institutional Animal Care and Use Committee of the Laboratory Animal Research Center (IACUC no. 2017-00044). Five-week-old female BALB/c mice ($n = 40$) were obtained from Central Lab Animal Incorporation (Seoul, Korea). The experimental design and timeline are shown in Figure 1. The mice were randomly allocated to 4 groups ($n = 10$ /group): (1) negative control (non-OVA sensitization + PBS), (2) positive control (OVA sensitization + PBS), (3) *K. turicensis* CAU Y1706 (OVA sensitization + *K. turicensis* CAU Y1706), and (4) bepotastine besilate (OVA sensitization + bepotastine besilate). To develop AD in mice using skin sensitization, the skin of each mouse was shaved using clippers and hair elimination cream. The OVA grade V (50 mg/mL, Sigma-Aldrich, St. Louis, MO) with alum (Sigma-Aldrich) in PBS was

injected intraperitoneally on d 7, 21, 35, and 49, and epicutaneously sensitized for 8 wk according to established procedures (Nonaka et al., 2008). Freeze-dried *K. turicensis* CAU Y1706 (1×10^{10} cfu/mouse) was dissolved in 200 μ L of PBS and administered by oral gavage. The normal control group was treated with an equal volume of PBS for 8 wk. Bepotastine besilate (Sigma-Aldrich), the positive treatment control, was applied by oral administration once per day (0.5 mg/kg). After 8 wk, the mice were killed, and the dorsal skin, ileum, and blood were collected for analysis.

Blood and Serum Cytokine Analysis

Blood samples were obtained collected by retro-orbital sinus puncture via the medial canthus of the eye. Whole blood was collected in spray-dried EDTA tubes (Green Cross Laboratories, Yongin, Korea) for eosinophil percentage and eosinophil count analysis. Serum was obtained by coagulation for 1 h at 4°C and with centrifugation for 1 h at $5,000 \times g$ at 4°C for IgE and cytokine (IL-4, IL-5, IL-10, IL-12, IL-13, TNF- α , and IFN- γ) analyses. Cytokines were determined using an ELISA kit (R&D systems, Minneapolis, MN) per the manufacturer's instructions (<https://www.rndsystems.com/products/elisas>). The absorbance at 450 nm was measured using an Infinite 200 PRO NanoQuant microplate reader (Tecan, Männedorf, Switzerland).

Microbiome Analysis

Fecal samples from 16-wk-old individual mice were collected in sterile 2-mL microcentrifuge tubes that were immediately placed on ice and then stored at -80°C . The DNA was extracted using a FastDNA SPIN kit for bacterial DNA (MP Biomedicals, Santa Ana, CA) according to the manufacturer's instructions. The 16S rRNA gene V3–V4 region was amplified by PCR and analyzed using MiSeq-based high-throughput sequencing (Illumina, San Diego, CA). After sequencing, the adapter sequences were removed using Scythe (v 0.994; <https://github.com/vsbuffalo/scythe>) and Sickle programs (<https://github.com/vsbuffalo/scythe>). The sequences were trimmed to remove short reads (<36 bp), extra-long tails, chimeric reads, and noise sequences by CD-HIT-OTU (<http://weizhong-lab.ucsd.edu/cd-hit-otu>; Li et al., 2012). The minimal quality score and the length of the merged reads were >20 and 300 bp, respectively. The remaining representative sequences were clustered and operational taxonomic units were defined at a cutoff of 97% similarity using CD-HIT-OTU. The microbiome analysis was carried out using QIIME (Quantitative Insight Into Microbial Ecology; v.1.9.1; <http://qiime.org/>) software.

Figure 1. Experimental design and timeline from the induction of atopic dermatitis in mice by ovalbumin (OVA) sensitization to the analysis.

Histological Analysis

The dorsal skin and ileum of the mice were used for histological analysis. Tissues were fixed in 10% formalin in PBS and embedded in paraffin. The tissue were cut with a microtome at thicknesses varying from 4 to 5 μm and stained with hematoxylin and eosin, or with toluidine blue and Congo red stains for mast cell and eosinophil evaluations, respectively. All of the fields of the toluidine blue-stained ileum section for mast cell counts were observed by a DM 4000B microscopy (Leica) at 400 $\times$ magnification. Five random fields of toluidine blue-stained dorsal skin sections were selected for mast cell quantification, and 20 random fields of Congo red-stained dorsal skin sections were picked for eosinophil quantification.

Statistics

The statistical analysis was performed using GraphPad Prism (v.7.0, GraphPad Software Inc., La Jolla, CA) software, and the data are shown as means $\pm$ the standard error of the mean. To identify relative differences in microbial phyla and genera, comparisons between groups were based on statistically significant differences by nonparametric Kruskal-Wallis test. Moreover, significant differences in cytokine, blood, and histologic analysis were calculated by one-way ANOVA for comparison of more than 2 groups, and *P*-values of less than 0.05 were considered significant.

RESULTS

Isolation of *K. turicensis* CAU Y1706

Kazachstania turicensis CAU Y1706 formed cream-colored, circular, and glossy colonies after 1 d of cultivation on TSA plates (Difco, Becton, Dickinson Co., Sparks, MD) at 30°C. The cells were oval-shaped and approximately 1.6 to 2.3 μm in diameter and 2.6 to 3.5 μm in length (Figure 2). *Kazachstania turicensis* CAU Y1706 used D-glucose, D-galactose, D-saccharose, D-trehalose, D-raffinose, D-fructose, D-mannose, or 2-keto-D-gluconate as a carbon source. However, the strain did not use glycerol, L-arabinose, D-xylose, adonitol, xylitol, inositol, D-sorbitol, α -methyl-D-glucoside, *N*-acetyl-D-glucosamine, D-cellobiose, D-lactose, D-maltose, D-melezitose, D-ribose, D-mannitol, D-turanose, or D-lyxose.

Effect of *K. turicensis* CAU Y1706 on Cytokine Levels

The effects of *K. turicensis* CAU Y1706 on serum cytokine production was examined. The serum concentrations of Th2 cytokines, such as IL-4 and IL-5, were also significantly lower in the *K. turicensis* CAU Y1706 groups as well as the bepotastine besilate group compared with the positive control group (Figures 3a and b). Moreover, the serum concentrations of regulatory T cell (**Treg**) cytokines, such as IL-10 and IL-1 β , were

lower in the *K. turicensis* CAU Y1706 group compared with the positive control group (Figures 3c and 3g). However, the levels of Th1 cytokines IFN- γ , TNF- α , and IL-12 were higher in the *K. turicensis* CAU Y1706 group than in the positive control group (Figures 3d–3f). These results demonstrate that *K. turicensis* CAU Y1706 enhanced the Treg-mediated suppression of the Th2 immune responses.

Effects of *K. turicensis* CAU Y1706 on Blood and Serum IgE Levels

Serum IgE levels were high in the positive control group, and were significantly reduced in the *K. turicensis* CAU Y1706 and bepotastine besilate groups (Figure 4a). Furthermore, the eosinophil count, and the eosinophil, neutrophil, and basophil percentages were significantly reduced in the *K. turicensis* CAU Y1706 group compared with the positive control and bepotastine besilate groups (Figures 4b–4e).

Effects of *K. turicensis* CAU Y1706 on Gut Microbiota Modulation

To identify microbial diversity in the gut, bacterial DNA was extracted from fecal samples and the 16S rRNA gene was sequenced following PCR amplification. A total of 3,231,137 bacterial sequence reads were obtained, with an average of 80,778 sequence reads and an average read length of 599 bp (± 9.2 bp) per sample. The diversity and richness of the bacterial community were statistically resolved by operational taxonomic units (defined as 97%), as well as by Shan-

non and Simpson indexes (Figures 5a and 5b). The *K. turicensis* CAU Y1706 group demonstrated a greater richness, evenness, and diversity in gut microbiota than did the positive control group. Differences in microbiota profiles at the genera level were assessed using an unweighted UniFrac-based 3-dimensional principal coordinate analysis plot (Figure 5c). These results show that the gut microbiota of the *K. turicensis* CAU Y1706 group was significantly different from that of the positive control group.

At the phylum level, 11 bacterial phyla were identified in the groups, and the 3 most predominant were *Firmicutes*, *Bacteroidetes*, and *Verrucomicrobia* (Figure 5d). These phyla represented a total of 94 genera, 4 of which were significantly abundant in all of the groups (Figures 5e–5h). Within *Firmicutes*, *Fecalibacterium* was present at lower levels in the negative control and the *K. turicensis* CAU Y1706 group than in the positive control group ($P < 0.0001$). Contrary, *Ruminococcus* was present at higher levels in the negative control and the *K. turicensis* CAU Y1706 group than in the positive control group ($P < 0.05$). *Akkermansia* of phylum *Verrucomicrobia* was more abundant in the negative control and the *K. turicensis* CAU Y1706 group than in the positive control group ($P < 0.0001$). *Lactobacillus* had more richness in the negative control, *K. turicensis* CAU Y1706, and bepotastine besilate group than in the positive control group ($P < 0.0001$). These results show that the gut microbiota, including the abundance of genera, differed between the *K. turicensis* CAU Y1706 and positive control groups.

Effect of *K. turicensis* CAU Y1706 on Inflammation in the Skin and Gut

To investigate the anti-inflammatory effects of *K. turicensis* CAU Y1706, OVA-sensitized mice treated with *K. turicensis* CAU Y1706 were assessed based on a dermatitis score and by pathological analysis. The dermatitis scores were dramatically lower in OVA-sensitized mice treated with *K. turicensis* CAU Y1706 than in the positive control group (Figure 6a). This finding demonstrates that *K. turicensis* CAU Y1706 had a recovery effect on the skin lesions of AD mice. The local infiltration of mast cells in the dorsal skin and ileum lesions was examined using toluidine blue staining. The accumulation of mast cells in both lesion sites was repressed in the OVA-sensitized mice treated with *K. turicensis* CAU Y1706 (Figures 6b and 6c). In addition, the number of eosinophils was significantly lower in the OVA-sensitized mice treated with *K. turicensis* CAU Y1706 compared with the positive control. Congo red staining demonstrated decreased eosinophil

Figure 2. Scanning electron microscopy image of *Kazachstania turicensis* CAU Y1706.

infiltration in the dorsal skin lesions of the OVA-sensitized mice treated with *K. turicensis* CAU Y1706 as well as bepotastine besilate (Figure 6d). These results show that *K. turicensis* CAU Y1706 can suppress the infiltration of mast cells and eosinophils in the dorsal skin and ileum of AD mice.

DISCUSSION

In the past several years, probiotics have been shown to have a potential role in the treatment of AD. These studies have mostly used *Lactobacillus* and *Bifidobacterium* strains (Jeong et al., 2016). It was recently

Figure 3. Effect of oral administration of *Kazachstania turicensis* CAU Y1706 on cytokine concentrations. Serum was used to determine the levels of cytokines including (A) IL-4, (B) IL-5, (C) IL-10, (D) IL-12, (E) IFN-γ, (F) tumor necrosis factor (TNF)-α, and (G) IL-1β using an ELISA kit (R&D systems, Minneapolis, MN). Asterisks indicate differences between the means for the positive control group compared with ANOVA: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. Error bars indicate SEM.

Figure 4. Effect of oral *Kazachstania turicensis* CAU Y1706 administration on blood. (A) Serum IgE levels were measured by ELISA assay to determine allergic responses, and inflammation was evaluated by whole blood analysis consisting of (B) eosinophil percentages, (C) eosinophil counts, (D) neutrophil percentages, and (E) basophil percentages. Asterisks indicate differences between the means for the positive control group compared with ANOVA: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. Error bars indicate SEM.

reported that the ingestion of yogurt, which contains *Lactobacillus*, influences the nature of the gut microbiota and affects AD (Shoda et al., 2017).

Kefir is a traditional fermented milk beverage originating from the Caucasian mountains that is now used worldwide (Kakisus et al., 2011). As with other fermented dairy products, kefir has been associated with a variety of health benefits such as cholesterol metabolism, inhibition of angiotensin-converting enzyme, antimicrobial activity, tumor suppression, and modulation of the immune system including alleviation of allergy and asthma (Bourrie et al., 2016). It is prepared from gelatinous granules, namely kefir grains, which consist of the polysaccharide and protein matrix (Marshall et al., 1984). The grains denote a natural symbiosis of different microorganisms that includes yeasts, LAB, and occasionally acetic acid bacteria (Witthuhn et al., 2005). *Kazachstania* spp., which previously belonged to the genus *Saccharomyces* first described by Zubkova (1971), now comprises 32 species (Vaughan-Martini et al., 2011).

Kimchi, a traditional Korean dish made from fermented vegetables, has been used as a health food in light of its beneficial effects on human health (Park et

al., 2014). Many studies have documented the microbial communities of kimchi and their role in fermentation (Jeong et al., 2013; Hong et al., 2016).

In this study, we investigated the effects of *K. turicensis* CAU Y1706, which was isolated from kimchi, on the immune response, gut microbiota, and histopathology in an OVA-sensitized BALB/c mouse model. Compared with the positive control group, *K. turicensis* CAU Y1706-treated mice had significantly lower levels of Th2 (IL-4 and IL-5) and Treg cytokines (IL-10), and higher levels of Th1 cytokines (IL-12, TNF- α , and IFN- γ). These findings are consistent with the major mechanism in AD involving abnormal Th2-type immune responses associated with skin damage or exposure to microbial stimuli (Brandt and Sivaprasad, 2011) and with other *Lactobacillus* studies that have shown suppression of Treg-mediated Th2 response factors as revealed by low IgE, lymphocyte, eosinophil, and monocyte levels, but activation of the Th1 response related to higher neutrophil levels (Endo et al., 2011; Owaga et al., 2014).

To investigate the correlation between immune modulation and the gut microbial community, the feces of the mice were analyzed by Illumina sequencing. The gut microbiota is known to produce short-chain fatty

Figure 5. The microbial community of the gut microbiota from atopic dermatitis (AD)-induced mice. Microbial community richness and diversity were measured by (A) Shannon-Wiener and (B) Simpson indexes (defined as 97% similarity). Differences in the gut microbiota were estimated by (C) unweighted principal coordinate (PC) analysis 3-dimensional plots. Blue marks: negative control group; red marks: positive control group; green marks: *Kazachstania turicensis* CAU Y1706; purple marks: betotastine besilate group. The relative abundance of the genera (D) and the scatter plot show the frequency of statistically significant genera (E–H). Comparisons between the 4 groups revealed statistically significant differences by nonparametric Kruskal-Wallis test ($P < 0.05$).

acids (SCFA; Kim et al., 2015). The SCFA butyrate plays important roles in innate immunity, protects gut health, has anti-inflammatory effects, and stimulates Treg cell differentiation (Xu et al., 2016). Furthermore, *Bacteroides*, *Ruminococcus*, and *Akkermansia* are known to be butyrate-producing bacteria (Ríos-Covián et al., 2016), whereas *Lactobacillus* is associated with stimulation of butyrate production (Tsukahara et al., 2006).

Our data support such a mechanism as an abundance was observed of the butyrate-producing bacteria *Lactobacillus* spp., *Bacteroides* spp., *Ruminococcus* spp., and *Akkermansia* spp. in the gut microbiota of the *K. turicensis* CAU Y1706-treated mice. Among these, *Lactobacillus* spp. produce anti-inflammatory cytokines, such as IL-10 (Nermes et al., 2011; Kosiewicz et al., 2014), *Bacteroides* spp. induces Treg and IL-10, and

Ruminococcus spp. is known to correlate with high IgE levels (West et al., 2015). Interestingly, high levels of *Akkermansia* spp. were observed in the *K. turicensis* CAU Y1706 group. These strains are associated with energy metabolism and balance, but the details of how they function in this capacity have yet to be elucidated (Huang et al., 2016). By contrast, *Faecalibacterium prausnitzii*, which is known to be associated with exacerbated inflammation and altered Th2 immune responses (Sitkin and Pokrotnieks, 2018), was depleted in the *K. turicensis* CAU Y1706-treated group.

Our results suggest that the beneficial effects of *K. turicensis* CAU Y1706 are due to interactions between gut microbiota, SCFA, and immune modulation between Th1 and Th2 in AD. Taken together, these results suggest a potential application for oral administration of *K. turicensis* CAU Y1706 to ameliorate AD.

Figure 6. Effect of oral administration of *Kazachstania turicensis* CAU Y1706 on atopic dermatitis (AD) skin lesions in mice. (A) The AD lesions in dorsal skin were evaluated and dermatitis scores were calculated as the sum of the scores for 3 symptoms: erythema, dryness, and scratching. The paraffin block of (B) the dorsal skin and (C) the ileum section were stained by toluidine blue and the number of mast cells was counted using a microscope (scale bar = 100 μ m). Arrows indicate a mast cell. (D) The number of eosinophils in the dorsal skin was quantified by microscopic analysis after staining with Congo red. Asterisks indicate differences between the means for the positive control group compared with ANOVA: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. Error bars indicate SEM.

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