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Full Paper

Relevant fecal microbes isolated from mice with food allergy elicited intestinal cytokine/chemokine network and T-cell immune responses

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The objective of this study was to identify the relevant fecal microbes from mice with food allergy and investigate the impact of these microbes on intestinal epithelial cells and allergen-specific T-cell responses. A murine model of ovalbumin (OVA)-induced food allergy was employed. The profile of fecal microbiota was evaluated by the traditional plating method and next-generation sequencing (NGS) of the 16S ribosomal RNA gene. The density of fecal bacteria growth on RCM, TSA and LB plates was elevated in mice with food allergy, whereas the diversity of fecal bacteria was decreased. Additionally, the relative abundances of Prevotellaceae and *Prevotella* were increased. The isolated fecal strains, mostly belonging to *Enterococcus, Streptococcus* and *Vagococcus,* significantly reduced the viability of intestinal Caco-2 cells but increased the production of interleukin (IL)-8, C-C motif chemokine ligand (CCL)-2, CCL-5, CCL-20 and C-X-C motif chemokine ligand (CXCL)-1. Moreover, cell expansion and secretion of IL-2, interferon (IFN)-γ, IL-4 and IL-17 by mesenteric lymph node (MLN) cells were augmented, whereas the production of IL-10 and transforming growth factor (TGF)-β was diminished. Although individual fecal strains had varying degrees of impact on Caco-2 cells and MLN cells, these results precisely indicate a different profile of fecal microbiota between normal mice and allergic mice. Most important, the relevant fecal microbes involved in allergen-induced dysbiosis have the potential to induce intestinal cytokine/chemokine network and T-cell immune responses.

Key words: cytokine/chemokine network, dysbiosis, fecal microbiota, food allergy, T-cell response

INTRODUCTION

Food allergy is a common allergic disorder characterized by adverse immune and hypersensitivity reactions to a food allergen, resulting in a poor quality of life and a considerable public health and economic burden [1]. The prevalence of food allergy has been increasing worldwide in recent decades, reaching 5% among children and approximately 4% among adults [1]. The rise in prevalence has occurred in such a short time frame that genetics alone cannot explain it. Therefore, researchers have focused on identifying underlying mechanisms of food allergy development using animal models [2]. Although substantial differences are known to exist between human and murine allergic responses, murine models of food allergy provide crucial and valuable information on disease mechanisms [3]. Typically, food allergy is the result of allergen-specific IgE-mediated mast cell degranulation, but nonetheless, the etiology of food allergy remains unclear. A failure to establish or a breakdown in the maintenance of oral tolerance may be responsible. Generally, oral tolerance can be influenced by genetic predisposition, the route of allergen sensitization, the timing and dose of allergen exposure, enteric microbiota and the tissue milieu [1]. The integrity of the intestinal epithelium is also one of the first major regulatory mechanisms that prevent the development of food allergy. Damage of the intestinal epithelium can also facilitate allergen uptake, followed by the induction of allergic responses if local cytokine/chemokine signals are present [2].

Indeed, alterations in the gut microbiota may precede the development of allergy. Numerous studies have substantiated the importance of microbiota in modulating the development of the immune system, revealing a pathogenic potential of enteric microbiota in food allergy [4]. Recent evidence has shown that a lack of microbial exposure during infancy is responsible for food allergy [5]. Clinical epidemiologic studies have postulated that reduced microbial pressure in westernized countries underlies the increase in development of allergies [6]. In addition, establishment of oral tolerance to dietary antigens is closely associated with the presence of commensal microbes [7]. Therefore, a good definition

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of the gut microbiota and an understanding of the relationship to food allergy are essential in preventing food allergy.

The role of the gut microbiota in host health has been extensively studied in recent years using culture-independent molecular methods [8]. The next-generation sequencing (NGS) technology provides a large number of sequencing reads in a single run, resulting in a large sampling depth and the detection of low-abundance taxa. Thus, the results of studies using high-throughput sequencing technologies have modernized our understanding of the gut microbiota in healthy and disease conditions. The aim of this study was to investigate the differences in fecal microbiota between normal mice and ovalbumin (OVA)-induced allergic mice by using traditional plating methods and NGS technology. Furthermore, fecal microbes isolated from allergic mice were identified. The impact of relevant microbes on intestinal epithelial cells and allergen-specific T-cell responses was evaluated by analyzing cell viability and cytokine/chemokine production of Caco-2 cells and OVA-primed mesenteric lymph node (MLN) cells, respectively, co-cultured with individual strains.

MATERIALS AND METHODS

Chemicals, reagent and cell lines

All chemicals, including OVA (Grade V), and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Reagents for cell culture and bacterial culture were purchased from GE Healthcare Life Sciences (Marlborough, MA, USA) and BD Diagnostic Systems (Sparks, MD, USA), respectively. ELISA reagents and standards were purchased from eBioscience, Inc. (San Diego, CA, USA), and 10% neutral buffered formalin was purchased from Leica Biosystems Richmond Inc. (Richmond, IL, USA). The Caco-2 cell line (ATCC® HTB-37™) was maintained in DMEM (L-glutamine, high glucose; Corning Inc., Corning, NY, USA) supplemented with 10% fetal bovine serum.

Mice and ethics statement

Four- to five-week-old female BALB/c mice were obtained from the National Laboratory Animal Center in Taiwan. All mice were housed at the Laboratory Animal Facility of the National Taiwan Ocean University (NTOU). On arrival, mice were randomly transferred to plastic cages containing a sawdust bedding and quarantined for 1 week. They were housed in a controlled environment at 23 ± 2°C with 50 ± 20% humidity and a 12-hr light/dark cycle. Except on the days of allergen challenge (described below), food and water were supplied ad libitum. All experiments were conducted in accordance with the guidelines of the Laboratory Animal Facility Center of NTOU. All animal studies were approved by the NTOU Institutional Animal Care and Use Committee (NTOU-IACUC-107026).

Protocol of animal experiments

The employed experimental protocol has been reported previously [9]. Briefly, the mice (n=6) were divided randomly into two groups (Fig. 1): the naive (NA) and OVA-sensitized and challenged (VH) groups. Except for mice in the NA group, each mouse was sensitized with OVA by intraperitoneal injection using 0.1 mL of sensitization solution containing 50 µg of OVA and 1 mg of aluminum potassium sulfate on day 3 and later boosted with a double dose on day 17, followed by a repeated challenge with OVA by gavage every other day from day 31 to day 41. Mice were deprived of food 3 hr before challenge. Allergic diarrhea was apparent 30–60 min following the challenge, and it was assessed by scoring the severity of the fecal form from 0 to 3: 0, no fecal matter or solid state; 1, funicular form; 2, slurry; 3, watery state. The mice were euthanized 3 hr after the last OVA challenge, and the duodenal tissues were prepared for hematoxylin and eosin (H & E) staining and toluidine blue staining to determine the induction of allergic enteritis. Fresh fecal samples were collected before each challenge and after the last challenge. These samples from two mice with similar scores in the same group were pooled together for NGS analysis and bacterial culture.

Next-generation sequencing analysis

Fecal DNA extraction was performed using a QIAamp DNA Stool Mini Kit (QIAGEN, Germantown, MD, USA) according to the manufacturer's recommendations. Metagenomic studies were performed by analyzing the prokaryotic 16S ribosomal RNA (16S rRNA) gene. V3 and V4 hypervariable regions were selected for distinguishing the diverse microbial populations. The first PCR was set up with 2.5 µL of DNA, 0.2 µM V3 + V4 forward and reverse primers (forward, TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG; reverse, GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC) and 12.5 µL 2X Kapa HiFi HotStart ReadyMix (Kapa Biosystems, Wilmington, MA, USA) in 25 µL reactions. The PCR cycling conditions were 3 min at 95°C; 25 cycles of 30 sec at 95°C, 30 sec at 55°C and 30 sec at 72°C; and then 5 min at 72°C. The amplified DNA was purified with Agencourt AMPure XP Reagent beads (Beckman Coulter Inc., Brea, CA, USA). The second PCR was set up to add indexes to the amplified DNA by adding 5 µL of purified DNA to 25 µL 2X Kapa HiFi HotStart ReadyMix (Kapa Biosystems), 5 µL Nextera XT Index 1 and 2 primers (Illumina, San Diego, CA, USA) in 50 µL reactions. The second PCR reaction was set at 3 min at 95°C; 8 cycles of 30 sec at 95°C, 30 sec at 55°C and 30 sec at 72°C; and then 5 min at 72°C, and this was followed by another Agencourt AMPure XP Reagent beads purification (Beckman Coulter, Inc., Brea, CA, USA). We used qPCR (KAPA SYBR FAST qPCR Master Mix) to quantify each library using a Roche LightCycler 480 system and then pooled them equally, diluting them each to 4 nM, for sequencing with an illumina MiSeq NGS system (Illumina, San Diego, CA, USA). More than 100,000 paired-end sequencing reads (2 × 300 bp) were generated. The sequence data were then processed using the QIIME 2 package, version 2019.4 [10]. Forward and backward reads were joined, and the tool was used to pick closed-reference OTUs from the Greengenes database (May 2013 version). OTU representative sequences were selected afterwards with a 97% similarity threshold.

Culture of bacteria

A previously described plating method was employed with minor modifications [11]. After the last challenge, fresh fecal samples were collected, weighed, suspended and serially diluted with buffered peptone water and cultured in triplicate on reinforced clostridial medium (RCM), tryptic soy (TSA) and lysogeny broth (LB) agar plates under anaerobic conditions. After incubation for 48 hr, the number of colony-forming units (CFU) in the range of 25–250 per plate was counted manually, and the

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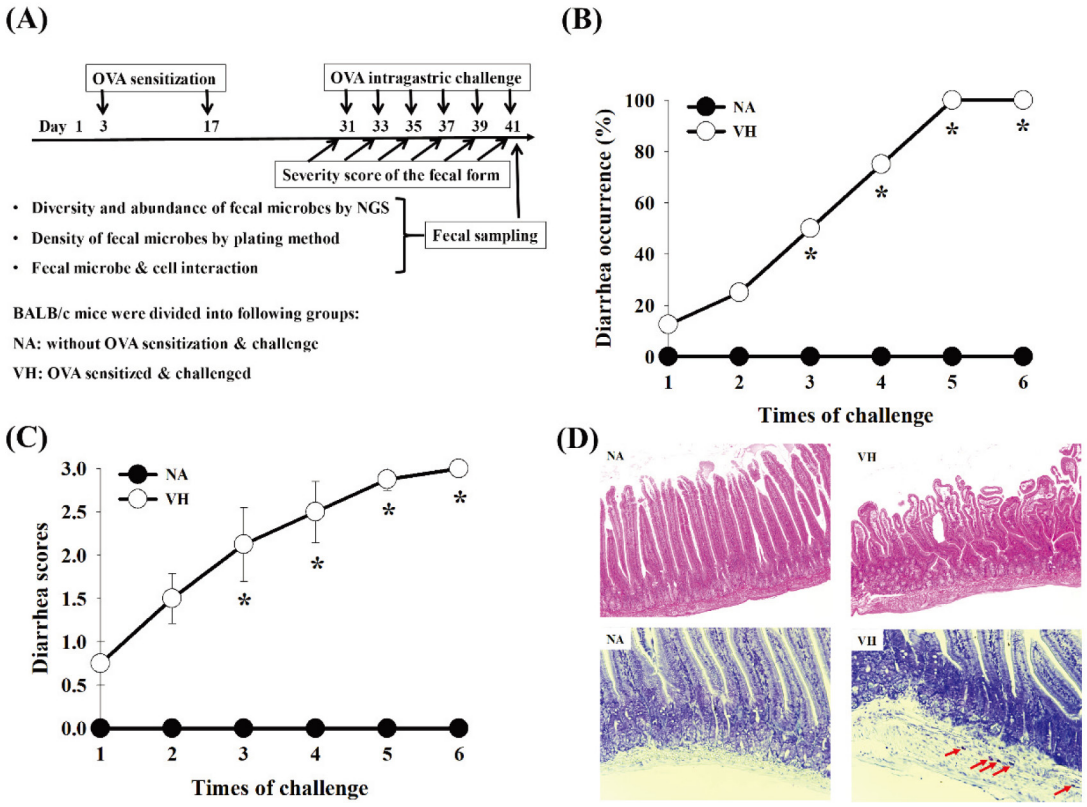

Fig. 1. Protocols of ovalbumin (OVA) sensitization and challenge and induction of allergic diarrhea.

(A) BALB/c mice (n=6) were divided randomly into two groups: the naive (NA) and OVA-sensitized and challenged (VH) groups. Mice in the OVA group were sensitized with OVA twice, followed by 6 OVA challenges. After each OVA challenge, fresh fecal samples were collected for bacterial culture and next-generation sequencing (NGS) analysis. The isolated colonies were prepared for sequence analysis and cell-microbe interaction experiments. (B) Occurrence and (C) severity of allergic diarrhea were observed for 60 min following each challenge, and allergic diarrhea was assessed by scoring the severity as described in the Materials and Methods. Each line indicates the mean score of two individual mice with almost identical scores throughout the experiment. Results are representative of three independent experiments. *$p<0.05$ compared withthe NA group. (D) The duodenal tissues were isolated for histopathological examination. Representative H & E-stained and toluidine blue-stained sections are shown (original magnification, ×100 and ×200, respectively). The red arrows indicate infiltrated mast cells.

density of microbes was expressed as CFU/g of feces. Colonies from these agar plates were isolated for further sequence analysis.

Identification of isolated fecal microbes

Based on the results of plate counts, five colonies each on RCM, TSA and LB plates cultured with fecal samples from the VH group were isolated. Total DNA was extracted from the 15 colonies using a High-Speed Plasmid Mini Kit (Geneaid Biotech Ltd., Sijhih City, Taiwan). The 16S rRNA gene was amplified by PCR with the universal primers 16F27 (50-AGAGTTTGATCCTGGCTCAG-30) and 16R1522 (50-AAGGAGGTGATCCAGCCGCA-30) [12]. Each amplified PCR product with the size of 1.5 kb was sequenced using a 3730 DNA analyzer (Applied Biosystems, Foster City, CA, USA), and the obtained sequences were compared with microbial sequences available in GenBank using BLAST (http://www.ncbi.nlm.nih.gov/BLAST). The results of the similarity analysis based on 16S rRNA sequences for the isolated strains are shown in Table 1. The identified strains were retained for further cell-microbe interaction experiments.

Cell-microbe interaction experiments

Caco-2 cells (ATCC® HTB-37™) were seeded into 24-well plates and grown for 7 days. The retained strains were cultured in broth for 24 hr, and the bacterial pellets were washed with sterile phosphate-buffered saline (PBS) three times for co-cultured assays. Caco-2 cells were then incubated for 14 hr in fresh medium containing isolated fecal microbes (1×10^7 CFU/mL for each strain). A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) stock solution (5 mg/mL in PBS) was added to each well (10 µL/well) and incubated for 4 hr. At the end of incubation, the formed formazan was dissolved with 10% dimethyl sulfoxide, and then the optical density (OD) was read at 570 nm. Viability (%) = (OD value of treated cells/OD value of untreated cells) × 100%. On the other hand, the supernatants of cultured Caco-2 cells were collected for the measurement of interleukin (IL)-8, C-C motif chemokine ligand (CCL)-2, CCL-5, CCL-20 and C-X-C motif chemokine ligand (CXCL)-1 production by enzyme-linked immunosorbent assay (ELISA; Thermo Fisher Scientific Inc., Waltham, MA, USA, and BioLegend, San Diego, CA, USA) according to the supplier's instructions.

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Table 1. Similarity analysis of isolated fecal strains based on 16S rRNA gene sequences

Isolate	Strain	Similarity (%)
RCM1	*Enterococcus faecalis* strain NBRC 100480	100
RCM2	*Enterococcus rivorum* strain S299	99
RCM3	*Enterococcus moraviensis* strain NBRC 100710	99
RCM4	*Vagococcus lutrae* strain m1134/97/1	99
RCM5	*Enterococcus crotali* strain ETRF1	99
TSA1	*Enterococcus plantarum* strain CCM 7889	99
TSA2	*Enterococcus hirae* ATCC 9790	99
TSA3	*Enterococcus wangshanyuanii* strain MN05	99
TSA4	*Enterococcus canis* strain NBRC 100695	99
TSA5	*Enterococcus faecium* strain NBRC 100486	99
LB1	*Streptococcus danieliae* strain ERD01G	99
LB2	*Streptococcus alactolyticus* strain ATCC 43077	99
LB3	*Streptococcus sanguinis* strain JCM 5708	99
LB4	*Vagococcus lutrae* strain m1134/97/1	99
LB5	*Enterococcus faecalis* strain NBRC 100480	99

Each obtained sequence pair was compared with microbial sequences available in GenBank using BLAST.

MLNs from sacrificed mice of the VH group were collected and processed into single-cell suspensions. The MLN cells (5×10^6 cells/mL) were seeded into 24- and 96-well culture plates (0.1 mL/well), treated with individual fecal strains (1×10^7 CFU/mL for each strain) and then re-stimulated with OVA (50 µg/mL) for 24 and 72 hr. Cell viability was determined by MTT assay as described above. The supernatants of cultured MLN cells were collected for IL-2, IL-4, IL-10, IL-17, interferon (IFN)-γ and transforming growth factor (TGF)-β measurement by ELISA (Thermo Fisher Scientific Inc., Waltham, MA, USA, and BioLegend, San Diego, CA, USA) according to the supplier's instructions.

Statistical analysis

The data are expressed as the mean ± standard error of the mean (SEM) for each treatment group. One-way ANOVA was used to test for differences within each group. Dunnett's two-tailed t-test was used to assess statistical differences between the treatment group and the control group. The level of significance was set at $p<0.05$.

RESULTS

In order to investigate the association between allergic diarrhea and dysbiosis, a murine model of food allergy was employed for the induction of food allergy as described in the Materials and Methods and Fig.1A. Throughout the experiment, normal fecal conformation was observed in the mice of the NA group. However, diarrhea occurrence and diarrhea scores of mice in the VH group gradually increased and reached the highest possible score of 3 in the sixth challenge (Fig. 1B and C). Additionally, abnormal villous edema, crypt hyperplasia and mast cell infiltration were observed in the duodenal tissues harvested from mice of the VH group (Fig. 1D). These results are similar to those reported in a previous study employing the same murine model, indicating successful induction of allergic enteritis [13].

The profile of fecal microbiota was first evaluated by plating methods. Before each challenge, fresh fecal samples were serially diluted and cultured on RCM, TSA and LB agar plates to determine the density of fecal microbes. As shown in Fig. 2A, the density of fecal microbe growth on these agar plates was significantly increased with each successive challenge. For a comprehensive understanding of the diversity and composition of the fecal microbiota, metagenomic studies were performed by analyzing the 16S rRNA genes extracted from fecal samples. As rarefaction curves are a representation of the species richness for a given number of individual samples, the curves of the VH and NA groups indicated that the species richness of the samples from the VH group was lower than that of the samples from the NA group (Fig. 2B). This was also evidenced by the results of alpha diversity analysis (Fig. 2C). The relative abundance of Prevotellaceae and *Prevotella* was increased in the samples from the VH group (Fig. 2D and E), indicating that development of allergic enteritis may be associated with the growth of specific microbes.

Furthermore, fecal strains of allergic mice grown on RCM, TSA and LB plates were isolated and identified. As shown in Table 1, the sequences of colonies mainly belonged to *Enterococcus*, *Streptococcus* and *Vagococcus*. The impact of these microbes on Caco-2 cells and OVA-primed MLN cells was evaluated by analyzing cell viability and cytokine/chemokine production. The isolated strains significantly reduced the viability of Caco-2 cells (Fig. 3A), indicating that these strains had the potential to damage intestinal integrity. As IL-8 is a crucial chemokine in the recruitment of immune cells from intravascular to interstitial sites [14], the secretion of IL-8 from Caco-2 cells co-cultured with the isolated strains was investigated. All of the isolated strains were able to promote the secretion of IL-8 (Fig. 3B). Because CCL-2, CCL-5, CCL-20 and CXCL-1 secreted by enterocytes are known to elicit inflammation [15], the level of these chemokines in the supernatants of cultured Caco-2 cells was measured. All of these strains could elicit the secretion of CCL-2, CCL-5, CCL-20 and CXCL-1 by Caco-2 cells (Fig. 4). Interestingly, these strains had different degrees of effect on augmenting cytokine/chemokine production.

As the employed model of food allergy is mediated by allergen-specific T cells [1, 9], the impact of isolated strains on the viability of OVA-specific MLN cells and the major cytokines secreted by

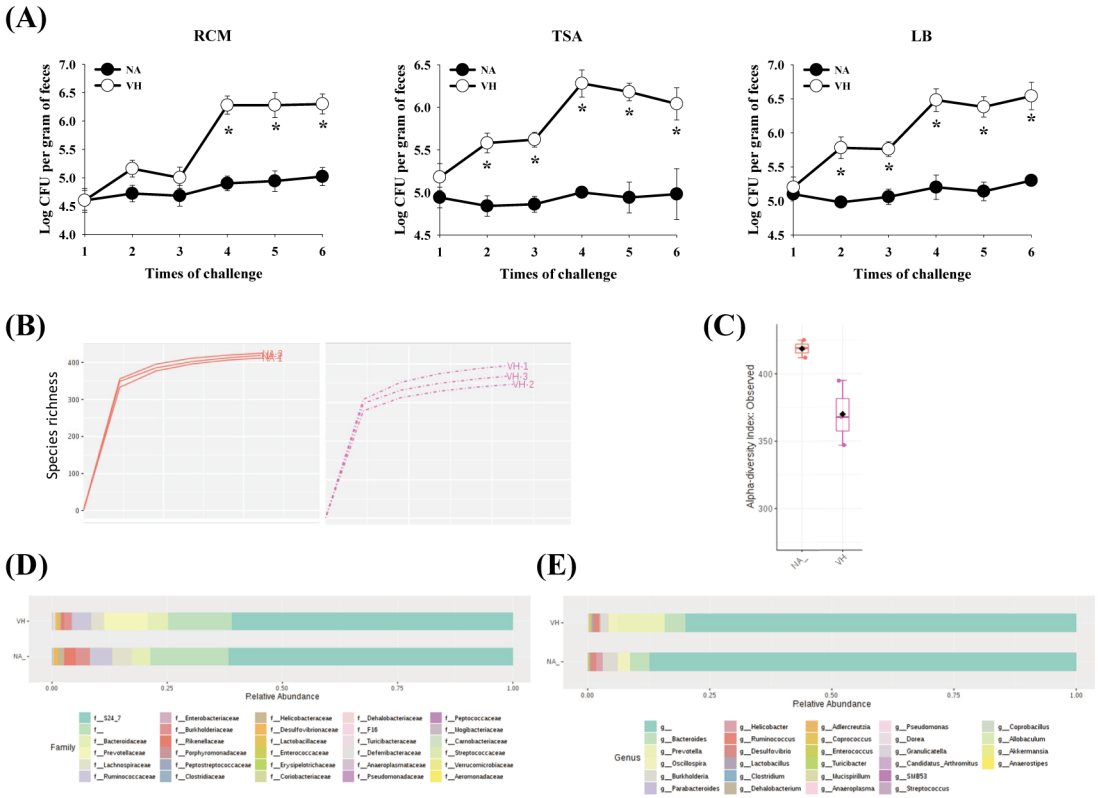

Fig. 2. Plating method and next generation sequencing (NGS) analysis of fecal microbiota from allergic mice.

(A) Before each challenge, fresh fecal samples from each group were weighed and incubated in triplicate on reinforced clostridial medium (RCM), tryptic soy (TSA) and lysogery broth (LB) agar plates. Results are representative of three independent experiments, each in triplicate. Data are expressed as the mean ± SEM of triplicate experiments. *p<0.05 compared with the naive (NA) group. After the last challenge, fresh fecal samples were collected for NGS of the 16S ribosomal RNA gene. (B) Rarefaction curves for bacterial species richness in the feces of mice. Each line indicates the OTU richness of mixed fecal samples from two individual mice in the same group. (C) Box plots illustrating the alpha diversity indices of the bacterial microbiomes of the NA and OVA-sensitized and challenged (VH) samples. Median values and interquartile ranges are indicated in the plots. (D & E) Microbial composition of fecal samples from each group. Each bar represents the relative abundance of each bacterial taxon within a group. Taxa assignments at the family and genus level are shown.

T helper (Th) subsets was investigated. The results of the MTT assay showed that re-stimulation with OVA significantly elevated the viability of OVA-primed MLN cells compared with that with PBS alone, revealing the induction of an allergen-specific immune response by re-stimulation with OVA (Fig. 5A). However, higher viability was observed when the re-stimulated MLN cells were co-cultured with the isolated strains (Fig. 5A). Concordantly, these strains substantially upregulated IL-2 production by MLN cells (Fig. 5B). As IL-2 is the major cytokine for T cell clonal expansion [16], the isolated strains had distinct degrees of impact on evoking allergen-induced T cell expansion.

Because a typical food allergy is a disorder of Th1/Th2 imbalance, the levels of IFN-γ and IL-4 (the major cytokines secreted by Th1 and Th2 cells, respectively) were measured [17]. These strains noticeably promoted both IFN-γ and IL-4 production (Fig. 5C). Accordingly, the impact of the isolated strains on intestinal T-cell responses was not restrained to the results of modulation of the Th1/Th2 immune balance. As Treg cells and Th17 cells have opposite effects on the elicitation of intestinal inflammation, the levels of IL-10, TGF-β (the major inhibitory cytokines secreted by Treg cells) and IL-17 (the major cytokines secreted by Th17 cells) were measured [17]. Co-culture with these strains vastly elevated IL-17 production (Fig. 6A). However, these strains showed limited or even inhibitory effects on IL-10 and TGF-β production (Fig. 6B and C).

DISCUSSION

Although emerging evidence suggests that the increasing prevalence of food allergies is associated with compositional and functional changes in the gut microbiota [18], the direct impact of relevant microbes involved in allergen-induced dysbiosis on the induction of allergic enteritis has remained unclear. In the present study, we demonstrated the role of enteric microbiota in intestinal cytokine/chemokine network and allergen-induced T-cell responses. This notion has been substantiated by several lines of evidence. Firstly, the density of fecal bacteria growth on RCM, TSA and LB plates gradually increased in conjunction with the induction of allergic diarrhea. Secondly, less diversity and increased relative abundances of fecal Prevotellaceae and *Prevotella* were observed in allergic mice. Thirdly, co-culture with the relevant fecal strains suppressed the viability of Caco-2

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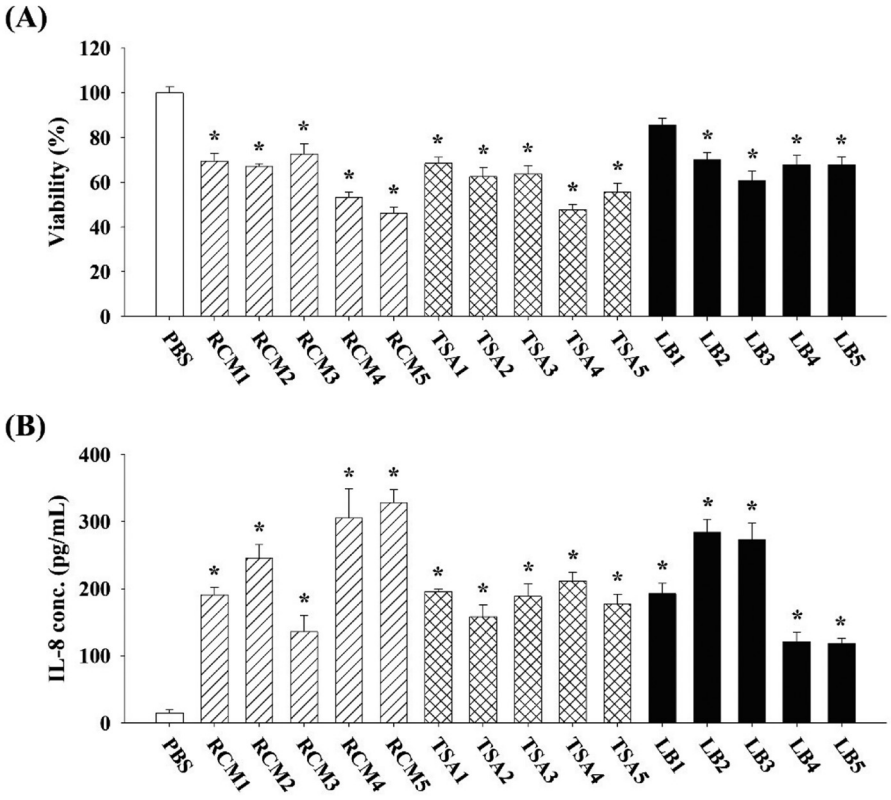

Fig. 3. Impact of isolated fecal strains on the viability of Caco-2 cells and the production of IL-8. Caco-2 cells (1×10^5 cells/well) were seeded into 24-well plates, grown for 7 days and then incubated for 18 hr in a fresh medium containing isolated fecal microbes (1×10^7 CFU/mL for each strain). (A) Cell viability was examined by MTT assay. The supernatants were collected for the measurement of (B) IL-8 production by ELISA. Results are representative of three independent experiments, each in triplicate. Data are expressed as the mean ± SEM of triplicate experiments. *p<0.05 compared with the phosphate-buffered saline (PBS)-treated group.

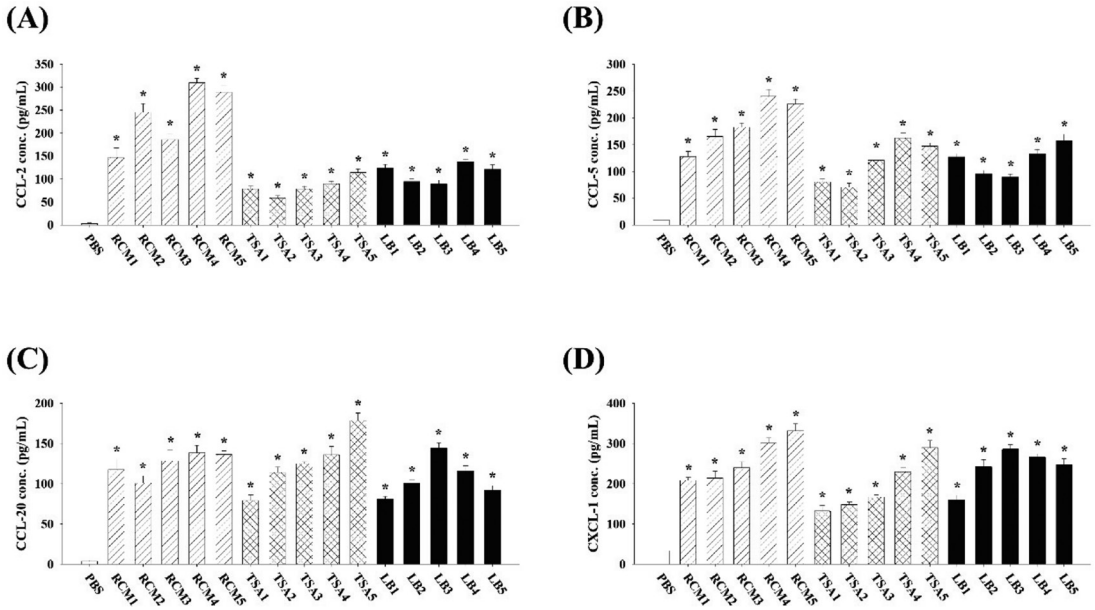

Fig. 4. Impact of isolated fecal strains on the production of CCL-2, CCL-5, CCL-20 and CXCL-1 by Caco-2 cells. Caco-2 cells (1×10^5 cells/well) were seeded into 24-well plates and then incubated for 18 hr in a fresh medium containing isolated fecal microbes (1×10^7 CFU/mL for each strain). The supernatants were collected for the measurement of (A) CCL-2, (B) CCL-5, (C) CCL-20 and (D) CXCL-1 production by ELISA. Results are representative of three independent experiments, each in triplicate. Data are expressed as the mean ± SEM from triplicate experiments. *p<0.05 compared with the phosphate-buffered saline (PBS)-treated group.

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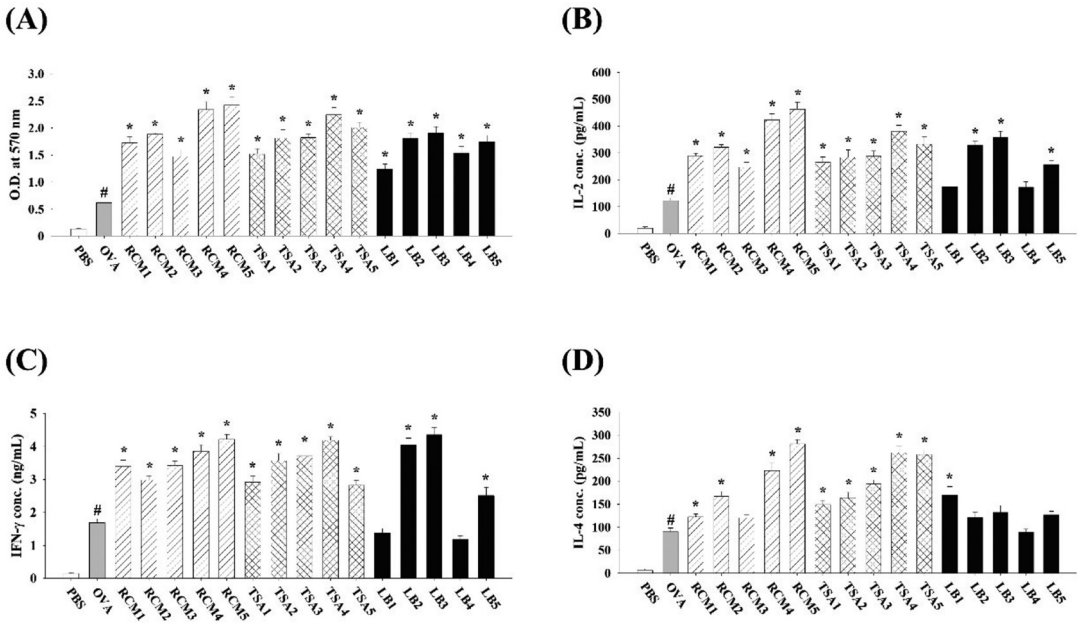

Fig. 5. Impact of isolated fecal strains on allergen-specific mesenteric lymph node (MLN) cell expansion. Mice in the OVA-sensitized and challenged (VH) group were treated as described in the Materials and Methods. The mice were sacrificed after the last challenge, and their MLNs were harvested. The MLN cells (5×10^6 cells/mL) were treated with each identified fecal strain (1×10^7 CFU/mL) and re-stimulated with OVA (50 μg/mL). After culture for 24 or 72 hr, (A) cell viability was examined by MTT assay, and (B) the production of IL-2, (C) IFN-γ and (D) IL-4 in supernatants was quantified by ELISA. Results are representative of three independent experiments, each in triplicate. Data are expressed as the mean ± SEM of triplicate experiments. $^{\#}p<0.05$ compared with the phosphate-buffered saline (PBS) treatment. *$p<0.05$ compared with the OVA re-stimulation.

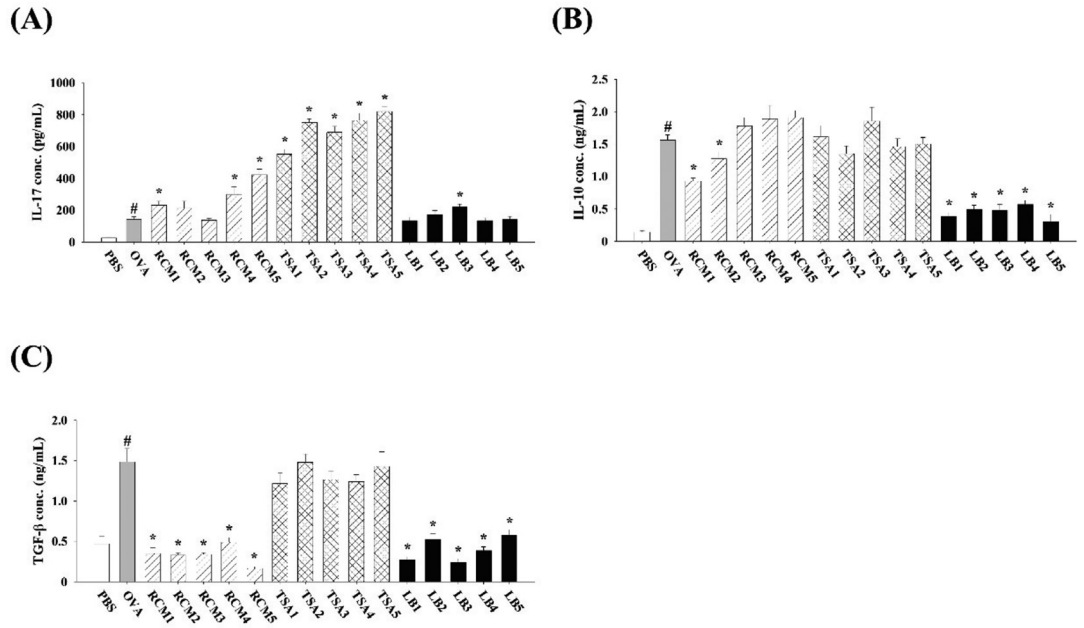

Fig. 6. Impact of isolated fecal strains on allergen-induced IL-17, IL-10 and TGF-β secretion by mesenteric lymph node (MLN) cells. Mice in the OVA-sensitized and challenged (VH) group were treated as described in the Materials and Methods. The mice were sacrificed after the last challenge, and their MLNs were harvested. The MLN cells (5×10^6 cells/mL) were treated with each identified fecal strain (1×10^7 CFU/mL) and re-stimulated with OVA (50 μg/mL). After culture for 72 hr, the levels of (A) IL-17, (B) IL-10 and (C) TGF-β in the supernatants were quantified by ELISA. Results are representative of three independent experiments, each in triplicate. Data are expressed as the mean ± SEM of triplicate experiments. $^{\#}p<0.05$ compared with the phosphate-buffered saline (PBS) treatment. *$p<0.05$ compared with the OVA re-stimulation.

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cells and elicited cytokine/chemokine production, including IL-8, CCL-2, CCL-5, CCL2- and CXCL-1. Finally, the relevant fecal strains enhanced allergen-induced T cell expansion and Th1-, Th2- and Th17-immune responses but diminished Treg immunity as evidenced by the deterioration of T cell subset-associated cytokines.

In mice, it appears that even minor differences in the gut microbiota can have substantial effects on experimental models of disease. Inbred mice with the same genetic background obtained from two different suppliers featured different dominant microbial communities [19]. Changes in microbial composition were further induced by variations in diet or by stress [2]. Therefore, inconsistencies within animal experiments may be related to the observed differences in the bacterial composition. In the current study, mice were obtained from the same supplier. Offspring of different mothers were randomly grouped, and all offspring were fed with the same diet. Mice in the same group show a similar profile of enteric microbiota. To avoid the factor of individual variation, we further isolated enteric strains for *in vitro* co-culture with Caco-2 cells and OVA-primed MLN cells. Interestingly, the isolated strains of *Enterococcus*, *Streptococcus* and *Vagococcus* decreased the viability of Caco-2 cells and increased the production of cytokines/chemokines, but not inhibitory cytokines.

In agreement with the results of NGS conducted in the current study, it has been reported that the abundance of *Prevotella* was increased in infants with food allergy [6]. It has also been demonstrated that *Prevotella* would stimulate epithelial cells to produce IL-8 and CCL20, which can promote mucosal Th17 immune responses [20]. As the results of the current study show elevated levels of IL-17, IL-8 and CCL20 in allergic mice, an increased abundance of *Prevotella* is considered a potential mechanism mediating allergic enteritis. With respect to the isolated fecal strains, the information pertaining to the biological activity of *Vagococcus* is limited, although *Vagococcus fluvialis* L-21 is able to stimulate some immune-related genes associated with the early inflammatory responses in leucocytes of fish [21]. The immunostimulatory properties of representative species of commonly dominant human small intestinal microbial communities, including six streptococcal strains and one *Enterococcus* strain, have been investigated. In agreement with the results of the current study, the different streptococci induced varying levels of IL-8 production. Moreover, the *Enterococcus* strain was demonstrated to be a potent inducer of cytokines [22]. On the other hand, the immunostimulatory activity of enterococci, especially *Enterococcus faecalis* and *Enterococcus faecium*, has been widely investigated, as they are considered potential probiotics. For example, *E. faecalis* CECT7121 could induce dendritic cell activation and skew the activation of T cells towards the production of IFN-γ [23]. Recently, it has been reported that gut-derived *E. faecium* from ulcerative colitis patients promotes colitis in a genetically susceptible mouse host [24].

With respect to the cytokine/chemokine network, CCL-2 is known to mediate Th17 cell migration during acute allergic airway inflammation [25]. CCL-5 is considered a major regulator of local immune responses, and it targets immune cells to sites of inflammation. Previous research has shown that CCL-5 is elevated along with other cytokines in tissue samples from atopic patients, where it is thought to have a role in recruitment of T cells and eosinophils [26]. It has been substantiated that depletion of the microbiota leads to the activation of goblet-cell-mediated antigen passage, which results in the production of IL-17 and CXCL-1 and an influx of leukocytes, reflecting a state of acute inflammation [27]. During repeated allergen exposure, the release of chemokines, including CCL-20, induces the accumulation of antigen-specific Th2 cells in the intestinal mucosa [28]. Additionally, IL-8 and CCL-20 expressed by intestinal epithelial cells are known to be regulated by interaction with bacterial microbes [29]. Involvement of the cytokine/chemokine network in enteritis of allergic mice has been mentioned in previous studies. Elevated levels of CCL-2, CCL-5, CCL-20 and CXCL-1 were observed in mice sensitized and orally challenged with OVA [28, 30–32]. In a study employing the same food allergy model as used in the current study, increased intestinal IL-4, IL-17 and IFN-γ production was observed in allergic mice [33]. Consistently, a similar profile of cytokine/chemokine production and T-cell responses was observed in the current *in vitro* system of Caco-2 cells and MLN cells co-cultured with fecal bacteria. Based on the findings of the current study and above mentioned literature, we suggest that the relevant fecal strains are potentially involved in the induction of allergic enteritis via at least two pathways. In the first pathway, these strains could pass across the intestinal mucosa, potentially by damaging enterocytes, and then act on immune cells within gut-associated lymphoid tissues. In the second pathway, elicitation of cytokine/chemokine secretion from intestinal epithelial cells would lead to the recruitment of effector T cells and inflammatory cells, followed by the initiation of mucosal inflammation. This hypothesis is also supported by the data regarding the cytokine profiles within MLN cells co-cultured with the relevant strains.

Although the current data obtained *in vitro* with individual strains are unlikely to adequately represent reactions to mixtures of gut microbiota *in vivo*, illustrating the impact of dominant species on intestinal epithelial cells and MLN cells may help to identify the underlying mechanisms that influence intestinal homeostasis. Further investigation is required to elucidate ligand-receptor interaction among fecal microbes, intestinal epithelial cells and immune cells, which will help to elucidate why the fecal strains had varying degrees of impact on Caco-2 cells and MLN cells. By phenotyping mice with allergic diarrhea, we clearly substantiated that relevant microbes involved in allergen-induced dysbiosis play an important role in intestinal cytokine/chemokine network and allergen-induced T-cell responses. These results will be essential for future studies to evaluate whether changes in microbiota composition as observed in this study can prospectively achieve induction of a phenotype protected from food allergy development.

CONFLICT OF INTEREST

No potential conflicts of interest were reported by the authors.

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