ABSTRACT

We aimed to investigate associations of dietary diversity (DD) with gut microbial diversity and the development of hen’s egg allergy (HEA) in infants. We enrolled 68 infants in a high-risk group and 32 infants in a control group based on a family history of allergic diseases. All infants were followed from birth until 12 months of age. We collected infant feeding data, and DD was defined using 3 measures: the World Health Organization definition of minimum DD, food group diversity, and food allergen diversity. Gut microbiome profiles and expression of cytokines were evaluated by bacterial 16S rRNA sequencing and real-time reverse transcriptase-polymerase chain reaction. High DD scores at 3 and 4 months were associated with a lower risk of developing HEA in the high-risk group, but not in the control group. In the high-risk group, high DD scores at 3, 4, and 5 months of age were associated with an increase in Chao1 index at 6 months. We found that the gene expression of IL-4, IL-5, IL-6, and IL-8 were higher among infants who had lower DD scores compared to those who had higher DD scores in high-risk infants. Additionally, high-risk infants with a higher FAD score at 5 months of age showed a reduced gene expression of IL-13. Increasing DD within 6 months of life may increase gut microbial diversity, and thus reduce the development of HEA in infants with a family history of allergic diseases.

Keywords: Diet; Egg allergy; Food allergy; Infant; Gut microbiome; Immune tolerance
INTRODUCTION

The prevalence of immediate-type food allergy (FA) ranges from 2% to 10% in all ages with an increasing trend during the last two to three decades and is affected by regions, age, ethnicity, birth season, frequency of dietary exposure, and cooking methods (1-3). Although the standard management strategy for FA is avoidance of food, it can cause many problems such as poor quality of life, social difficulties, and psychological stress on patients and their families (2). Therefore, it is important to identify risk factors for the development of FA and implement preventive measures for high-risk infants.

Previous studies have shown that early introduction of solid foods has a protective effect on the development of FA and food sensitization (1,4,5), and recent guidelines recommended the introduction of complementary foods within the first six months of age (5,6). Dietary diversity (DD), the number of various food items consumed during infancy, has been also reported to protect allergic diseases including FA and asthma (7,8). Recently, Venter et al. (8) demonstrated that increased DD at six and nine months of age reduced the risk of FA development during childhood in a large-scale birth cohort study. They also showed that the likelihood of FA over ten years of life was decreased by additional intake of food items by six months or allergenic foods by one year, suggesting a positive role of early and diverse food introduction in the prevention of FA (8). Early Ag exposure through the gastrointestinal tract is associated with the prevention of allergic sensitization and the acquisition of oral tolerance by stimulating goblet cells to facilitate the maintenance of preexisting Treg cells and produce the IL-10 (9). Additionally, gut epithelial barrier dysfunction and allergic sensitization are induced by low diversity of fecal microbiota (10,11).

Although additional intake of diverse foods during infancy is inversely related to the odds of FA, the exact mechanism of this association remains unclear. Furthermore, the effects of dietary habits on immunologic alterations or allergy outcomes may differ between infants with and without risk factors for allergies (4,12,13). Therefore, we investigated whether DD during infancy could lower the risk of developing IgE-mediated FA in infants with a family history of allergic diseases. Additionally, the association of cytokines with DD during the first year of life was investigated.

MATERIAL AND METHODS

Study population and clinical evaluation

In this prospective birth cohort study, we screened 157 pregnant women at ≥28 weeks gestation and finally enrolled 68 infants in a high-risk group and 32 infants in a control group. At the time of enrollment, parents completed a questionnaire regarding basic demographic information, and underwent skin prick tests (SPTs). On the basis of family history of allergic diseases and SPT responses, the high-risk group was defined when they met one of two criteria: (1) at least one parent had both a positive SPT and a history of asthma or allergic rhinitis; and (2) at least one parent or sibling had physician-diagnosed atopic dermatitis (AD). The control group was determined when both parents had neither an allergy history nor a positive SPT.

All infants were serially followed up from birth until twelve months of age. We evaluated the presence of AD and FA, and obtained dietary data at two, six, and twelve months of age. We collected stool samples at six months of age. When the parents reported any suspicious
allergic symptoms, the infants were brought to the outpatient clinic and examined by an allergy specialist. Diagnosis of FA was based on a positive oral food challenge test or convincing history of adverse reactions within 2 h of food ingestion plus positive serum specific IgE (sIgE, ≥0.1 kU/L). Immediate reactions included urticaria, edema, cough, wheezing, vomiting, diarrhea, hypotension and altered mentality. This study was approved by the Institutional Review Board (IRB No. SMC-2016-12-111), and written informed consent was obtained from all the parents prior to participation in this study.

**Dietary data and diversity**

The families were surveyed using a standardized questionnaire regarding when the infants first started eating ten different food groups (grains, vegetables, fruits, meat, fish, eggs, dairy, wheat, peanuts, and legumes/nuts) every month from three months to twelve months. The food group diversity (FGD) was calculated by summing the number of ten food groups introduced in the child’s diet (maximum count of ten). The World Health Organization (WHO) DD was defined as the sum of the number of food groups such as grains, legumes/nuts, dairy, flesh foods, eggs, vitamin A-rich fruits and vegetables, and other fruit and vegetables (maximum count of seven) ([8](https://doi.org/10.4110/in.2022.22.e17)). The food allergen diversity (FAD) score was calculated by summing the number of main food allergens such as milk, egg, wheat, fish, soy, peanut, tree nuts, and sesame (maximum count of eight) included at each time point ([14](https://immunenetwork.org)).

**SPTs**

A skin prick test (SPT) was performed on the volar aspects of the forearms using the following nine inhalant allergens in pregnant women and their husbands: *Dermatophagoides pteronyssinus*, *D. farinae*, tree pollen mixture I (*Alnus glutinosa*, *Corylus avellana*, *Populus sp.*, *Ulmus scabra*, and *Salix caprea*), birch pollen, ragweed, weed pollen mixture (*Artemisia vulgaris*, *Urtica dioica*, *Tanaxacum vulgare*, and *Plantago lanceolata*), grass pollen mixture (*Holcus lanatus*, *Dactylis glomerata*, *Lolium perenne*, *Phleum pratense*, *Poa pratensis*, and *Festuca pratensis*), cat dander, and cockroach. Histamine was used as a positive control and normal saline as a negative control. All of the above allergens were provided by Allergopharma, Reinbek, Germany. The SPT was regarded as positive if the wheal diameter was ≥3 mm and controls showed adequate reactions.

**Blood tests**

The 25(OH) D levels were measured by a Unicel DxI 800® chemiluminescent immunoassay (Beckman Coulter Inc., Brea, CA, USA), and IgE levels were determined by the Immuno-CAP (Thermo Fisher Scientific, Waltham, MA, USA) system. Genomic DNA was extracted from peripheral blood leukocytes and was genotyped for three filaggrin (*FLG*) null variants (3321delA, K4022X, and S3296X), which are common among Koreans, by direct DNA sequencing as described previously ([15](https://doi.org/10.4110/in.2022.22.e17)).

**Measurements of cytokine mRNA expression**

Peripheral blood mononuclear cells were purified from peripheral blood using standard Ficoll-Paque™ PLUS (GE Healthcare, Chicago, IL, USA) gradient centrifugation according to the instructions of the manufacturer. RNeasy Mini Kits (Qiagen, Germantown, MD, USA) were used for RNA extraction according to the manufacturer’s protocol. RNA was reverse-transcribed into cDNA using the SuperScript VILO MasterMix (Invitrogen, Carlsbad, CA, USA). Real-time RT-PCR was performed and analyzed by the dual-labeled fluorogenic probe method using an ABI Prism 7900 sequence detector (Applied Biosystems, Foster City, CA, USA). Primers and probes for 18S, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, TSLP, TNF-α, and TGF-β were purchased from Applied Biosystems.
**Bacterial 16S rRNA sequencing of fecal samples**

The hypervariable regions V3–V4 of the bacterial 16S rRNA gene was amplified on stool samples collected at six months of age and sequenced on the Illumina MiSeq platform (Illumina, San Diego, CA, USA). The 16S rRNA gene sequence data were analyzed using the Quantitative Insights Into Microbial Ecology software package (v1.9.1) (16). Using qualified sequences (Phred ≥Q20), the operational taxonomic units (OTUs) were identified and quantified using the open reference method that maps sequences with 97% identity to known sequence in the Greengenes database (v13_8) using UCLUST alignment algorithms and the EzBioCloud database (http://www.ezbiocloud.net) (17-19).

The alpha diversity was carried out using the phylogenetic distance and the detected number of species metrics. The microbial beta-diversity was compared using the Bray-Curtis dissimilarity coefficient. The linear discriminant analysis (LDA) effect size was used to identify biologically and statistically significant differences in OTU relative abundance according to DD (20). An LDA score of >3 (among OTUs with >1% relative abundance in any group) was used to determine significant differences in abundance of OTUs.

**Statistical analysis**

Data were analyzed using SPSS for Windows (version 27.0; SPSS, Chicago, IL, USA) and Graphpad Prism (version 9.00; Graphpad Software, San Diego, CA, USA). A χ² test and Fisher’s exact test were applied to determine the differences in the proportions based on normal or non-normal distribution. A Mann-Whitney U test was used to compare continuous variables between two groups.

Univariable and multivariable logistic regression analyses were conducted to determine the effect of DD scores on the development of FA in high-risk infants. Variables for adjustment included sex, family history of allergic diseases, mode of delivery, FLG mutation, season of birth, maternal education levels, monthly household income, the presence of AD during the twelve months after birth, the presence of older siblings, antibiotic treatment during the first two months of life, the age of introduction of solid foods, and serum concentration of 25(OH)D. Variables with a p-value <0.1 in univariable analysis were chosen for the multivariable analysis. A p-value <0.05 was considered significant.

**RESULTS**

**Clinical characteristics of study population**

Among subjects who met the eligibility criteria, 54 out of 68 infants in the high-risk group and 27 out of 32 infants in the control group completed the study (Table 1). Overall, 8/54 (14.8%) and 1/27 (3.7%) infants developed FA in the high-risk group and the control group, respectively (p=0.259). Hen’s egg allergy (HEA) developed in eight infants, while four out of eight had cow’s milk allergy and one had soy allergy, as well. Since all patients with FA had HEA, we focused on HEA in the analyses. No infants were diagnosed with FA before six months of age.

**Effects of DD on the development of egg allergy**

The FGD, WHO DD, and FAD scores are shown in Fig. 1. In the high-risk group, univariable analysis showed that the diagnosis of AD was associated with the development of HEA in the first year of life (odds ratio [OR], 28.8; 95% confidence intervals [CIs], 3.13–264.52) (Table 2).
After adjusting for the mode of delivery, the diagnosis of AD, and the use of antibiotics before two months of age, the multivariable analysis revealed that high FGD, WHO DD, and FAD scores at three and four months of age were less likely to develop HEA in the high-risk group (Table 3 and Fig. 2). A FAD score ≥1 at five months of age was also associated with a decreased risk of developing HEA among high-risk infants (adjusted OR, 0.06; 95% CI, 0–0.77) (Table 3 and Fig. 2). However, there was no association of FGD, WHO DD, and FAD scores at six months to twelve months with the development of HEA. In the control group, there were no significant factors or DD scores influencing the development of HEA in the univariable and multivariable analyses (all p>0.05).

**Association of DD scores with gut microbial diversity**
The fecal Chao1 indices at six months of age were higher in high-risk infants who had higher FGD, WHO DD, and FAD scores at three months and four months of age compared to those who had lower FGD, WHO DD, and FAD scores (all p<0.05) (Fig. 3). Additionally, Chao1 indices were also higher in high-risk infants with a higher FAD score at five months of age.
than those with a lower FAD score (p=0.004) (Fig. 3). Higher Shannon indices were also found in high-risk infants with higher WHO DD scores at four months of age and FAD scores at four and five months than those with lower WHO DD and FAD scores (all p=0.038) (Fig. 3). However, there were no differences in Chao1 or Shannon indices according to DD score at six months.

Figure 1. FGD (A), WHO DD (B), and FAD (C) scores at each time point. FGD score was defined as the sum of the number of ten different food groups (grains, legumes/nuts, dairy, flesh foods, eggs, vitamin A-rich fruits, and vegetables). WHO DD score and FAD score were calculated by summing of the number of seven food groups (grains, legumes/nuts, dairy, flesh foods, eggs, vitamin A-rich fruits and vegetables, and other fruit and vegetables) and eight food allergen groups (milk, egg, wheat, fish, soy, peanut, tree nuts, and sesame), respectively.
months to twelve months. Additionally, no differences were observed in Chao1 or Shannon indices between infants with higher and lower DD scores in the control group.

**Association of DD scores with cytokine expressions**

When DD scores from three months to six months of age were divided into two groups according to the median values, mRNA expression of IL-4, IL-5, IL-6, and IL-8 were higher in high-risk infants who had lower DD scores compared to those who had higher DD scores.
Additionally, high-risk infants with a higher FAD score at five months of age showed a reduced gene expression of IL-13 (p=0.029) (Table 4).

**DISCUSSION**

In a birth cohort study, the Protection Against Allergy Study in Rural Environment/EFRAIM, Roduit et al. (7) demonstrated that increased DD in the first year of life is inversely associated
with doctor-diagnosed FA with a dose-response effect. Venter et al. (8) also showed that high infant DD decreased the likelihood of developing FA over the first decade of life in the Food Allergy and Intolerance Research birth cohort. Both studies analyzed data from a large study population in Europe during a long-term period and emphasized the importance of DD during infancy for prevention of FA. To our knowledge, the present study was the first research in Asia to investigate the association between DD during infancy and the development of HEA. Our findings in Korean infants are consistent with those two prior studies, indicating that DD should be considered in relation with FA for Asian populations as well. We also found that high-risk infants with high DD scores showed an increased gut microbial diversity and a downregulation of IL-4, IL-5, IL-6, IL-8, and IL-13 compared to

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**Figure 3.** Gut microbial diversity in infants at age 3 (A) and 4 (B) months according to the food group diversity, at age 3 (C) and 4 (D) months according to World Health Organization dietary diversity and at age 3 (E), 4 (F) and 5 (G) months according to food allergen diversity. The central box in each box plot indicates the interquartile range and median, while the upper and lower lines indicate the maximum and minimum values, respectively.
Table 4. Relative gene expression of cytokines at 12 months of age according to dietary diversity scores in high-risk infants

| Cytokines | Food group diversity score | WHO diet diversity score | Food allergen diversity score |
|-----------|-----------------------------|--------------------------|-------------------------------|
|           | 0 (0–7.34) | 0 (0–9.04) | 0 (0–9.05) | 0 (0–9.72) | 0 (0–9.72) | 0 (0–9.72) | 0.066 | 0.066 | 0.066 | 0.066 | 0.066 | 0.066 |
| IL-4      | 11.57 (0.00–15.48) | 14.00 (2.26–15.48) | 14.00 (2.26–15.48) | 0 (0–7.34) | 0 (0–9.04) | 0 (0–9.04) | 0 (0–9.04) | 0 (0–9.04) | 0 (0–9.04) | 0 (0–9.04) | 0 (0–9.04) | 0 (0–9.04) |
| IL-5      | 0.046 | 0 (0–7.34) | 0 (0–9.04) | 0 (0–9.04) | 0 (0–9.04) | 0 (0–9.04) | 0 (0–9.04) | 0 (0–9.04) | 0 (0–9.04) | 0 (0–9.04) | 0 (0–9.04) | 0 (0–9.04) |
| IL-6      | 5.35 (1.89–5.83) | 1.90 (0.96–2.93) | 1.90 (0.96–2.93) | 1.90 (0.96–2.93) | 1.90 (0.96–2.93) | 1.90 (0.96–2.93) | 1.90 (0.96–2.93) | 1.90 (0.96–2.93) | 1.90 (0.96–2.93) | 1.90 (0.96–2.93) | 1.90 (0.96–2.93) | 1.90 (0.96–2.93) |
| IL-8      | 1.19 (0.00–1.83) | 1.15 (0.00–1.83) | 1.15 (0.00–1.83) | 1.15 (0.00–1.83) | 1.15 (0.00–1.83) | 1.15 (0.00–1.83) | 1.15 (0.00–1.83) | 1.15 (0.00–1.83) | 1.15 (0.00–1.83) | 1.15 (0.00–1.83) | 1.15 (0.00–1.83) | 1.15 (0.00–1.83) |
| IL-10     | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) |
| IL-13     | 1.08 (0.00–1.83) | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) |
| TSLP      | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) |
| TNF-α     | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) |
| TGF-β     | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) | 1.06 (0.00–1.83) |

*1 at 3 months, 1 at 4 months, 3 at 5 months, and 4 at 6 months of age; †1 at 3 months, 1 at 4 months, 3 at 5 months, and 5 at 6 months of age; ‡1 at 3 months, 1 at 4 months, 3 at 5 months, and 6 at 6 months of age.
route of exposure to food allergens, and other factors \((21,22)\). In our cohort, eight of 54 infants in the high-risk group (14.8%) developed an HEA. Among those eight patients with HEA, four and one were also allergic to cow’s milk and soybean, respectively. Interestingly, we found that DD of the three different measures along with microbial diversity, but before the introduction of egg proteins, were contributed to reducing the development of HEA among high-risk infants.

Treg cells play a significant role in immune system regulation toward FA \((9)\). Therefore, before the introduction of allergenic foods, the gut microenvironment capacity to promote Treg cell induction plays a key role in developing an acquired oral tolerance against food Ags \((9)\). In this regard, gut microbiota development during this period is important, because gut microbiota interact with the innate and adaptive immune system within the mucosa through microbe-derived metabolites to support the induction of tolerance \((21)\). In a study by Roduit et al., \((7)\) children with low DD had a reduced expression of Foxp3 and an increased expression of \(\epsilon\) germline transcripts, which are markers of Treg cells and Ab isotype switching to IgE. Meanwhile, it has been reported that gut microbiota development in infants is influenced by the mode of delivery, feeding patterns, and antibiotics usage \((23-25)\). Based on our observations, the protective effect of DD is significantly associated with gut microbial diversity.

In our study of Korean infants, we found that vegetables, cereals, and cow’s milk introduced at three to five months of age may contribute to gut microbial diversity at age six months. Oral tolerance to hen’s eggs seems to be acquired through introduction of egg protein between six and twelve months of age, although we did not intervene for preventive purposes. Our observation about timing of food introduction is compatible with recent recommendations by the experts in the US and Canada to introduce allergenic foods such as eggs and peanuts around six months of age, though not before four months of age \((26)\). Unfortunately, we did not investigate how altered gut microbiota affects host immune status to induce tolerance to food allergens.

Of note, DD during early infancy contributed to gut microbial diversity and a decrease in development of HEA only in the high-risk group. In other words, our control group did not show a positive association between DD and gut microbial diversity. This finding is not surprising, because microbiota responses to dietary intervention are known to vary considerably between subjects; therefore, subjects with low and high microbiota richness often respond differently to dietary intervention \((23)\). Similar to our study, early introduction of hen’s egg in high-risk infants with eczema decreased the prevalence of HEA \((1,27)\), whereas a randomized trial of introduction of allergenic food at three months of age in standard-risk infants did not show a statistically significant prevention of HEA in the intention-to-treat analysis \((13)\). We do not yet understand how the immunologic status of high-risk infants with a family history of allergic diseases differs from that of the control group and how it changes according to feeding patterns. It means that either DD or gut microbiota is not the only factor to induce oral tolerance to allergenic foods in the control group. There may be a complex interplay among multiple factors and/or hidden factors in the prevention of FA. Further research is necessary to elucidate the development of host immunity in relation to gut microbiota and dietary patterns.

Since all patients with FA in the present study had HEA, the results of this study cannot be generalized to types of FA other than eggs and should be interpreted carefully. In addition, our sample size was small, and a long-term follow-up was not performed after infancy.
However, the management of FA during the first year of life is also a huge burden on parents (2). Therefore, the results of the present study are clinically meaningful in that we analyzed the relationship between DD and the development of HEA for the first time in Asia, showing the protective effects of DD against HEA in high-risk infants regardless of ethnicity. Our study also demonstrates the importance of DD in three to five-month-old infants at an earlier age than previous studies, along with its association with gut microbial diversity and cytokine profiles.

In conclusion, our results suggest that greater food diversity within the first 6 months of life may increase gut microbial diversity and reduce the development of IgE-mediated HEA during infancy. Exposure of the gastrointestinal tract to various foods early in life could be beneficial to establish oral immune tolerance and prevent HEA in children with a family history of allergic diseases.

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