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Original Article

Dietary Diversity during Early Infancy Increases Microbial Diversity and Prevents Egg Allergy in High-Risk Infants

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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

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Conflict of Interest
The authors declare no potential conflicts of interest.

Abbreviations
AD, atopic dermatitis; CI, confidence interval; DD, dietary diversity; FA, food allergy; FAD, food allergen diversity; FGD, food group diversity; FLG, filaggrin; HEA, hen's egg allergy; OR, odds ratio; OTU, operational taxonomic unit; sIgE, specific IgE; SPT, skin prick test; WHO DD, World Health Organization definition of minimum dietary diversity.

Author Contributions
Conceptualization: Lee BR, Ahn K, Kim J; Data curation: Lee BR, Jung HI, Kim SK, Kwon M, Kim H, Jung M, Kyung Y, Kim BE, Choi SJ, Oh SY, Ahn K, Kim J; Formal analysis: Lee BR, Baek SY, Kim S, Bae J, Ahn K, Kim J; Resources: Choi SJ, Oh SY, Ahn K, Kim J; Supervision: Ahn K, Kim J; Writing - original draft: Lee BR, Ahn K, Kim J; Writing - review & editing: Lee BR, Jung HI, Kim SK, Kwon M, Kim H, Jung M, Kyung Y, Kim BE,Choi SJ, Oh SY, Baek SY, Kim S, Bae J, Ahn K, Kim J.

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 prevent 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). 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).

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*, *Taraxacum 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 ImmunoCAP (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).

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 by 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 χ^2 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**).

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Table 1. Clinical characteristics of subjects

Characteristics	High-risk group (n = 54)	Control group (n = 27)	p-value
Sex (male)	16 (29.6)	8 (29.6)	0.606
Mode of delivery (cesarean section)	23 (42.6)	8 (29.6)	0.258
Season of birth			0.105
Spring	15 (27.8)	11 (40.7)	0.239
Summer	13 (24.1)	1 (3.7)	0.028
Autumn	9 (16.7)	4 (14.8)	1.000
Winter	17 (31.5)	11 (40.7)	0.409
Preterm birth	3 (5.6)	3 (11.1)	0.395
Presence of atopic dermatitis			
At age 2 mon	3 (5.6)	1 (3.7)	1.000
At age 6 mon	12 (22.2)	2 (7.4)	0.125
At age 12 mon	15 (27.8)	3 (11.1)	0.089
Presence of older siblings	21 (38.9)	8 (29.6)	0.413
Maternal education level			0.938
College degree	48 (88.9)	26 (96.3)	0.658
High school degree or less	6 (11.1)	1 (3.7)	0.658
Use of systemic antibiotics			
At age <2 mon	8 (14.8)	7 (25.9)	0.534
At age 3–6 mon	14 (25.9)	18 (66.7)	0.302
At age 7–12 mon	34 (63.0)	22 (81.5)	0.631
Introduction of solid foods before 6 mon	33 (61.1)	16 (59.3)	1.000
Food allergy			
Egg	8 (14.8)	1 (3.7)	0.259
Cow's milk	4 (7.4)	0	0.296
Soy	1 (1.9)	0	1.000
Filaggrin gene variants	8 (14.8)	1 (3.7)	0.259
3321delA			1.000
AA	54 (100.0)	26 (100.0)	
Aa	0	0	
aa	0	0	
K4022X			0.485
AA	53 (98.1)	26 (100.0)	
Aa	1 (1.9)	0	
aa	0	0	
S3296X			1.000
AA	54 (100.0)	26 (100.0)	
Aa	0	0	
aa	0	0	

Data are presented as number (%).

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

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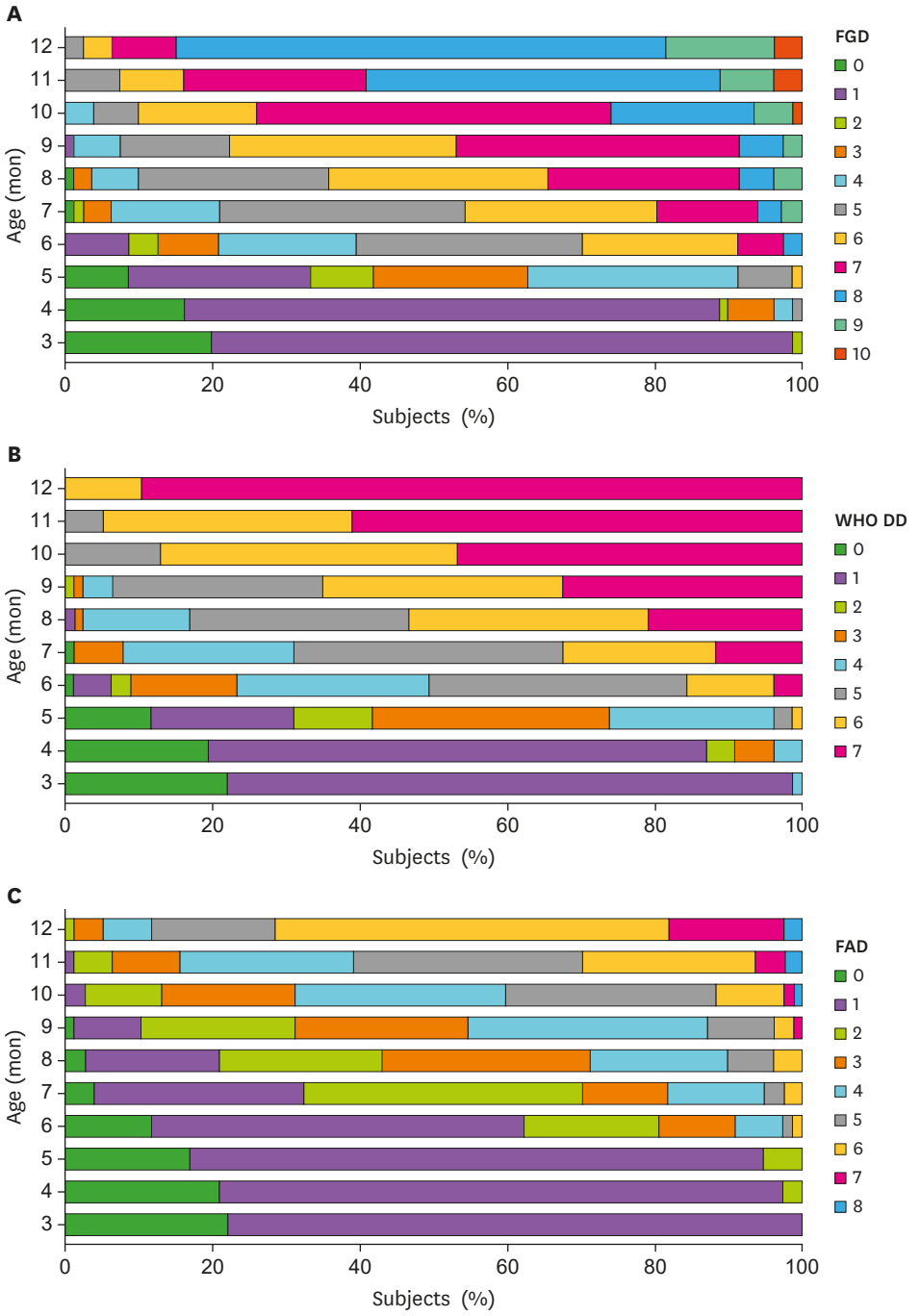

Figure 1. FGD (A), WHO DD (B), and FAD (C) scores at each time point. FGD score was defined as the sum of the number of ten different food groups (grains, legumes/nuts, dairy, flesh foods, eggs, vitamin A-rich fruits, and vegetables). WHO DD score and FAD score were calculated by summing of the number 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.

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

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Table 2. Univariable analyses for baseline characteristics influencing development of egg allergy in high-risk infants

Variables	Food allergy	
	OR (95% CI)	p-value
Male sex	0.85 (0.15–4.75)	0.849
C-sec delivery	0.16 (0.02–1.37)	0.094
FLG mutation	0.73 (0.15–3.48)	0.692
Births in autumn and winter	2.75 (0.79–9.55)	0.111
Low levels of maternal education (≤high school degree)	1.46 (0.14–15.10)	0.749
Low income household (<US $3,400/mon)	3.50 (0.74–16.56)	0.114
Atopic dermatitis in the first year of life	28.80 (3.13–264.52)	0.003
Presence of older siblings	3.13 (0.66–14.79)	0.151
Use of antibiotics before 2 mon of age	4.92 (0.89–27.10)	0.067
Introduction of solid foods after 6 mon of age	1.71 (0.38–7.72)	0.488
25(OH)D <20 ng/mL	0.88 (0.09–8.49)	0.913

Table 3. Multivariable analyses for diet diversity scores influencing development of egg allergy in high-risk infants*

Variables	Adjusted OR (95% CI)	p-value
Food group diversity score		
≥1 at 3 mon	0.02 (0–0.56)	0.021
≥1 at 4 mon	0.02 (0–0.51)	0.019
≥3 at 5 mon	0.51 (0.04–5.82)	0.587
≥4 at 6 mon	0.24 (0.03–2.18)	0.204
≥5 at 7 mon	0.39 (0.04–4.12)	0.436
≥6 at 8 mon	0.13 (0.01–1.14)	0.065
≥6 at 9 mon	0.29 (0.03–3.15)	0.308
≥7 at 10 mon	0.67 (0.07–5.97)	0.718
≥8 at 11 mon	0.86 (0.12–6.42)	0.885
≥8 at 12 mon	0.20 (0.01–3.07)	0.251
WHO diet diversity score		
≥1 at 3 mon	0.03 (0–0.52)	0.016
≥1 at 4 mon	0.03 (0–0.43)	0.011
≥3 at 5 mon	0.70 (0.06–8.67)	0.785
≥5 at 6 mon	0.43 (0.03–5.83)	0.528
≥5 at 7 mon	4.03 (0.33–49.07)	0.275
≥6 at 8 mon	0.60 (0.07–5.32)	0.649
≥6 at 9 mon	0.44 (0.05–4.31)	0.481
≥6 at 10 mon	1.88 (0.12–29.61)	0.652
≥7 at 11 mon	1.55 (0.16–15.14)	0.707
≥7 at 12 mon	0.22 (0.01–6.87)	0.391
Food allergen diversity score		
≥1 at 3 mon	0.03 (0–0.52)	0.016
≥1 at 4 mon	0.03 (0–0.43)	0.011
≥1 at 5 mon	0.06 (0–0.77)	0.031
≥1 at 6 mon		1.000
≥5 at 7 mon	0.63 (0.07–5.68)	0.683
≥6 at 8 mon	0.22 (0.02–2.00)	0.177
≥6 at 9 mon	0.33 (0.04–3.01)	0.324
≥6 at 10 mon	0.96 (0.10–8.71)	0.968
≥7 at 11 mon	1.98 (0.22–17.84)	0.543
≥7 at 12 mon	1.37 (0.10–18.69)	0.813

*Adjusted for mode of delivery, atopic dermatitis in the first year of life, and use of antibiotics before 2 months of age.

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

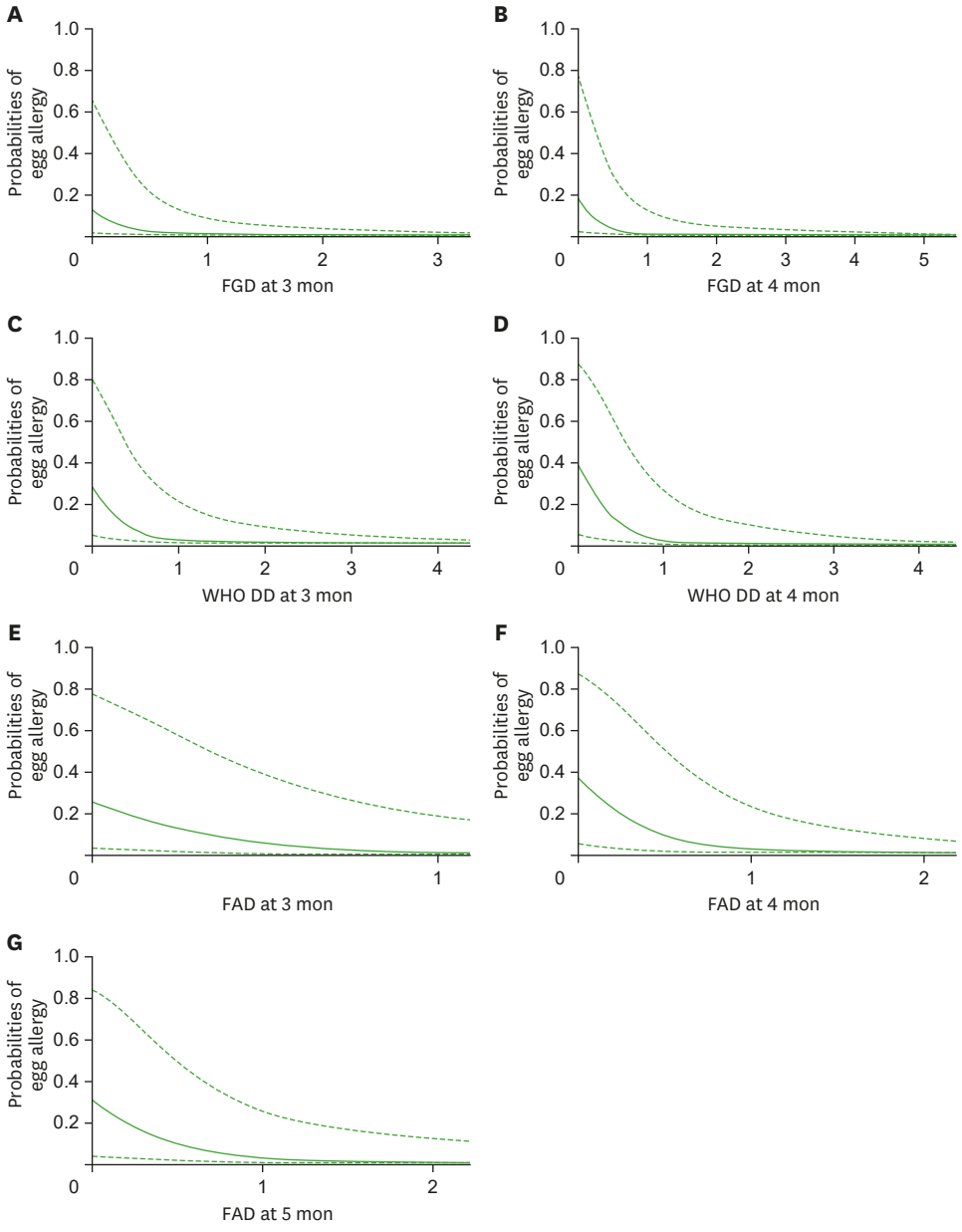

Figure 2. The food group diversity at age 3 (A) and 4 (B) months, World Health Organization dietary diversity at age 3 (C) and 4 (D) months, and food allergen diversity at age 3 (E), 4 (F), and 5 (G) months versus egg allergy in the high-risk group. Multivariable analyses (holding delivery mode = vaginal delivery, use of antibiotics before 2 months of age = no, and having atopic dermatitis ever = no) were performed. The solid line represents the predicted probabilities of developing egg allergy, and dashed lines represent the 95% confidence intervals.

(**Table 4**). 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

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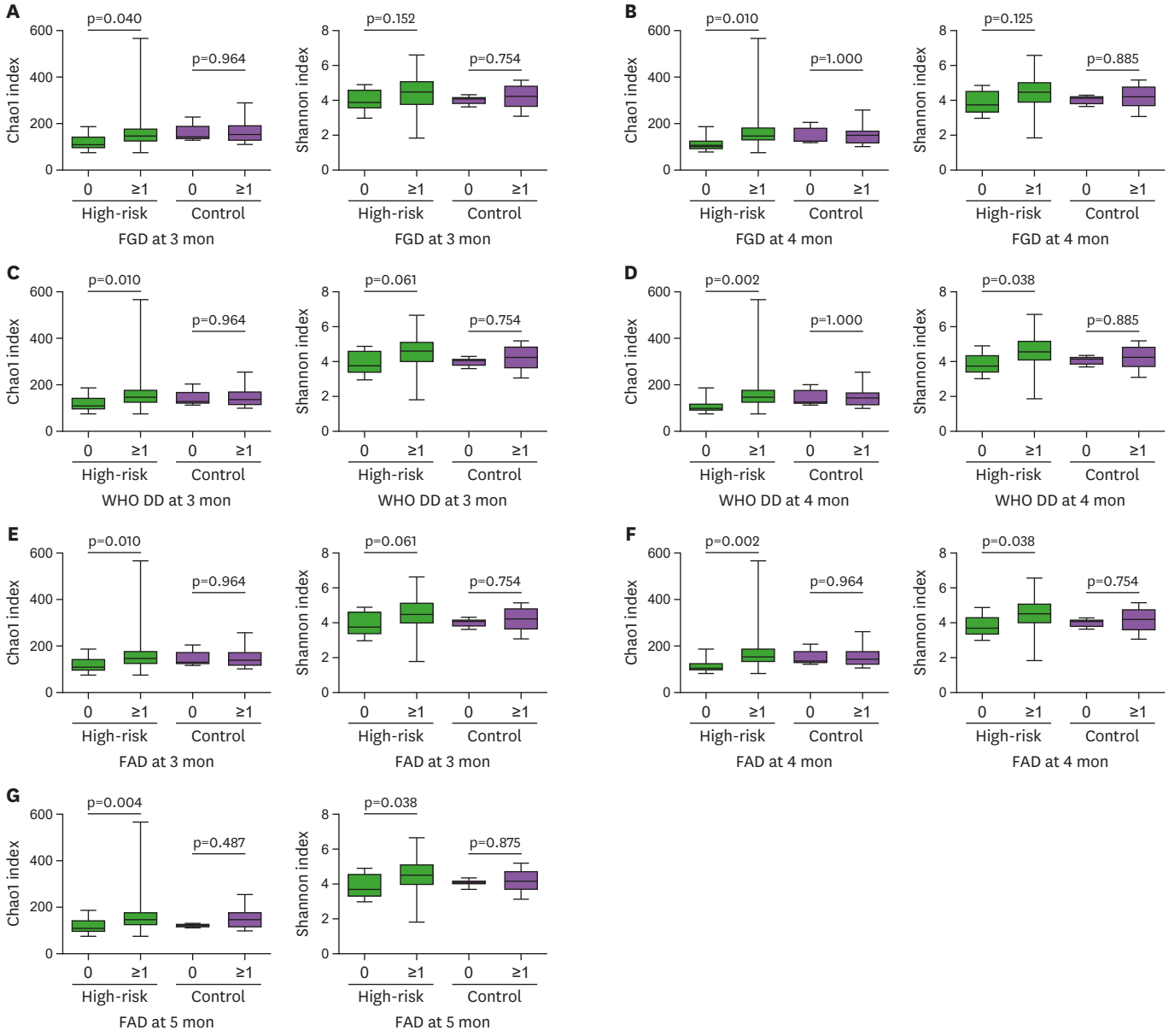

Figure 3. Gut microbial diversity in infants at age 3 (A) and 4 (B) months according to the food group diversity, at age 3 (C) and 4 (D) months according to World Health Organization dietary diversity and at age 3 (E), 4 (F) and 5 (G) months according to food allergen diversity. The central box in each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

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

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		
	<Median*	≥Median*	p-value	<Median†	≥Median†	p-value	<Median‡	≥Median‡	p-value
IL-4									
At 3 mon	11.57 (0.00–15.48)	0 (0–9.04)	0.080	14.00 (2.26–15.48)	0 (0–8.77)	0.006	14.00 (2.26–15.48)	0 (0–8.77)	0.006
At 4 mon	14.08 (0.00–15.77)	0 (0–9.02)	0.029	14.00 (2.26–15.48)	0 (0–8.77)	0.006	14.00 (2.26–15.48)	0 (0–8.77)	0.006
At 5 mon	0 (0–14.00)	0 (0–8.96)	0.294	0 (0–13.28)	0 (0–9.05)	0.560	13.92 (0.00–14.62)	0 (0–9.02)	0.035
At 6 mon	4.53 (0300–14.21)	0 (0–9.30)	0.369	0 (0–11.27)	2.83 (0.00–9.72)	0.778	11.57 (2.26–14.48)	0 (0–9.72)	0.181
IL-5									
At 3 mon	7.49 (0.70–18.74)	0 (0–7.34)	0.032	7.49 (0.00–18.74)	0 (0–7.34)	0.104	7.49 (0.00–18.74)	0 (0–7.34)	0.104
At 4 mon	7.55 (0.00–20.00)	0 (0–7.06)	0.046	7.49 (0.00–18.74)	0 (0–7.34)	0.104	7.49 (0.00–18.74)	0 (0–7.34)	0.104
At 5 mon	7.43 (0.00–14.33)	0 (0–3.65)	0.354	3.72 (0.00–13.29)	0 (0–4.72)	0.105	7.43 (0.00–14.95)	0 (0–8.16)	0.305
At 6 mon	0 (0–9.40)	0 (0–9.51)	0.942	0 (0–12.88)	0 (0–5.79)	0.240	11.25 (7.46–21.38)	0 (0–7.34)	0.008
IL-6									
At 3 mon	1.90 (0.77–6.76)	2.75 (1.49–4.20)	0.838	3.93 (1.19–11.56)	2.54 (1.41–3.91)	0.347	3.93 (1.19–11.56)	2.54 (1.41–3.91)	0.347
At 4 mon	1.93 (0.96–7.03)	2.65 (1.42–4.12)	0.753	3.93 (1.19–11.56)	2.54 (1.41–3.91)	0.347	3.93 (1.19–11.56)	2.54 (1.41–3.91)	0.347
At 5 mon	3.71 (1.90–6.37)	1.88 (1.04–3.34)	0.012	3.55 (1.89–5.83)	1.90 (1.13–3.53)	0.041	1.93 (0.96–7.03)	1.65 (1.42–4.12)	0.732
At 6 mon	3.83 (0.89–6.87)	2.25 (1.43–3.78)	0.445	2.37 (1.15–5.83)	2.64 (1.15–5.83)	0.533	3.93 (1.20–6.76)	2.54 (1.41–4.20)	0.528
IL-8									
At 3 mon	2.89 (0.72–5.28)	1.08 (0.71–2.77)	0.149	3.82 (1.59–5.51)	1.05 (0.66–2.61)	0.012	3.82 (1.59–5.51)	1.05 (0.66–2.61)	0.012
At 4 mon	3.13 (1.24–2.76)	1.06 (0.66–2.76)	0.045	3.82 (1.59–5.51)	1.05 (0.66–2.61)	0.012	3.82 (1.59–5.51)	1.06 (0.66–2.73)	0.013
At 5 mon	1.24 (0.68–4.42)	1.06 (0.67–2.25)	0.200	1.20 (0.58–4.32)	1.09 (0.71–2.54)	0.401	3.13 (1.24–5.43)	1.06 (0.66–2.76)	0.049
At 6 mon	3.33 (0.52–4.75)	1.08 (0.79–2.67)	0.186	1.20 (0.90–4.12)	0.99 (0.61–2.54)	0.165	3.82 (1.71–5.51)	1.08 (0.66–2.77)	0.031
IL-10									
At 3 mon	5.34 (3.79–10.42)	6.27 (2.28–14.33)	0.903	6.59 (4.98–10.42)	5.59 (2.25–14.60)	0.701	6.59 (4.98–10.42)	5.59 (2.25–14.60)	0.701
At 4 mon	5.62 (4.95–11.20)	5.74 (2.23–14.05)	0.775	6.59 (4.98–10.42)	5.59 (2.25–14.60)	0.701	6.59 (4.98–10.42)	5.59 (2.25–14.60)	0.701
At 5 mon	5.62 (3.30–11.65)	5.74 (2.20–14.61)	0.713	6.59 (3.35–11.61)	5.40 (2.22–14.61)	0.815	7.57 (4.95–11.20)	5.45 (2.27–14.60)	0.632
At 6 mon	6.59 (2.88–10.82)	5.60 (2.31–13.78)	0.940	6.59 (2.49–11.85)	5.59 (2.44–15.03)	0.709	6.84 (5.12–11.23)	5.59 (2.31–13.78)	0.693
IL-13									
At 3 mon	1.73 (0.10–2.35)	1.17 (0.00–1.85)	0.496	2.12 (0.37–2.50)	1.17 (0.00–1.81)	0.120	2.12 (0.37–2.50)	1.15 (0.00–1.83)	0.120
At 4 mon	1.98 (0.00–2.38)	1.12 (0.00–1.80)	0.333	2.12 (0.37–2.50)	1.15 (0.00–1.83)	0.120	1.98 (0.00–2.46)	1.15 (0.00–1.83)	0.120
At 5 mon	1.48 (0.00–2.44)	1.15 (0.00–1.83)	0.400	1.48 (0.12–2.47)	1.15 (0.00–1.83)	0.228	2.27 (1.48–2.55)	1.13 (0.00–1.81)	0.029
At 6 mon	1.88 (6.30–2.76)	1.15 (0.00–1.85)	0.108	1.28 (0.00–2.30)	1.15 (0.00–1.85)	0.587	1.88 (0.37–2.35)	1.17 (0.00–1.87)	0.531
TSLP									
At 3 mon	2.84 (1.21–3.88)	1.67 (1.09–2.72)	0.155	2.84 (1.55–3.88)	1.63 (1.09–2.72)	0.056	2.84 (1.55–3.88)	1.63 (1.09–2.72)	0.056
At 4 mon	3.35 (1.31–3.94)	1.65 (1.10–2.64)	0.064	2.84 (1.55–3.88)	1.63 (1.09–2.72)	0.056	2.84 (1.55–3.88)	1.65 (1.12–2.79)	0.066
At 5 mon	1.94 (1.22–3.31)	1.65 (1.15–2.97)	0.738	1.82 (1.17–3.05)	1.67 (1.15–3.01)	0.987	2.32 (1.31–3.94)	1.65 (1.10–2.88)	0.137
At 6 mon	2.34 (1.51–3.50)	1.65 (1.13–2.88)	0.145	1.93 (1.33–33.06)	1.60 (1.10–3.00)	0.584	2.84 (1.57–4.24)	1.67 (1.13–2.92)	0.178
TNF-α									
At 3 mon	1.22 (0.83–1.88)	1.17 (0.49–1.83)	0.860	1.37 (1.04–2.11)	1.12 (0.78–1.78)	0.245	1.37 (1.04–2.11)	1.12 (0.78–1.78)	0.245
At 4 mon	1.28 (1.00–1.83)	1.15 (0.78–1.83)	0.567	1.37 (1.04–2.11)	1.12 (0.78–1.78)	0.245	1.37 (1.04–2.11)	1.12 (0.78–1.78)	0.245
At 5 mon	1.09 (0.96–1.64)	1.38 (0.67–1.85)	0.789	1.08 (0.96–1.74)	1.29 (0.70–1.84)	0.796	1.46 (1.00–2.14)	1.15 (0.78–1.74)	0.214
At 6 mon	1.00 (0.52–2.05)	1.19 (0.81–1.83)	0.557	1.12 (0.85–1.84)	1.28 (0.78–1.76)	0.798	1.65 (1.07–2.11)	1.15 (0.78–1.78)	0.300
TGF-β									
At 3 mon	4.33 (3.33–6.24)	3.76 (2.79–5.24)	0.347	4.33 (3.39–4.24)	3.76 (2.78–5.24)	0.280	4.33 (3.39–4.24)	3.76 (2.78–5.24)	0.280
At 4 mon	4.49 (3.81–6.70)	3.69 (2.81–5.16)	0.164	4.33 (3.39–6.24)	3.76 (2.78–5.24)	0.280	4.33 (3.39–6.24)	3.76 (2.78–5.24)	0.280
At 5 mon	4.03 (3.21–5.75)	4.03 (3.21–5.75)	0.302	4.16 (3.19–5.76)	3.82 (2.58–4.93)	0.284	4.49 (3.25–6.70)	3.75 (2.78–4.98)	0.209
At 6 mon	3.47 (2.74–5.12)	4.09 (2.99–5.33)	0.698	4.16 (2.86–5.64)	3.82 (2.93–4.93)	0.646	5.77 (3.59–7.12)	3.82 (2.88–4.98)	0.135

*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, 1 at 5 months, and 1 at 6 months of age.

those with low DD scores. In addition to the results from prior studies in Europe (7,8), our study suggests that infant DD should begin as early as three months to four months of age, and a decreased risk of developing HEA may be mediated by gut microbial diversity during infancy. Our observation provides an important basis for understanding the mechanisms of FA development and establishing preventive measures during infancy.

FA is a consequence of immune dysregulation and loss of normal immune tolerance, conditions that are influenced by host immune status, age, gut microbiome, barrier defects,

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 Cε 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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