Neonatal gut microbiota and risk of developing food sensitization and allergy

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Background: Food sensitization (FS) develops in early infancy and is a risk factor for subsequent food allergy (FA). Recent evidence suggests relationships of gut microbiota with FS and FA. However, little is known about the role of neonatal gut microbiota in the pathobiology of these manifestations.
Objectives: We sought to characterize gut microbiota in children using an enterotyping approach and determine the association of gut microbiota and the enterotypes with the development of FS and FA.
Methods: We combined gut microbiome and fecal short-chain fatty acid data from 2 longitudinal birth-cohort studies in Japan, clustered the microbiome data from children who were 1 week to 7 years old and their mothers and identified enterotypes. We also determined the associations of gut microbiota and enterotypes with risks of developing FS and FA across the 2 studies using multivariable regression models.
Results: Data from the 2563 microbiomes identified 6 enterotypes. More gut bacteria (eg, *Bifidobacterium*) in 1-month-old children showed significant relationships with the development of FS and FA than in 1-week-old children. Enterotypes at 1 month old consisted of *Bacteroides*-dominant, *Klebsiella*-dominant, and *Bifidobacterium*-dominant enterotypes. *Bifidobacterium*-dominant enterotypes with the highest fecal propionate concentration had the lowest risks of developing FS and FA, especially of hen egg white sensitization. *Bifidobacterium*-dominant enterotypes had lower risks at 2 years old in one study (vs *Bacteroides*-dominant enterotype, adjusted odds ratio [adjOR]: 0.10, 95% CI: 0.01-0.78; vs *Klebsiella*-dominant enterotype, adjOR: 0.10, 95% CI: 0.01-0.77) and at 9 months old in the other study (vs *Bacteroides*-dominant enterotype, adjOR: 0.33, 95% CI: 0.11-0.91).
Conclusions: In these birth-cohort studies, gut microbiome clustering identified distinct neonatal enterotypes with differential risks of developing FS and FA. (J Allergy Clin Immunol 2025;155:932-46.)

Key words: *16S rRNA gene sequencing, bifidobacterium, enterotype, food allergy, gut microbiota, hen egg white, neonate, propionate, sensitization, short-chain fatty acids*

Food sensitization, characterized by the presence of IgE antibodies targeting specific food antigens (allergens), is a major risk factor for developing food allergy and other allergic diseases,[1,2] and the prevalence of food sensitization and allergy has risen over several decades.[3,4] Food sensitization develops in early infancy, and the prevalence increases from 10% to 20% at 6 months old[5,6] to 20% to 30% by 5 years old[5,7,8] in Westernized countries. To develop a food sensitization and allergy prevention strategy, understanding the maturation of the immune system during the neonatal period—a window of opportunity[9]—is necessary.

From the early postnatal period, gut microbiota dramatically changes under the influence of various factors (eg, presence of siblings, delivery mode, feeding pattern) and their integrated effects,[10-15] resulting in greater interindividual variations (ie, higher heterogeneity) than in other age groups.[16] Also, ethnicity[17] and region[18] contribute to the differences in gut microbiota, even in early infancy. Growing evidence, mainly in non-Asian populations from North America and Europe, has suggested the importance of gut microbiota and their metabolites (eg, short-chain fatty acids [SCFAs]) during this period not only in the maturation of the immune system,[19,20] but also in the susceptibility to subsequent diseases.[21,22] For example, recent studies have suggested the association of gut microbiota and fecal SCFAs with risks of developing food sensitization and allergy in infants[23-29] and the significant[19,30]or nonsignificant[28,31] association in neonates. Furthermore, factors that influence gut microbiota, gut microbiota itself, and disease risks form a complex interplay. To unravel the heterogeneity and the complex interplay, emerging evidence highlights the importance of identifying gut microbial subgroups—enterotypes.[32,33] For example, a birth-cohort study has identified enterotypes from longitudinally collected gut microbiome data with different risks of food sensitization among infants.[34] However, despite its clinical and research significance, little is known about the association of neonatal enterotypes with food sensitization and allergy—let alone in Asian populations and Asian countries.

To address these knowledge gaps, we analyzed data from 2 independent birth-cohort studies in Japan[35,36] to identify

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Abbreviations used
adjOR: Adjusted odds ratio
CHIBA: Chiba High-risk Birth-cohort for Allergy
DMM: Dirichlet multinomial mixtures
HEW: Hen egg white
rRNA: Ribosomal RNA
SCFA: Short-chain fatty acid

enterotypes, by unsupervised data clustering, and the longitudinal associations of gut microbiota and enterotypes with the subsequent development of food sensitization and allergy.

METHODS

Study designs and participants

This study included data from 2 longitudinal birth-cohort studies conducted in Japan. Details of the study design, setting, participants, data and specimen collection, testing, and statistical analysis may be found in the Methods in this article's Online Repository (available at www.jacionline.org). Briefly, the Chiba High-risk Birth-cohort for Allergy (CHIBA) study[36] is a high-risk birth cohort of children with a family history of any allergic diseases—atopic dermatitis, asthma, allergic rhinitis, and/or food allergy. The Katsushika study[35,37] was originally a 2×2 factorial, randomized, non–treatment-controlled trial to evaluate the prevention of allergic diseases by skincare and/or symbiotics in neonates and infants. This study has reported that skincare, symbiotics, or their combination did not decrease the risk of food sensitization at 9 months old.[35] After the skincare and/or symbiotics period, the participants continued to be followed. Of the 4 groups, we included participants assigned to the 2 symbiotic-free groups in this study. The CHIBA study used a family history of allergic disease as an inclusion criterion, while the Katsushika study did not. The CHIBA study included preterm cases (<37 weeks), while the Katsushika study excluded preterm cases. Both studies were approved by the Bioethics Review Committee, Chiba University, Japan (approval nos. 754 and 2067, respectively), and protocols of the Katsushika study were registered at the University Hospital Medical Information Network (JPRN-UMIN000010838). Written informed consent was obtained from the guardians.

Data and specimen collection

The timing of visit and specimen collections in the 2 studies is summarized in Fig E1 (see this article's Online Repository at www.jacionline.org). Clinical data were collected via structured questionnaires from their guardians and consultation with a pediatric allergist and transmitted electronically by clinical coordinators. Serum specimens were tested for IgE levels. Total IgE level was measured by latex-enhanced immunoturbidimetry. IgE specific for hen egg white (HEW), ovomucoid, cow's milk, peanut, and buckwheat was measured by the ImmunoCAP system (Phadia KK, Tokyo, Japan). Fecal specimens were tested for gut microbiome and fecal-SCFA and SCFA-precursor profiling. The details of extraction of bacterial genomic DNA using an enzyme method[38,39] and fecal SCFAs and SCFA precursors are described in the Methods in this article's Online Repository.

Gut microbiome profiling

The details of gut microbiome profiling are described in the Methods in this article's Online Repository. Briefly, PCR amplicons of the 16S rRNA gene V_1-V_2 region were sequenced using MiSeq (Illumina, San Diego, Calif) across multiple runs (see Table E1 of this article's Online Repository at www.jacionline.org). Of the filter-passed reads, 3000 randomly selected high-quality reads per sample were used for operational taxonomic unit clustering using vsearch[40] with a sequence identity threshold of 97% and UniFrac analysis. All samples had Good's coverage of >0.95. The operational taxonomic units were assigned against the 16S database based RefSeq 16S records[41] (downloaded January 2020) using the GLSEARCH program.[42]

Fecal-SCFA and SCFA-precursor profiling

The details of SCFA (ie, formate, acetate, propionate, and butyrate) and SCFA-precursor (ie, lactate and succinate) profiling are described in the Methods in this article's Online Repository. Briefly, the SCFA and SCFA-precursor profiling was conducted using gas chromatography-tandem mass spectrometry platforms on a GCMS-TQ8030 triple quadrupole mass spectrometer (Shimadzu, Kyoto, Japan). The data were processed, and the concentration was calculated by LabSolutions Insight (Shimadzu).

Outcome measures

The primary outcome of interest was the development of food sensitization, defined as a serum food-specific IgE level of 0.70 kU/L or higher. The secondary outcome was the development of food allergy. Food allergy was diagnosed by 1 pediatric allergy specialist in each study based on the Japanese guideline for food allergy[43,44] and was defined as positive food sensitization and compatible clinical history of immediate-onset reaction (ie, a reaction that occurs within 2 hours of the food ingestion).

Statistical analysis

The analytic workflow is summarized in Fig 1. The details of the method are found in the Methods in this article's Online Repository. Briefly, first, in the exploratory part, to characterize a temporal change of gut microbiota, we clustered all gut microbiome data from children and their mothers in both the CHIBA and Katsushika studies into enterotypes[32,33] by unsupervised Dirichlet multinomial mixtures (DMM) clustering.[45] We determined an optimal number of clusters based on a Laplace approximation and a contribution of each bacterial genus to enterotyping by computing the sum of the mean absolute difference between relative abundance derived from the DMM models using the selected-number components and a single component. We also identified the most dominant bacterial genus in each enterotype based on relative abundance derived from the DMM model using the selected-number components. We determined the temporal change of the enterotypes and examined a separation of the gut microbiome data among enterotypes by a principal coordinate analysis using an unweighted UniFrac distance and the degree of separation among enterotypes by using permutational multivariate analysis of variance. We also determined gut environmental maturation by computing a Shannon index[15] and microbiota

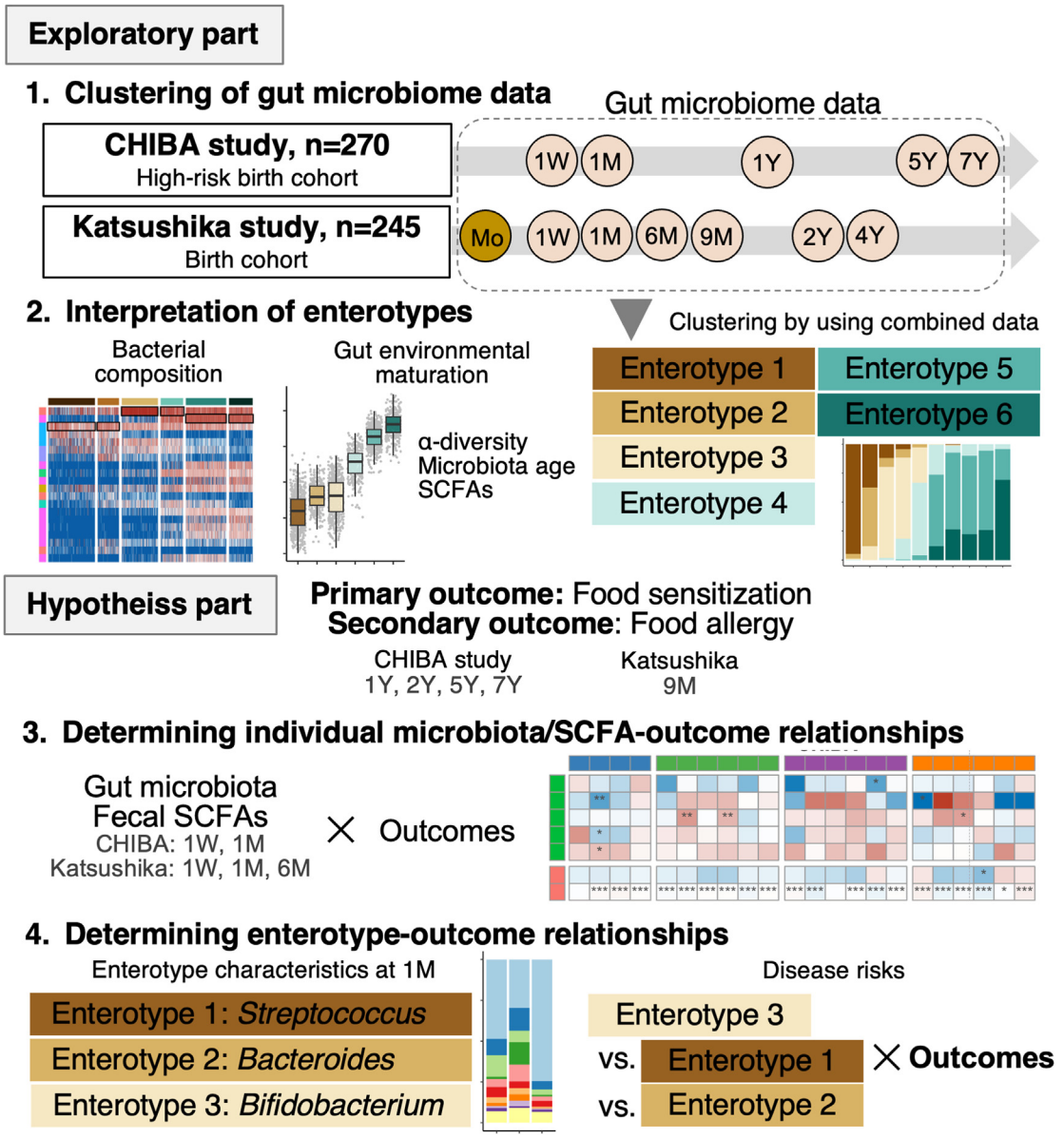

FIG 1. Analytic workflow. **(1)** Clustering of gut microbiome data: By using gut microbiome data from 2 birth-cohort studies of 270 children in the CHIBA study and 245 children as well as 240 of their mothers (*Mo*) in the Katsushika study, we generated 6 clusters by using a DMM model. **(2)** Interpretation of enterotypes: To interpret the gut environmental characteristics of the 6 enterotypes, we developed a heatmap on major bacterial genera and box plots on gut environmental maturation factors (ie, Shannon index, gut microbiota age, and SCFA and SCFA-precursor concentration). **(3)** Determining individual microbiota/SCFA-outcome relationships: The primary and secondary outcomes of this study were the development of food sensitization and allergy, respectively. To determine the individual relationships between gut microbiota and fecal SCFAs from 1 week old (*1W*) to 6 months old (*6M*) and the outcomes, we summarized log-fold change of the levels by negative binomial (for gut microbiota) or linear (for fecal SCFA) regression models in heatmaps. **(4)** Determining enterotype-outcome relationships: To interpret gut environmental characteristics of the 3 enterotypes at 1M, we developed bar plots on major bacterial genera and box plots on gut environmental maturation factors. Additionally, to examine the clinical significance between the enterotypes at 1M, we determined associations of the 3 enterotypes with risks of developing food sensitization and allergy by constructing logistic regression models. *Y*, Year old.

age by a random-forest-based sparse model consisting of selected 19 genera[46] and using fecal-SCFA and SCFA-precursor concentrations.[47] Second, to determine relationships of parental, demographic, nutritional, and environmental factors to gut microbiota at each age, we applied the compositions (ie, a matrix of bacterial genus abundance for each participant) to ordination analysis. We also projected these factors onto the ordination space with maximizing their correlation (r) by using an envfit model and

summarized r^2 in a heatmap. To interpret the relationships of these factors to relative abundance in each bacterial genus, we summarized estimated differences using negative binomial (for genera with <20% of subjects having zero) or zero-inflated negative binomial (for genera with ≥20% or <75% of subjects having zero) regression models for binary factors or Spearman ρ for ordinal factors in heatmaps. We also estimated the longitudinal relationships of the factors to each gut microbiota abundance by using mixed-effect models. Third, in the hypothesis-testing part, we examined the relationships between relative abundance in each bacterial genus from 1 week old to 6 months old (ie, ages prior to outcome measurement) and the outcomes (ie, development of food sensitization and allergy) as well as total and specific IgE levels. Therefore, we estimated differences using multivariable negative binomial or zero-inflated negative binomial regression models. In the multivariable models, we adjusted for potential confounders (ie, maternal history of allergic diseases, presence of older siblings, delivery mode, and exclusive breast-feeding at 1 month old, as well as active skincare in the Katsushika study) based on clinical plausibility and *a priori* knowledge.[13,36,48,49] We also estimated differences in fecal-SCFA levels for the outcomes and IgE levels by multivariable linear regression models using the $\log_2$-transformed levels. Fourth, to determine a complex interplay between the potential confounders, gut microbiota, and fecal SCFAs, we computed their pairwise relationships by Spearman ρ using microbiota abundances and SCFA levels. Fifth, to interpret characteristics of the enterotypes at the age of interest, we compared the difference in relative abundances by using the largest linear discriminant analysis effect size[50] and gut environmental maturation factors among the enterotypes. Lastly, to determine associations of the enterotypes with risks of developing food sensitization and allergy, we constructed mixed-effects logistic regression models by adjusting for the potential confounders as fixed effects and fecal collection site and 16S rRNA gene sequencing run as random effects. For sparse binary data in the food sensitization or allergy, the models were corrected by Firth method to reduce a bias of maximum likelihood estimate, as appropriate.[51] Additionally, for the sensitivity analysis, we computed E-values[52] to determine the robustness of causal inference to potential unmeasured confounding. We performed the statistical analysis using R version 4.1.2 (R Foundation, Vienna, Austria). We considered a 2-tailed P value <.05 as statistically significant. We corrected for multiple hypothesis testing using the Benjamini-Hochberg false discovery rate[53] with false discovery rate <.05 considered statistically significant.

RESULTS

Two hundred seventy participants in the CHIBA study and 245 participants in the Katsushika study provided ≥1 fecal specimen. In the CHIBA and Katsushika studies, the participant characteristics differed in the proportion of vaginal delivery, asphyxia, maternal diabetes, antibiotic medication, and factors related to the inclusion or exclusion criteria (eg, preterm, maternal history of allergic diseases, and active skincare; P <.05) (Table I). A total of 2563 fecal specimens were collected: 1061 and 1262 from children in the CHIBA and Katsushika studies, respectively, with 240 from mothers in the Katsushika study at enrollment (Fig E1).

DMM modeling-based enterotypes reflected the age-dependent maturation of gut microbiota

By applying an unsupervised clustering approach to the combined gut microbiome data from the 2 studies, a 6-class model provided an optimal fit (see Fig E2 of this article's Online Repository at www.jacionline.org). The most common enterotype changed from enterotype 1 to 4 at 1 week old toward 2 years old. After 4 years old, the most common enterotype was enterotype 5, while that in their mothers was enterotype 6 (Fig 2, *A*). This trend did not change when the enterotype transitions in the 2 studies were visualized separately (see Fig E3 of this article's Online Repository at www.jacionline.org). The genera with the highest relative abundance derived from the DMM model were *Streptococcus* in enterotypes 1 and 2, *Bifidobacterium* in enterotypes 3 and 4, and *Blautia* in enterotypes 5 and 6 (Fig 2, *B*; see Fig E4 of this article's Online Repository at www.jacionline.org). The overall gut microbiome community in each subject was also clustered toward the 6 enterotypes ($R^2 = 0.31$, P <.001) (Fig 2, *C*). In gut environmental maturation, the Shannon index (Fig 2, *D*) and microbiota age (Fig 2, *E*; see Figs E5-E7 of this article's Online Repository at www.jacionline.org) matured from enterotype 1 to 6. Likewise, the fecal SCFAs and SCFA-precursor concentrations also shifted from enterotype 1 to 6 (Fig 2, *F*).

Siblings, delivery mode, and feeding pattern were related to gut microbiota in early childhood

We next investigated the relationship of children's demographics and parental, nutritional, and environmental factors to gut microbial composition at each age. The presence of older siblings and vaginal delivery were related to gut microbial composition in the neonatal period, and breast-feeding was related to those in the infantile period in the 2 studies (Fig 3, *A*). In their relationships to gut microbiota abundance, children with household older siblings had higher *Bifidobacterium* and lower *Escherichia* abundances in the 2 studies (Fig 3, *B*). Likewise, children who were born vaginally had higher *Bifidobacterium* and *Bacteroides* abundances than those born by cesarean delivery in the 2 studies (Fig 3, *C*). More breast-feeding significantly correlated with a lower *Lactococcus* abundance in the neonatal and infantile periods and a higher *Staphylococcus* abundance at 1 month old in the 2 studies (Fig 3, *D*). However, most of the other relationships differed between the 2 studies, especially in the preschool and school ages (Fig 3). We also determined the longitudinal relationships of the factors with gut microbiota (see Fig E8 of this article's Online Repository at www.jacionline.org). In the 2 studies, the presence of older siblings was related to a higher *Bifidobacterium* abundance, and vaginal delivery was related to a higher *Bacteroides* abundance. In contrast, the longitudinal relationship of more breast-feeding differed between the 2 cohorts, especially in *Rothia*: decreases in the CHIBA study and increases in the Katsushika study.

Individual relationships between gut microbiota, fecal SCFAs, and outcomes showed high heterogeneity and complex interplay

We next investigated the relationships between gut bacterial genus abundances or fecal-SCFA levels from 1 week old to 6 months old (ie, ages prior to outcome measurement) and the development of the outcomes—food sensitization and allergy

TABLE I. Baseline participant characteristics

Participant characteristics	Overall (N = 515)	CHIBA (n = 270)	Katsushika (n = 245)	*P* value*
Sex female	263 (51)	136 (50)	127 (52)	.72
Vaginal delivery	384 (74)	191 (71)	193 (79)	.034
Preterm (<37 wk)	12 (2)	12 (4)	0 (0)	.001
Low birth weight (<2500 g)	23 (5)	16 (6)	7 (3)	.13
Asphyxia (Apgar score at 5 min < 7)	6 (1)	0 (0)	6 (2)	.011
Maternal age at recruitment (y)	34 (31-38)	35 (31-38)	34 (31-38)	.42
Maternal BMI before pregnancy (kg/m^2)	20.8 (19.5-22.6)	20.7 (19.4-22.7)	20.8 (19.5-22.5)	1.00†
Maternal BMI before delivery (kg/m^2)	24.9 (23.2-26.6)	24.7 (23.1-26.5)	25.0 (23.4-26.8)	.34†
Maternal BMI gain during pregnancy (kg/m^2)	3.9 (3.0-4.6)	3.8 (2.9-4.5)	3.9 (3.1-4.8)	.081†
Maternal history of allergic diseases‡	305 (59)	234 (86)	71 (29)	< .001
Maternal diabetes	23 (5)	18 (7)	5 (2)	.017
Maternal hypertension	14 (3)	6 (2)	8 (3)	.59
Maternal active smoking during pregnancy	11 (2)	7 (3)	4 (2)	.55
Maternal passive smoking during pregnancy	176 (34)	98 (39)	78 (32)	.13
Maternal antibiotic medication	129 (25)	31 (11)	98 (40)	< .001
Paternal history of allergic diseases‡	285 (55)	200 (74)	70 (27)	< .001
Household older siblings	236 (46)	116 (43)	120 (49)	.18
Active skincare	124 (24)	0 (0)	124 (51)	< .001

Values are median (interquartile range) or n (%).
*Tested by the Fisher exact test, unless otherwise indicated.
†Tested by the Wilcoxon rank sum test.
‡Defined as a history of atopic dermatitis, food allergy, and asthma in addition to allergic rhinitis and hay fever, except for a history of urticaria.

—as well as IgE levels in each study. Regarding their relationships by age, gut bacterial genera at 1 month old showed more significant associations than those at 1 week old across the 2 studies (Fig 4; see Fig E9 of this article's Online Repository at www.jacionline.org). Likewise, in the Katsushika study, gut bacterial genera at 6 months old showed significant relationships. In contrast, fecal SCFAs at all ages showed few significant relationships with the outcomes or IgE levels (see Figs E10 and E11 of this article's Online Repository at www.jacionline.org). Despite the comparably significant relationships at 6 months old in the Katsushika study, we focused on the data at 1 month old in the following analyses since previous research has reported that 10% to 20% developed food sensitization by 6 months old.[5,6] Focusing on individual bacterial genera at 1 month old, the same genera with the significant relationships were identified between the outcomes and IgE levels (eg, CHIBA: *Bifidobacterium*, *Lactococcus*, *Staphylococcus*; Katsushika: *Bifidobacterium*), while most of the genera were not shared between the 2 studies, except for *Bifidobacterium*. *Bifidobacterium* had the most significant relationships although the relationships showed both increases and decreases for the development of the outcomes or increase of IgE levels. Additionally, the gut bacterial genera, with the significant relationships to the outcomes (eg, *Bifidobacterium*, *Lactococcus*, *Staphylococcus*) (Figs 4 and E9), had significant correlations with other bacterial genera as well as the potential confounders and fecal SCFAs (see Fig E12 of this article's Online Repository at www.jacionline.org). Collectively, these data suggest differences in the heterogeneity of the microbiota-outcome relationships across the studies and the associations of interplay between microbiota and SCFAs with the outcomes.

Characteristics of enterotypes at 1 month old revealed a matured phenotype of enterotype 3

To reduce the heterogeneity and understand the interplay in the microbiota-outcome relationships, we focused on enterotypes at 1 month old (ie, enterotype 1, 2, or 3). We determined their distinct clinical and gut environmental characteristics that were consistent between the 2 studies (Tables II and III and Fig 5). For example, neonates with enterotype 1 (CHIBA: 31%; Katsushika: 43%) were characterized by higher proportions of vaginal delivery and the presence of older siblings as well as more breast-feeding. Neonates with enterotype 2 (CHIBA: 57%; Katsushika: 45%) were characterized by lower proportions of vaginal delivery and the presence of older siblings as well as less breast-feeding. Neonates with enterotype 3 (CHIBA: 12%; Katsushika 12%) were characterized by higher proportions of vaginal delivery and the presence of older siblings as well as less breast-feeding. In gut microbial composition, enterotypes 1, 2, and 3 were characterized by a higher abundance of *Bacteroides, Klebsiella*, and *Bifidobacterium*, respectively (Fig 5, *A*). Based on (1) *a priori* knowledge—the association of vaginal delivery,[48,54,55] the presence of older siblings,[49,56] or less breast-feeding[36] with the lower disease risk; (2) immune suppressive roles of *Bifidobacterium;*[19,57-60] and (3) the significant relationships of *Bifidobacterium* with the outcomes (Fig 4), we set enterotype 3 as an "enterotype of interest." Then, we compared it with enterotype 1 or 2 in the following analyses. In gut environmental maturation, although enterotype 3 had a lower Shannon index than enterotype 2 in the CHIBA study (Fig 5, *B*), enterotype 3 had a higher microbiota age than enterotype 1 or 2 (Fig 5, *C*). Additionally, enterotype 3 had higher propionate concentrations than enterotype 1 in the 2 studies (Fig 5, *D*). Enterotype 3 also had higher propionate in the CHIBA study and acetate concentrations in the 2 studies than enterotype 2 (Fig 5, *D*).

Enterotype 3 at 1-month-old was associated with lower risks of food sensitization and allergy

Lastly, we determined the associations of the enterotypes at 1 month old with the risks of developing food sensitization and allergy. Regarding food sensitization (Table IV) in the CHIBA study, compared to enterotype 1, enterotype 3 had lower risks

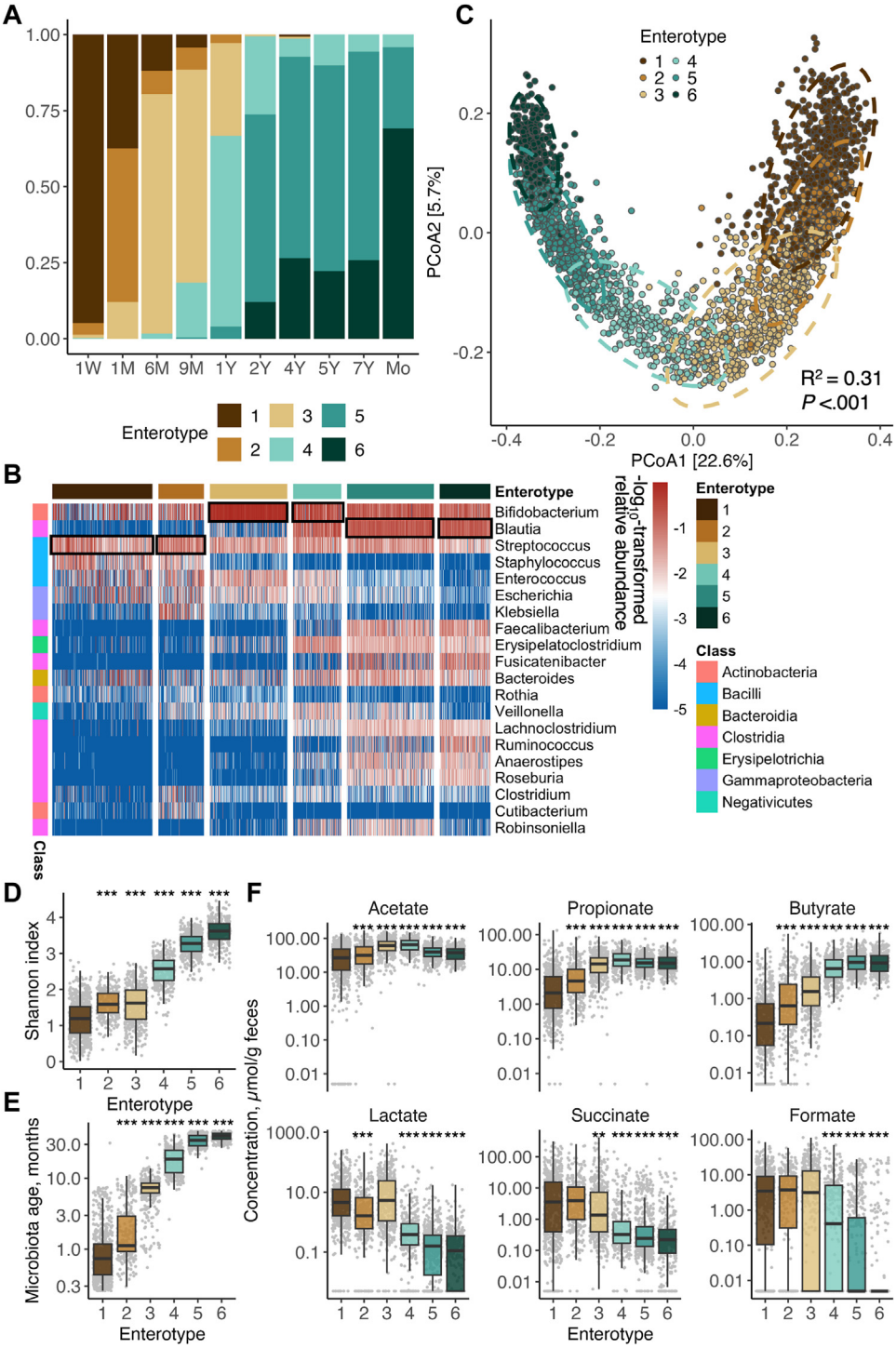

FIG 2. Enterotypes based on DMM modeling and their temporal changes. **(A)** The bar graphs show the proportion of the 6 enterotypes based on DMM modeling at each age and their temporal changes. **(B)** The heat map shows the relative abundance of the 20 bacterial genera with the highest contribution to enterotyping. The contribution was determined by computing the sum of the mean absolute difference between relative abundance derived from the DMM models using the selected-number components (k = 6) and a single component (k = 1). The *black boxed* area indicates the bacterial genera with the highest relative abundance derived from the DMM model in each enterotype. **(C)** Principal coordinate analysis (*PCoA*) plot using the unweighted UniFrac distance reveals the degree of separation among enterotypes. Ellipses with *dashed lines* represent the 95% CI. The R^2 and *P* values were computed by permutational multivariate analysis of variance. **(D-F)** The box plots show median with interquartile range of **(D)** the alpha diversity (the Shannon index); **(E)** microbiota age (generated by random-forest-based sparse 19-genus model); and **(F)** fecal-SCFA and SCFA-precursor concentration in each enterotype. **P* < .05, ***P* < .01, ****P* < .001 by the Wilcoxon rank-sum test compared to enterotype 1. The analyses in this figure were performed by using the combined data from the CHIBA and Katsushika studies.

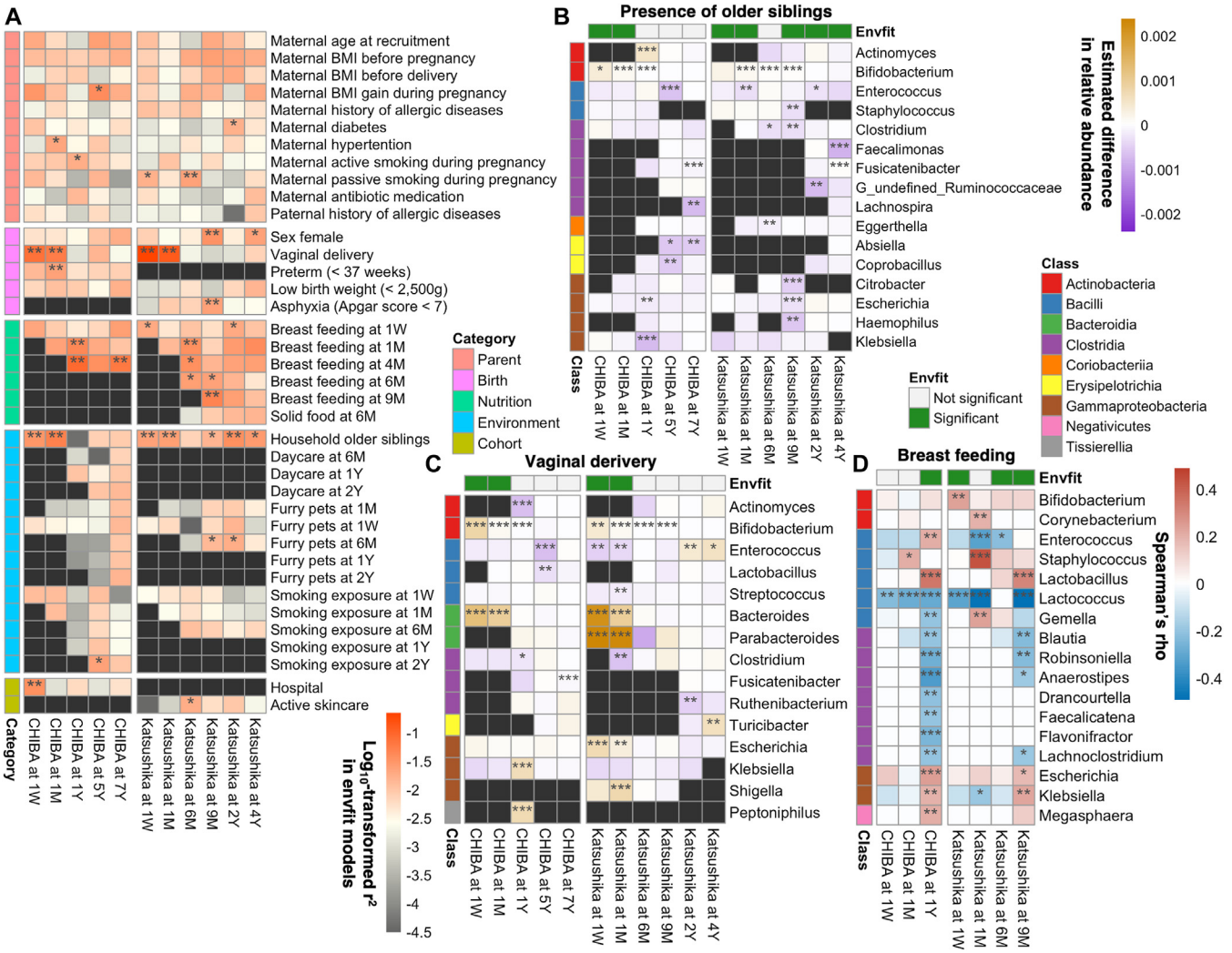

FIG 3. Factors related with gut microbiota composition and relative abundance at different ages. **(A)** The heatmap shows effect sizes of covariates (r^2) to gut microbial composition at genus level determined by the *envfit* function in the "vegan" R package (*P < .05, **P < .01). Dark gray cells represent no data. **(B,C)** The heatmaps show an estimated fold change in a relative abundance by using negative binomial or zero-inflated negative binomial regression models (**B,** the presence of older siblings compared to the absence of older siblings; **C,** vaginal delivery compared to cesarean delivery; *false discovery rate [*FDR*] < 0.05, **FDR < 0.01, ***FDR < 0.001). Genera with FDR < 0.01 at any age were shown. (**D**) The heatmap shows Spearman ρ between the data for the same age for a degree of breast-feeding and a gut bacterial genus abundance (*FDR < 0.05, **FDR < 0.01, ***; FDR <. 0001 by Spearman rank correlation analysis). Due to the absence of breast-feeding data at 1 year old in the CHIBA study, we utilized the data at 4 months old. *BMI*, Body mass index.

of developing HEW sensitization at 1 year old (adjusted odds ratio [adjOR]: 0.34; 95% CI: 0.11-1.04; P = .058) and 2 years old (adjOR: 0.10; 95% CI: 0.01-0.78; P = .029; E-value = 5.93). Additionally, compared to enterotype 2, enterotype 3 also had a lower risk of developing HEW sensitization at 2 years old (adjOR: 0.10; 95% CI: 0.01-0.77; P = .027; E-value = 5.84). Consistently, in the Katsushika cohort, enterotype 3 had lower risks of developing HEW sensitization at 9 months old (compared to enterotype 1: adjOR: 0.33; 95% CI: 0.11-0.91; P = .040; E-value = 2.86; compared to enterotype 2: adjOR: 0.41; 95% CI: 0.13-1.11; P = .092). However, in the CHIBA and Katsushika studies, enterotype 3 was not significantly associated with lower risks of developing sensitization to other food allergens (eg, ovomucoid, cow's milk, peanut, buckwheat, and any food), except for a lower risk of developing sensitization to any food allergens at 9 months old compared to enterotype 1 in the Katsushika study (adjOR: 0.31; 95% CI: 0.19-0.86; P = .030; E-value = 2.96). Furthermore, regarding food allergy (see Table E2 of this article's Online Repository at www.jacionline.org), although the associations of enterotype 3 with the risks of developing food allergy did not reach statistical significance, enterotype 3 was associated with lower risks at 2 years old compared to enterotype 1 (OR: 0.13; 95% CI: 0.00-1.10; P = .064) and enterotype 2 (OR: 0.16; 95% CI: 0.00-1.27; P = .094).

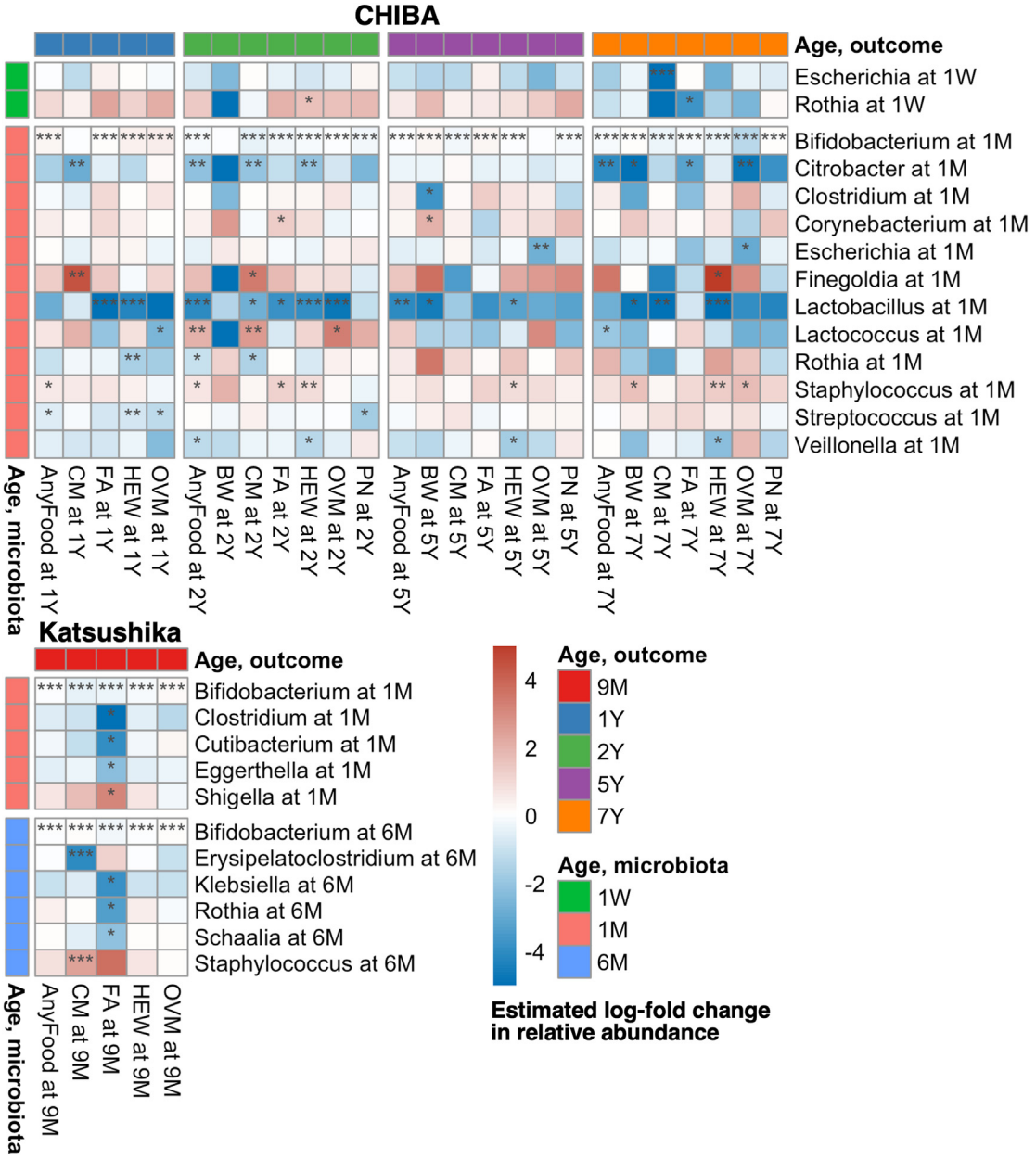

FIG 4. Relationship between gut microbiota at each age and the development of food sensitization and allergy. The heatmaps visualize an estimated fold change in relative abundance at the genus level for the development of food sensitization and food by negative binomial or zero-inflated negative binomial regression models (*FDR < 0.05, **FDR < 0.01, ***FDR < 0.001). *BW*, Buckwheat; *CM*, cow's milk; *FA*, food allergy; *OVM*, ovomucoid; *PN*, peanut.

DISCUSSION

By using data from 515 Japanese children and their 240 mothers in the 2 independent birth-cohort studies, we identified 6 enterotypes describing their temporal changes in gut microbiota composition. We next examined the relationship of factors such as the presence of older siblings, delivery mode, and the degree of breast-feeding with gut microbial composition. Additionally, we determined the individual relationships between gut microbiota (eg, *Bifidobacterium* at 1 month old) and the development of food sensitization and allergy. We also found that neonates with a *Bifidobacterium*-dominant enterotype had lower risks of developing food sensitization, especially to HEW, and allergy in each birth-cohort study.

Recent research has indicated the association of infantile gut microbiota (>3 months old) and its metabolites SCFAs with the risk of developing food sensitization and allergy[24-28,34] and the importance of an unsupervised clustering approach for understanding their complex relationship[23-27] (eg, enterotyping[3,24]). The neonatal enterotypes identified here are in agreement with these studies. For example, birth- or early-life–cohort studies

TABLE II. Participant characteristics according to 3 enterotypes at 1 month old in the CHIBA study

Subject characteristic	Enterotype 1 (n = 67; 31%)	Enterotype 2 (n = 121; 57%)	Enterotype 3 (n = 26; 12%)	*P* value*
Sex female	30 (45)	58 (48)	15 (58)	.53
Vaginal delivery	55 (82)	76 (63)	21 (81)	.015
Preterm (<37 wk)	4 (6)	6 (5)	1 (4)	.91
Low birth weight (<2500 g)	3 (4)	11 (9)	0 (0)	.23
Asphyxia (Apgar score at 5 min < 7)	0 (0)	0 (0)	0 (0)	1.00
Maternal age at recruitment (y)	35.8 (30.8-37.8)	34.1 (30.3-37.2)	35.4 (32.0-38.2)	.22†
Maternal BMI before pregnancy (kg/m^2)	20.5 (19.5-22.4)	20.8 (19.2-22.8)	20.8 (19.2-22.0)	.92†
Maternal BMI before delivery (kg/m^2)	24.6 (23.1-26.2)	24.8 (23.0-26.5)	24.2 (22.7-25.8)	.43†
Maternal BMI gain during pregnancy (kg/m^2)	3.5 (2.7-4.5)	3.9 (3.1-4.5)	3.6 (2.5-4.4)	.34†
Maternal history of allergic diseases‡	57 (85)	109 (90)	23 (88)	.61
Maternal diabetes	8 (12)	6 (5)	1 (4)	.18
Maternal hypertension	1 (1)	4 (3)	0 (0)	.83
Maternal active smoking during pregnancy	1 (1)	3 (2)	0 (0)	1.00
Maternal passive smoking during pregnancy	25 (37)	42 (35)	5 (19)	.21
Maternal antibiotic medication	5 (7)	14 (12)	4 (15)	.47
Paternal history of allergic diseases‡	49 (73)	92 (76)	19 (73)	.88
Breast-feeding at 1 wk				.20
Exclusive	6 (9)	7 (6)	1 (4)	
Some	59 (88)	114 (94)	24 (92)	
No	2 (3)	0 (0)	1 (4)	
Breast-feeding at 1 mo				< .001
Exclusive	11 (16)	14 (12)	1 (4)	
Almost	54 (81)	100 (83)	16 (62)	
Some	1 (1)	2 (2)	3 (12)	
Little	0 (0)	3 (2)	5 (19)	
No	1 (1)	2 (2)	1 (4)	
Solid food intake at 1 wk	0 (0)	0 (0)	0 (0)	1.00
Solid food intake at 1 mo	0 (0)	0 (0)	0 (0)	1.00
Household older siblings	32 (48)	45 (37)	17 (65)	.017
Furry pets at 1 wk	11 (16)	27 (22)	5 (19)	.68
Furry pets at 1 mo	11 (16)	23 (19)	4 (15)	.86
Smoking exposure at 1 wk	16 (24)	30 (25)	5 (19)	.88
Smoking exposure at 1 mo	9 (13)	23 (19)	3 (12)	.58
Hospital, Chiba Medical Center	48 (72)	101 (83)	16 (62)	.021
Active skincare	0 (0)	0 (0)	0 (0)	1.00

Values are median (interquartile range) or n (%).
*Tested by the Fisher exact test, unless otherwise indicated.
†Tested by the Kruskal-Wallis test.
‡Defined as a history of atopic dermatitis, food allergy, and asthma in addition to allergic rhinitis and hay fever, except for a history of urticaria.

have reported that a perturbation in gut SCFA-producing bacteria (eg, *Clostridia* and *Negativicutes*) during infancy was associated with risks of developing food sensitization and/or allergy.[24-26,28] Another birth-cohort study has reported that children with higher fecal propionate levels also had a lower risk of developing food sensitization.[27] Additionally, recent research has identified enterotypes based on longitudinally collected gut microbiome data, as our study, and has shown the association of having an enterotype characterized by a lower *Bacteroides* abundance from 3- to 12 months old with a higher risk of developing peanut sensitization at 2 years old.[34] The current study builds on these earlier findings, mainly on non-Asian populations from North America and Europe,[23-27] and extends their findings by identifying neonatal enterotypes with differential risks of developing food sensitization and allergy in Asian populations. A better understanding of neonatal enterotypes may facilitate understanding the maturation of the immune system and developing potential prevention strategies (eg, the use of biotics[61]) against food sensitization and allergy.

The current study found the individual relationships between gut microbiota at 1 month old and the development of food allergy and sensitization as well as IgE levels. Our findings are consistent with the growing evidence of the concept of the "window of opportunity" that assigns an early postnatal period as a critical period for lifelong host-microbial and immune homeostasis.[9] For example, recent reports have revealed the association of neonatal gut microbiota and fecal metabolites with the susceptibility to developing allergic diseases.[19,29-31] A birth-cohort study has shown that cesarean delivery–associated gut microbial scores at 1 week old and 1 month old[31] and gut microbiota age at 1 month old[29] were not significantly associated with the risks of developing allergic sensitization at 12 to 18 months old. In contrast, there are other recent birth-cohort studies showing the association of children with higher fecal 12,13-diHOM concentration at 1 month old with developing risks of atopy or eczema at 2 years old or asthma at 4 years-= old.[19,30] Although the importance of the neonatal gut microbiota and its role in immune development

TABLE III. Participant characteristics according to 3 enterotypes at 1 month old in the Katsushika study

Subject characteristic	Enterotype 1 (n = 101; 43%)	Enterotype 2 (n = 106; 45%)	Enterotype 3 (n = 28; 12%)	*P* value*
Sex female	46 (46)	55 (52)	18 (64)	.20
Vaginal delivery	86 (85)	73 (69)	26 (93)	.004
Preterm (<37 wk)	3 (3)	3 (3)	0 (0)	1.00
Low birth weight (<2500 g)	1 (1)	5 (5)	0 (0)	.22
Asphyxia (Apgar score at 5 min < 7)	35.1 (31.6-38.4)	33.8 (31.3-37.4)	33.9 (31.1-39.8)	.50†
Maternal age at recruitment (y)	20.6 (19.3-21.5)	21.2 (19.6-22.5)	22.2 (19.6-24.9)	.026†
Maternal BMI before pregnancy (kg/m^2)	24.6 (23.1-26.2)	25.4 (23.5-26.9)	25.7 (24.7-28.4)	.042†
Maternal BMI before delivery (kg/m^2)	3.8 (3.1-4.7)	4.0 (3.1-4.7)	3.5 (3.1-4.8)	.86†
Maternal BMI gain during pregnancy (kg/m^2)	30 (30)	29 (27)	6 (21)	.72
Maternal history of allergic diseases‡	1 (1)	2 (2)	1 (4)	.48
Maternal diabetes	2 (2)	4 (4)	2 (7)	.33
Maternal hypertension	0 (0)	4 (4)	0 (0)	.19
Maternal active smoking during pregnancy	32 (32)	32 (30)	11 (39)	.65
Maternal passive smoking during pregnancy	41 (41)	38 (36)	13 (46)	.54
Maternal antibiotic medication	35 (35)	36 (34)	11 (39)	.88
Paternal history of allergic diseases‡	46 (46)	55 (52)	18 (64)	.20
Breastfeeding at 1 wk				.085
Exclusive	45 (45)	35 (33)	7 (25)	
Some	56 (55)	71 (67)	21 (75)	
No	0 (0)	0 (0)	0 (0)	
Breast-feeding at 1 mo				< .001
Exclusive	57 (56)	43 (41)	7 (25)	
Almost	26 (26)	24 (23)	3 (11)	
Some	18 (18)	34 (32)	12 (43)	
Little	0 (0)	5 (5)	6 (21)	
No	0 (0)	0 (0)	0 (0)	
Solid food intake at 1 wk	0 (0)	0 (0)	0 (0)	1.00
Solid food intake at 1 mo	0 (0)	0 (0)	0 (0)	1.00
Household older siblings	55 (54)	48 (45)	14 (50)	.41
Furry pets at 1 wk	31 (19)	49 (22)	11 (20)	.76
Furry pets at 1 mo	19 (19)	29 (27)	6 (21)	.34
Smoking exposure at 1 wk	20 (20)	22 (21)	6 (21)	.98
Smoking exposure at 1 mo	18 (11)	34 (15)	7 (13)	.48
Hospital, Katsushika Medical Hospital	101 (100)	106 (100)	28 (100)	1.00
Active skincare	56 (55)	53 (50)	13 (46)	.63

Values are median (interquartile range) or n (%).
*Tested by the Fisher exact test, unless otherwise indicated.
†Tested by the Kruskal-Wallis test.
‡Defined as a history of atopic dermatitis, food allergy, and asthma in addition to allergic rhinitis and hay fever, except for a history of urticaria.

is widely recognized, the exact mechanisms remain unclear. Recent evidence suggests a potential mechanism—the vulnerability of the neonatal gut epithelial barrier function.[62] For example, a birth-cohort study has reported that a higher level of zonulin, an epithelial tight junction regulator, in the cord blood —a surrogate marker of intestinal barrier function—was associated with the risk of developing HEW sensitization at 1 year age.[63] Collectively, these conflicting results suggest the importance of further validation in the neonatal gut environment.

Although the exact mechanisms underlying the observed enterotypes warrant further investigation, the current study indicates that neonates with enterotype 3 at 1 month old, characterized by the highest *Bifidobacterium* abundance and fecal propionate level, had the lowest risks of developing food sensitization and allergy. Regarding clinical characteristics, neonates with enterotype 3 had a higher proportion of the presence of older siblings and vaginal delivery, as well as less breast-feeding. Consistently, previous research has reported that the presence of older siblings and vaginal delivery were associated with a higher *Bifidobacterium* abundance in the early infantile period[10,14,64,65] and lower risks of developing food allergy.[48,49,54-56] In contrast, recent research has shown the conflicting association of breast-feeding with a *Bifidobacterium* abundance in the early infantile period[13,66,67] and the development of food allergy.[36,68-73] Although the apparent discrepancy may be attributable to the difference in the target population, setting, measurements, outcomes, and any combination of these factors, the current study identified enterotypes that also had distinct characteristics in the clinical factors and different risks of food allergy and sensitization. Taken together, these studies, including ours, indicate the intimate relationship of these clinical factors with the gut environment and the development of food sensitization and allergy.

Regarding gut environmental characteristics of enterotype 3 —the highest *Bifidobacterium* abundance and fecal propionate level—studies using murine models have reported that *Bifidobacterium* prevented or suppressed the development of food allergy via regulatory T cells, IL-10, and mast cells.[57-59] A recent birth-cohort study has also shown that a higher *Bifidobacterium*

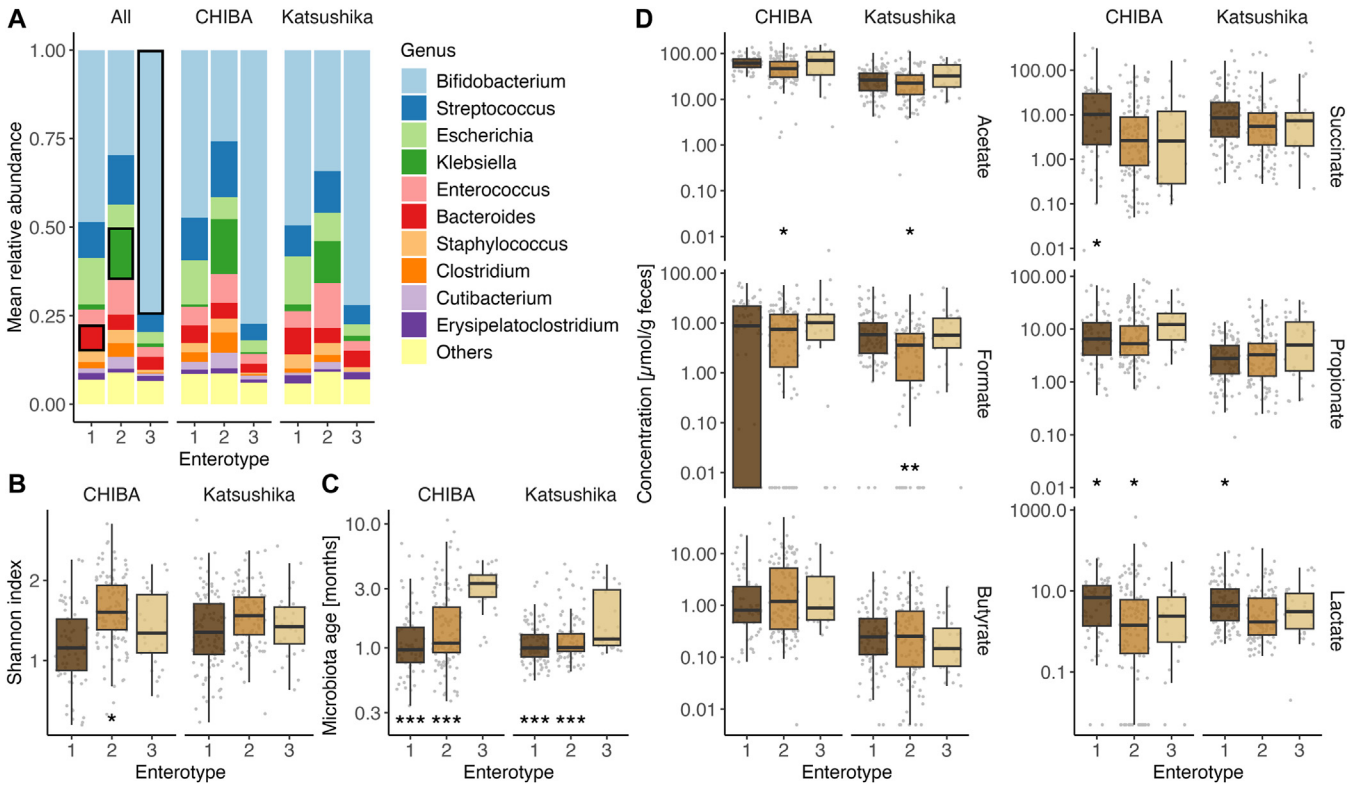

FIG 5. Characteristics of the gut environment in each enterotype at 1 month age. **(A)** The bar plots show mean relative abundances of the 10 bacterial genera at 1 month old with the highest relative abundance. The *black boxed* area indicates bacterial genera with the largest linear discriminant analysis effect size within the enterotype (ie, dominant bacterial genera in each enterotype). **(B-D)** The box plots represented by median with interquartile range show the Shannon index, microbiota age (generated by random-forest-based sparse 19-genus model), and fecal-SCFA and SCFA-precursor concentration (*$P < .05$, **$P < .01$, ***$P < .001$ by the Wilcoxon rank-sum test compared to enterotype 3).

abundance at 1 week old was related to more mature immunity at 3 years old (eg, higher capacity of mononuclear cells to produce cytokines [IL-6, IL-13, IL-1β]).[60] Another birth-cohort study that clustered neonatal gut microbiome data has shown the relationship of a subgroup with lower *Bifidobacterium* relative abundance to a higher risk of developing sensitization to multiple food and/or aeroallergens at 2 years old.[19] As for fecal propionate, a birth-cohort study has revealed that children with high fecal propionate levels at 1 year old had a lower risk of developing food sensitization by 6 years old.[27] Regardless of the complexity of the relationship between clinical factors, gut environment, and the development of food sensitization and allergy, our analysis revealed the association between the identified neonatal enterotypes (exposure) and food sensitization and allergy (outcomes) with adjustment for clinical factors that may influence the exposure and outcomes and, furthermore, confirmed consistent results in the 2 independent birth-cohort studies. Our data should thus advance research into developing prevention strategies based on an enterotyping approach, especially in neonates.

The present study has several potential limitations. First, only Asians who were born and raised in the urban area of Japan participated in these studies. Although our results may not be generalized to other ethnic groups or regions, our results are essential, considering ethnic[17] and regional[18] differences in early-life gut microbiota and a publication bias toward non-Asian populations and North American and European countries. Second, because some detailed data were not available (eg, age at initiation of solid food intake), we did not determine the relationship to gut microbiota. Third, for gut bacterial DNA extraction, we used an enzyme method,[38,39] and our findings showed higher *Bifidobacterium* or *Streptococcus* abundance compared to the commonly used methods (eg, bead beating, DNA extraction kit). However, the previous literature has shown that the enzyme method was more similar to theoretical data compared to the commonly used methods.[39] Fourth, we used genus-level gut microbiome data by 16S rRNA gene sequencing with lower resolution than species-level data by shotgun sequencing. However, a recent birth-cohort study has shown similar results between genus- and species-level enterotyping by a DMM model.[13] Additionally, our results were supported by metabolome data containing fecal SCFAs and their precursors, one of the most important fecal metabolites. Fifth, this study used a diagnosis by 1 pediatric allergy specialist in each study to define food allergy because we thought that diagnosis by an oral food challenge was too invasive in birth-cohort studies. Lastly, for some outcomes, the number of children who developed the outcomes was limited. For the limited number, we did not adjust the effect of the fecal specimen collection site and sequencing run in all regression models due to the model instability. Additionally, in contrast to the association of enterotypes with risks of developing food sensitization, our study did not show significant differences in the associations with risks of developing food allergy. However, food sensitization defined

TABLE IV. Associations of enterotype 3 with development of sensitization compared to enterotype 1 or 2 at 1 month old

Allergen	Cohort	Age	Enterotype	Number of the outcome*	Profile size*	Unadjusted model†		Adjusted model‡		
						Odds ratio (95% CI)	*P* value	Odds ratio (95% CI)	*P* value	E-value§
Egg white	CHIBA	1 y	3	5 (19)	26	1	—	1	—	—
			vs 1	30 (46)	65	0.28 (0.08-0.78)	.021	0.34 (0.11-1.04)	.058	—
			vs. 2	36 (30)	120	0.55 (0.17–1.47)	.26	0.74 (0.25–2.23)	.60	—
		2 y	3	2 (8)	26	1	—	1	—	—
			vs 1	21 (33)	63	0.17 (0.03-0.64)	.022	0.10 (0.01-0.78)	.029	5.93 (1.51)
			vs 2	35 (31)	112	0.18 (0.03-0.67)	.026	0.10 (0.01-0.77)	.027	5.84 (1.53)
		5 y	3	1 (7)	15	1	—	1	—	—
			vs 1	10 (20)	50	−0.92 (−3.21 to 0.67)	.28	−0.82 (−3.11 to 0.79)	.35	—
			vs 2	16 (19)	85	−0.83 (−3.09 to 0.69)	.31	−0.75 (−3.02 to 0.81)	.38	—
		7 y‖	3	0 (0)	13	1	—	1	—	—
			vs 1	8 (19)	43	−1.87 (−6.75 to 0.34)	.11	−1.98 (−6.87 to 0.24)	.088	—
			vs 2	8 (11)	75	−1.22 (−6.11 to 0.96)	.33	−1.46 (−6.36 to 0.77)	.24	—
	Katsushika	9 mo	3	6 (24)	25	1	—	1	—	—
			vs 1	47 (48)	98	0.34 (0.12-0.89)	.036	0.33 (0.11-0.91)	.040	2.86 (1.28)
			vs 2	47 (45)	104	0.38 (0.13-0.99)	.060	0.41 (0.13-1.11)	.092	—
Ovomucoid	CHIBA	1 y	3	1 (4)	25	1	—	1	—	—
			vs 1	10 (17)	58	−1.26 (−3.53 to 0.28)	.12	−1.36 (−3.63 to 0.19)	.091	—
			vs 2	9 (9)	102	−0.52 (−2.78 to 1.02)	.55	−0.74 (−3.02 to 0.85)	.39	—
		2 y	3	1 (4)	26	1	—	1	—	—
			vs 1	7 (12)	60	−0.87 (−3.16 to 0.73)	.31	−0.85 (−3.14 to 0.76)	.33	—
			vs 2	16 (15)	110	−1.09 (−3.33 to 0.37)	.16	−1.08 (−3.33 to 0.43)	.18	—
		5 y	3	3 (20)	15	1	—	1	—	—
			vs 1	4 (8)	51	2.94 (0.52-15.15)	.19	2.95 (0.57-15.35)	.20	—
			vs 2	7 (9)	82	2.68 (0.52-11.19)	.19	2.48 (0.52-11.85)	.26	—
		7 y‖	3	0 (0)	13	1	—	1	—	—
			vs 1	4 (9)	43	−1.12 (−6.03 to 1.22)	.40	−1.39 (−6.31 to 0.96)	.29	—
			vs 2	4 (5)	75	−0.53 (−5.44 to 1.80)	.71	−1.15 (−6.08 to 1.23)	.40	—
	Katsushika	9 mo	3	1 (4)	25	1	—	1	—	—
			vs 1	19 (19)	98	−1.39 (−3.62 to 0.06)	.063	−1.20 (−3.46 to 0.30)	.13	—
			vs 2	13 (12)	104	−0.90 (−3.15 to 0.59)	.26	−0.71 (−2.98 to 0.84)	.40	—
Cow's milk	CHIBA	1 y	3	2 (8)	26	1	—	1	—	—
			vs 1	11 (17)	65	0.41 (0.06-1.68)	.27	0.22 (0.03-1.89)	.17	—
			vs 2	14 (12)	120	0.63 (0.09-2.44)	.55	0.35 (0.04-2.91)	.33	—
		2 y	3	3 (12)	26	1	—	1	—	—
			vs 1	12 (19)	63	0.55 (0.12-1.95)	.39	0.68 (0.16-2.84)	.60	—
			vs 2	22 (20)	112	0.53 (0.12-1.72)	.34	0.56 (0.15-2.17)	.40	—
		5 y	3	1 (7)	15	1	—	1	—	—
			vs 1	7 (14)	51	−0.49 (−2.80 to 1.16)	.59	−0.44 (−2.74 to 1.20)	.63	—
			vs 2	6 (7)	84	0.22 (−2.09 to 1.89)	.82	0.20 (−2.13 to 1.90)	.84	—
		7 y‖	3	0 (0)	13	1	—	1	—	—
			vs 1	5 (11)	44	−1.32 (−6.23 to 0.96)	.30	−1.32 (−6.23 to 0.97)	.30	—
			vs 2	4 (5)	75	−0.53 (−5.44 to 1.80)	.71	−0.64 (−5.56 to 1.73)	.65	—
	Katsushika	9 mo	3	1 (4)	25	1	—	1	—	—
			vs 1	8 (8)	97	−0.44 (−2.71 to 1.13)	.62	−0.67 (−3.01 to 1.00)	.46	—
			vs 2	7 (7)	104	−0.25 (−2.53 to 1.34)	.78	−0.21 (−2.54 to 1.50)	.83	—
Peanut	CHIBA	2 y	3	1 (4)	26	1	—	1	—	—
			vs 1	4 (6)	63	−0.25 (−2.59 to 1.50)	.79	−0.08 (−2.47 to 1.78)	.94	—
			vs 2	3 (3)	112	0.61 (−1.75 to 2.47)	.56	0.71 (−1.70 to 2.65)	.51	—
		5 y‖	3	0 (0)	15	1	—	1	—	—
			vs 1	3 (6)	51	−0.81 (−5.73 to 1.61)	.57	−0.87 (−5.79 to 1.54)	.53	—
			vs 2	6 (7)	83	−0.96 (−5.85 to 1.27)	.47	−1.05 (−5.96 to 1.23)	.43	—
		7 y	3	1 (8)	13	1	—	1	—	—
			vs 1	6 (14)	42	−0.39 (−2.72 to 1.31)	.67	−0.16 (−2.49 to 1.57)	.87	—
			vs 2	4 (5)	75	0.65 (−1.71 to 2.44)	.53	0.83 (−1.55 to 2.69)	.44	—
Buckwheat	CHIBA	2 y‖	3	0 (0)	26	1	—	1	—	—
			vs 1	2 (3)	63	−0.77 (−5.71 to 1.79)	.60	−0.44 (−5.43 to 2.35)	.78	—
			vs 2	1 (1)	112	0.34 (−4.66 to 3.30)	.84	0.50 (−4.54 to 3.63)	.78	—
		5 y¶	3	0 (0)	15	1	—	1	—	—
			vs 1	2 (4)	51	−0.45 (−5.40 to 2.13)	.77	−0.55 (−5.50 to 2.02)	.71	—
			vs 2	4 (5)	80	−0.60 (−5.51 to 1.72)	.67	−0.98 (−5.91 to 1.41)	.48	—
		7 y	3	1 (8)	13	1	—	1	—	—

(Continued)

TABLE IV. *(Continued)*

Allergen	Cohort	Age	Enterotype	Number of the outcome*	Profile size*	Unadjusted model†		Adjusted model‡		
						Odds ratio (95% CI)	*P* value	Odds ratio (95% CI)	*P* value	E-value§
			vs 1	3 (7)	42	0.30 (−2.10 to 2.22)	.77	0.30 (−2.11 to 2.25)	.77	—
			vs 2	3 (4)	74	0.90 (−1.50 to 2.80)	.41	0.88 (−1.57 to 2.88)	.43	—
Any food¶	CHIBA	1 y	3	7 (27)	26	1	—	1	—	—
			vs 1	32 (49)	65	0.38 (0.13-0.99)	.056	0.37 (0.13-1.08)	.069	—
			vs 2	42 (35)	120	0.68 (0.25-1.68)	.42	0.74 (0.26-2.07)	.57	—
		2 y	3	5 (19)	26	1	—	1	—	—
			vs 1	24 (38)	63	0.39 (0.12-1.10)	.091	0.34 (0.10-1.15)	.082	—
			vs 2	41 (37)	112	0.41 (0.13-1.10)	.098	0.35 (0.11-1.11)	.075	—
		5 y	3	3 (20)	15	1	—	1	—	—
			vs 1	14 (27)	51	0.66 (0.14-2.47)	.56	0.62 (0.15-2.61)	.51	—
			vs 2	20 (24)	85	0.81 (0.17-2.87)	.76	0.69 (0.17-2.85)	.61	—
		7 y	3	1 (8)	13	1	—	1	—	—
			vs 1	14 (32)	44	−1.38 (−3.66 to 0.20)	.091	−1.41 (−3.72 to 0.20)	.089	—
			vs 2	15 (20)	75	−0.76 (−3.03 to 0.79)	.37	−0.95 (−3.26 to 0.66)	.27	—
	Katsushika	9 mo	3	6 (24)	25	1	—	1	—	—
			vs 1	49 (50)	98	0.32 (0.11-0.82)	.024	0.31 (0.10-0.86)	.030	2.96 (1.38)
			vs 2	48 (46)	104	0.37 (0.13-0.95)	.050	0.41 (0.14-1.11)	.093	—

*Values are n or n (%).

†Unadjusted logistic regression model.

‡For the CHIBA study, estimated by mixed-effects multivariate logistic regression models adjusted for potential confounders (ie, maternal history of allergic diseases, presence of older siblings, delivery mode, and exclusive breast-feeding at 1 month old) accounting for patient clustering by hospital and 16S rRNA gene sequencing run. For the Katsushika study, estimated by multivariate logistic regression models adjusted for the potential confounders with active skincare.

§The E-value (with its lower 95% CI bound) represents how strongly unmeasured confounder(s) represents how strongly a set of unmeasured confounders would be associated with the exposure and outcome to fully eliminate the observed association.

||Logistic regression models were corrected by Firth method to reduce a bias of maximum likelihood estimate for complete separation in the outcome. In the corrected models, fixed-effect models were used for both studies.

¶Defined by having 1 or more positive values (>0.70 kU/L) of egg white-, ovomucoid-, and cow's milk–specific IgE for 1 y and 9 mo or of egg white-, ovomucoid-, cow's milk–, peanut- and buckwheat-specific IgE for 2, 5, and 7 years.

by objective parameters may be precedent to the development of food allergy in children and thus potentially a more important outcome in developing prevention strategies.

Conclusion

By applying an unsupervised clustering approach to the gut microbiome data of 2 birth-cohort studies from neonate to school ages, we identified 6 enterotypes. We also revealed that factors (eg, the presence of older siblings, vaginal delivery, breast-feeding) were significantly related to gut microbiota composition. Additionally, we identified the individual relationships between gut microbiota abundance (eg, *Bifidobacterium* at 1 month old) and the development of food sensitization and allergy. Furthermore, neonates with *Bifidobacterium*-dominant enterotype had the lowest risks of developing food sensitization, especially to HEW, and allergy. Our findings should pave the way for further studies into the detailed mechanisms underlying the link between neonatal gut microbiota and food sensitization and allergy (eg, evaluation of host immune responses through transcriptome and proteome data). Furthermore, these findings should offer an evidence base for developing prevention strategies based on modifying gut microbiota[61] during the early postnatal period.

Declaration of AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used ChatGPT and Grammarly to improve readability and language of the manuscript. After using these tools/services, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

DISCLOSURE STATEMENT

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Key messages

- **In 2 birth-cohort studies in Japan, gut microbiome data collected from 1 week old to 7 years old identified 6 enterotypes with distinct proportions with aging of the children and gut environmental characteristics.**
- **More gut bacteria at 1 month old had significant associations with the development of food sensitization and allergy compared to those at 1 week old.**
- **Of the 3 enterotypes at 1 month old, *Bifidobacterium*-dominant enterotype neonates, with the highest fecal propionate concentration, had the lowest risks of food sensitization and allergy, especially HEW sensitization.**

REFERENCES

1. Alduraywish SA, Lodge CJ, Campbell B, Allen KJ, Erbas B, Lowe AJ, et al. The march from early life food sensitization to allergic disease: a systematic review and meta-analyses of birth cohort studies. Allergy 2016;71:77-89.
2. Waserman S, Bégin P, Watson W. IgE-mediated food allergy. Allergy Asthma Clin Immunol 2018;14:55.
3. Rönmark E, Bjerg A, Perzanowski M, Platts-Mills T, Lundbäck B. Major increase in allergic sensitization in schoolchildren from 1996 to 2006 in northern Sweden. J Allergy Clin Immunol 2009;124:357-63,63.e1-15.
4. Jackson KD, Howie LD, Akinbami LJ. Trends in allergic conditions among children: United States, 1997-2011. NCHS Data Brief 2013;(121):1-8.
5. Chiu CY, Huang YL, Tsai MH, Tu YL, Hua MC, Yao TC, et al. Sensitization to food and inhalant allergens in relation to atopic diseases in early childhood: a birth cohort study. PLoS One 2014;9:e102809.
6. Wärnberg Gerdin S, Lie A, Asarnoj A, Borres MP, Lødrup Carlsen KC, Färdig M, et al. Impaired skin barrier and allergic sensitization in early infancy. Allergy 2021;77:1464-76.
7. Salo PM, Arbes SJ Jr, Jaramillo R, Calatroni A, Weir CH, Sever ML, et al. Prevalence of allergic sensitization in the United States: results from the National Health and Nutrition Examination Survey (NHANES) 2005-2006. J Allergy Clin Immunol 2014;134:350-9.
8. Osborne NJ, Koplin JJ, Martin PE, Gurrin LC, Lowe AJ, Matheson MC, et al. Prevalence of challenge-proven IgE-mediated food allergy using population-based sampling and predetermined challenge criteria in infants. J Allergy Clin Immunol 2011;127:668-76.e1-2.
9. Renz H, Adkins BD, Bartfeld S, Blumberg RS, Farber DL, Garssen J, et al. The neonatal window of opportunity-early priming for life. J Allergy Clin Immunol 2018;141:1212-4.
10. Bäckhed F, Roswall J, Peng Y, Feng Q, Jia H, Kovatcheva-Datchary P, et al. Dynamics and stabilization of the human gut microbiome during the first year of life. Cell Host Microbe 2015;17:690-703.
11. Bokulich NA, Chung J, Battaglia T, Henderson N, Jay M, Li H, et al. Antibiotics, birth mode, and diet shape microbiome maturation during early life. Sci Transl Med 2016;8:343ra82.
12. Yassour M, Vatanen T, Siljander H, Hämäläinen AM, Härkönen T, Ryhänen SJ, et al. Natural history of the infant gut microbiome and impact of antibiotic treatment on bacterial strain diversity and stability. Sci Transl Med 2016;8:343ra81.
13. Stewart CJ, Ajami NJ, O'Brien JL, Hutchinson DS, Smith DP, Wong MC, et al. Temporal development of the gut microbiome in early childhood from the TEDDY study. Nature 2018;562:583-8.
14. Shao Y, Forster SC, Tsaliki E, Vervier K, Strang A, Simpson N, et al. Stunted microbiota and opportunistic pathogen colonization in caesarean-section birth. Nature 2019;574:117-21.
15. Roswall J, Olsson LM, Kovatcheva-Datchary P, Nilsson S, Tremaroli V, Simon MC, et al. Developmental trajectory of the healthy human gut microbiota during the first 5 years of life. Cell Host Microbe 2021;29:765-76.e3.
16. Yatsunenko T, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, Contreras M, et al. Human gut microbiome viewed across age and geography. Nature 2012;486:222-7.
17. Xu J, Lawley B, Wong G, Otal A, Chen L, Ying TJ, et al. Ethnic diversity in infant gut microbiota is apparent before the introduction of complementary diets. Gut Microbes 2020;11:1362-73.
18. Fallani M, Young D, Scott J, Norin E, Amarri S, Adam R, et al. Intestinal microbiota of 6-week-old infants across Europe: geographic influence beyond delivery mode, breast-feeding, and antibiotics. J Pediatr Gastroenterol Nutr 2010;51:77-84.
19. Fujimura KE, Sitarik AR, Havstad S, Lin DL, Levan S, Fadrosh D, et al. Neonatal gut microbiota associates with childhood multisensitized atopy and T cell differentiation. Nat Med 2016;22:1187-91.
20. Ruohtula T, de Goffau MC, Nieminen JK, Honkanen J, Siljander H, Hämäläinen AM, et al. Maturation of gut microbiota and circulating regulatory T cells and development of IgE sensitization in early life. Front Immunol 2019;10:2494.
21. Scheepers LEJM, Penders J, Mbakwa CA, Thijs C, Mommers M, Arts ICW. The intestinal microbiota composition and weight development in children: the KOALA Birth Cohort Study. Int J Obes 2015;39:16-25.
22. Vatanen T, Franzosa EA, Schwager R, Tripathi S, Arthur TD, Vehik K, et al. The human gut microbiome in early-onset type 1 diabetes from the TEDDY study. Nature 2018;562:589-94.
23. Azad MB, Konya T, Guttman DS, Field CJ, Sears MR, HayGlass KT, et al. Infant gut microbiota and food sensitization: associations in the first year of life. Clin Exp Allergy 2015;45:632-43.
24. Savage JH, Lee-Sarwar KA, Sordillo J, Bunyavanich S, Zhou Y, O'Connor G, et al. A prospective microbiome-wide association study of food sensitization and food allergy in early childhood. Allergy 2018;73:145-52.
25. Tanaka M, Korenori Y, Washio M, Kobayashi T, Momoda R, Kiyohara C, et al. Signatures in the gut microbiota of Japanese infants who developed food allergies in early childhood. FEMS Microbiol Ecol 2017;93:fix099.
26. Fazlollahi M, Chun Y, Grishin A, Wood RA, Burks AW, Dawson P, et al. Early-life gut microbiome and egg allergy. Allergy 2018;73:1515-24.
27. Roduit C, Frei R, Ferstl R, Loeliger S, Westermann P, Rhyner C, et al. High levels of butyrate and propionate in early life are associated with protection against atopy. Allergy 2019;74:799-809.
28. Chun Y, Grishin A, Rose R, Zhao W, Arditi Z, Zhang L, et al. Longitudinal dynamics of the gut microbiome and metabolome in peanut allergy development. J Allergy Clin Immunol 2023;152:1569-80.
29. Gao Y, Stokholm J, O'Hely M, Ponsonby AL, Tang MLK, Ranganathan S, et al. Gut microbiota maturity mediates the protective effect of siblings on food allergy. J Allergy Clin Immunol 2023;152:667-75.
30. Levan SR, Stamnes KA, Lin DL, Panzer AR, Fukui E, McCauley K, et al. Elevated faecal 12,13-diHOME concentration in neonates at high risk for asthma is produced by gut bacteria and impedes immune tolerance. Nat Microbiol 2019;4:1851-61.
31. Stokholm J, Thorsen J, Blaser MJ, Rasmussen MA, Hjelmsø M, Shah S, et al. Delivery mode and gut microbial changes correlate with an increased risk of childhood asthma. Sci Transl Med 2020;12:eaax9929.
32. Arumugam M, Raes J, Pelletier E, Le Paslier D, Yamada T, Mende DR, et al. Enterotypes of the human gut microbiome. Nature 2011;473:174-80.
33. Costea PI, Hildebrand F, Arumugam M, Bäckhed F, Blaser MJ, Bushman FD, et al. Enterotypes in the landscape of gut microbial community composition. Nat Microbiol 2018;3:8-16.
34. Tun HM, Peng Y, Chen B, Konya TB, Morales-Lizcano NP, Chari R, et al. Ethnicity associations with food sensitization are mediated by gut microbiota development in the first year of life. Gastroenterology 2021;161:94-106.
35. Dissanayake E, Tani Y, Nagai K, Sahara M, Mitsuishi C, Togawa Y, et al. Skin care and synbiotics for prevention of atopic dermatitis or food allergy in newborn infants: a 2 × 2 factorial, randomized, non-treatment controlled trial. Int Arch Allergy Immunol 2019;180:202-11.
36. Nakano T, Ochiai S, Suzuki S, Yamaide F, Morita Y, Inoue Y, et al. Breastfeeding promotes egg white sensitization in early infancy. Pediatr Allergy Immunol 2020;31:315-8.
37. Fikri B, Tani Y, Nagai K, Sahara M, Mitsuishi C, Togawa Y, et al. Soluble CD14 in breast milk and its relation to atopic manifestations in early infancy. Nutrients 2019;11:2118.
38. Kim SW, Suda W, Kim S, Oshima K, Fukuda S, Ohno H, et al. Robustness of gut microbiota of healthy adults in response to probiotic intervention revealed by high-throughput pyrosequencing. DNA Res 2013;20:241-53.
39. Mori H, Kato T, Ozawa H, Sakamoto M, Murakami T, Taylor TD, et al. Assessment of metagenomic workflows using a newly constructed human gut microbiome mock community. DNA Res 2023;30:dsad010.
40. Edgar RC. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 2010;26:2460-1.
41. O'Leary NA, Wright MW, Brister JR, Ciufo S, Haddad D, McVeigh R, et al. Reference sequence (RefSeq) database at NCBI: current status, taxonomic expansion, and functional annotation. Nucleic Acids Res 2016;44:D733-45.
42. Pearson WR, Lipman DJ. Improved tools for biological sequence comparison. Proc Natl Acad Sci U S A 1988;85:2444-8.
43. Japanese guideline for food allergy. Allergol Int 2011;60:221-36.

44. Urisu A, Ebisawa M, Ito K, Aihara Y, Ito S, Mayumi M, et al. Japanese guideline for food allergy 2014. Allergol Int 2014;63:399-419.
45. Holmes I, Harris K, Quince C. Dirichlet multinomial mixtures: generative models for microbial metagenomics. PLoS One 2012;7:e30126.
46. Subramanian S, Huq S, Yatsunenko T, Haque R, Mahfuz M, Alam MA, et al. Persistent gut microbiota immaturity in malnourished Bangladeshi children. Nature 2014;510:417-21.
47. Tsukuda N, Yahagi K, Hara T, Watanabe Y, Matsumoto H, Mori H, et al. Key bacterial taxa and metabolic pathways affecting gut short-chain fatty acid profiles in early life. ISME J 2021;15:2574-90.
48. Eggesbø M, Botten G, Stigum H, Nafstad P, Magnus P. Is delivery by cesarean section a risk factor for food allergy? J Allergy Clin Immunol 2003;112:420-6.
49. Kusunoki T, Mukaida K, Morimoto T, Sakuma M, Yasumi T, Nishikomori R, et al. Birth order effect on childhood food allergy. Pediatr Allergy Immunol 2012;23:250-4.
50. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, et al. Metagenomic biomarker discovery and explanation. Genome Biol 2011;12:R60.
51. Firth D. Bias reduction of maximum likelihood estimates. Biometrika 1993;80:27-38.
52. VanderWeele TJ, Ding P. Sensitivity analysis in observational research: introducing the E-value. Ann Intern Med 2017;167:268-74.
53. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc 1995;57:289-300.
54. Koplin J, Allen K, Gurrin L, Osborne N, Tang MLK, Dharmage S. Is caesarean delivery associated with sensitization to food allergens and IgE-mediated food allergy: a systematic review. Pediatr Allergy Immunol 2008;19:682-7.
55. Mitselou N, Hallberg J, Stephansson O, Almqvist C, Melén E, Ludvigsson JF. Cesarean delivery, preterm birth, and risk of food allergy: nationwide Swedish cohort study of more than 1 million children. J Allergy Clin Immunol 2018;142:1510-4.e2.
56. Metsälä J, Lundqvist A, Kaila M, Gissler M, Klaukka T, Virtanen SM. Maternal and perinatal characteristics and the risk of cow's milk allergy in infants up to 2 years of age: a case-control study nested in the Finnish population. Am J Epidemiol 2010;171:1310-6.
57. Zhang LL, Chen X, Zheng PY, Luo Y, Lu GF, Liu ZQ, et al. Oral *Bifidobacterium* modulates intestinal immune inflammation in mice with food allergy. J Gastroenterol Hepatol 2010;25:928-34.
58. Kim JH, Jeun EJ, Hong CP, Kim SH, Jang MS, Lee EJ, et al. Extracellular vesicle-derived protein from *Bifidobacterium longum* alleviates food allergy through mast cell suppression. J Allergy Clin Immunol 2016;137:507-16.e8.
59. Jin BY, Li Z, Xia YN, Li LX, Zhao ZX, Li XY, et al. Probiotic interventions alleviate food allergy symptoms correlated with cesarean section: a murine model. Front Immunol 2021;12:741371.
60. Rabe H, Lundell AC, Sjöberg F, Ljung A, Strömbeck A, Gio-Batta M, et al. Neonatal gut colonization by *Bifidobacterium* is associated with higher childhood cytokine responses. Gut Microbes 2020;12:1-14.
61. Kim JY, Kwon JH, Ahn SH, Lee SI, Han YS, Choi YO, et al. Effect of probiotic mix (*Bifidobacterium bifidum, Bifidobacterium lactis, Lactobacillus acidophilus*) in the primary prevention of eczema: a double-blind, randomized, placebo-controlled trial. Pediatr Allergy Immunol 2010;21:e386-93.
62. Sanidad KZ, Zeng MY. Neonatal gut microbiome and immunity. Curr Opin Microbiol 2020;56:30-7.
63. Yamaide F, Oniki N, Fikri B, Sato N, Nakano T, Shimojo N. Cord blood zonulin is associated with high-level sensitization to food allergen and food allergy development. Allergol Int 2024;73:338-9.
64. Penders J, Thijs C, Vink C, Stelma FF, Snijders B, Kummeling I, et al. Factors influencing the composition of the intestinal microbiota in early infancy. Pediatrics 2006;118:511-21.
65. Christensen ED, Hjelmsø MH, Thorsen J, Shah S, Redgwell T, Poulsen CE, et al. The developing airway and gut microbiota in early life is influenced by age of older siblings. Microbiome 2022;10:106.
66. Ma J, Li Z, Zhang W, Zhang C, Zhang Y, Mei H, et al. Comparison of gut microbiota in exclusively breast-fed and formula-fed babies: a study of 91 term infants. Sci Rep 2020;10:15792.
67. Fehr K, Moossavi S, Sbihi H, Boutin RCT, Bode L, Robertson B, et al. Breastmilk feeding practices are associated with the co-occurrence of bacteria in mothers' milk and the infant gut: the CHILD cohort study. Cell Host Microbe 2020;28:285-97.e4.
68. Katz Y, Rajuan N, Goldberg MR, Eisenberg E, Heyman E, Cohen A, et al. Early exposure to cow's milk protein is protective against IgE-mediated cow's milk protein allergy. J Allergy Clin Immunol 2010;126:77-82.e1.
69. Brew BK, Kull I, Garden F, Almqvist C, Bergström A, Lind T, et al. Breastfeeding, asthma, and allergy: a tale of two cities. Pediatr Allergy Immunol 2012;23:75-82.
70. Paton J, Kljakovic M, Ciszek K, Ding P. Infant feeding practices and nut allergy over time in australian school entrant children. Int J Pediatr 2012;2012:675724.
71. Muraro A, Dreborg S, Halken S, Høst A, Niggemann B, Aalberse R, et al. Dietary prevention of allergic diseases in infants and small children. Part III: critical review of published peer-reviewed observational and interventional studies and final recommendations. Pediatr Allergy Immunol 2004;15:291-307.
72. Munblit D, Peroni DG, Boix-Amorós A, Hsu PS, Van't Land B, Gay MCL, et al. Human milk and allergic diseases: an unsolved puzzle. Nutrients 2017;9:894.
73. Lodge CJ, Tan DJ, Lau MXZ, Dai X, Tham R, Lowe AJ, et al. Breastfeeding and asthma and allergies: a systematic review and meta-analysis. Acta Paediatr 2015;104:38-53.