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Original Article

Gut microbiota and fecal metabolites in sustained unresponsiveness by oral immunotherapy in school-age children with cow's milk allergy

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CM, cow's milk; CMA, cow's milk allergy; DBPCFC, double-blind placebo-controlled food challenge; EHCF, extensively hydrolyzed casein formula; FDR, false discovery rate; Mb, gut microbiota; OIT, oral immunotherapy; OTU, operational taxonomic unit; PC, principal component; PCA, principal component analysis; SCFA, short-chain fatty acids; SU, sustained unresponsiveness; WGCNA, weighted gene co-expression network analysis; WSM, water-soluble metabolite

ABSTRACT

Background: Oral immunotherapy (OIT) can ameliorate cow's milk allergy (CMA); however, the achievement of sustained unresponsiveness (SU) is challenging. Regarding the pathogenesis of CMA, recent studies have shown the importance of gut microbiota (Mb) and fecal water-soluble metabolites (WSMs), which prompted us to determine the change in clinical and gut environmental factors important for acquiring SU after OIT for CMA.
Methods: We conducted an ancillary cohort study of a multicenter randomized, parallel-group, delayed-start design study on 32 school-age children with IgE-mediated CMA who underwent OIT for 13 months. We defined SU as the ability to consume cow's milk exceeding the target dose in a double-blind placebo-controlled food challenge after OIT followed by a 2-week-avoidance. We longitudinally collected 175 fecal specimens and clustered the microbiome and metabolome data into 29 Mb- and 12 WSM-modules.
Results: During OIT, immunological factors improved in all participants. However, of the 32 participants, 4 withdrew because of adverse events, and only 7 were judged SU. Gut environmental factors shifted during OIT, but only in the beginning, and returned to the baseline at the end. Of these factors, milk- and casein-specific IgE and the *Bifidobacterium*-dominant module were associated with SU (milk- and casein-specific IgE; OR for 10 kU_A/L increments, 0.67 and 0.66; 95%CI, 0.41–0.93 and 0.42–0.90; *Bifidobacterium*-dominant module; OR for 0.01 increments, 1.40; 95%CI, 1.10–2.03), and these associations were observed until the end of OIT.
Conclusions: In this study, we identified the clinical and gut environmental factors associated with SU acquisition in CM-OIT.

Introduction

Cow's milk (CM) allergy (CMA) is one of the most common food allergies in children, reportedly in the range from 1.2 % to 17 % in a meta-analysis of infants and adults.[1] Fifty to sixty percent of the children have the possibility of naturally outgrowing their CMA by

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3–5 years of age.[2–5] However, the rest are forced to continue avoiding CM intake and live under the fear of severe or even fatal reactions because of accidental exposure to foods or drinks that contain CM, which is ubiquitous in the food supply.

As a treatment option for CMA, several randomized controlled trials have shown the effectiveness of oral immunotherapy (OIT) and the acceptably reduced risk of treatment-associated adverse effects (i.e., severe or fatal anaphylaxis) by exercising with great caution during OIT performed by specialists.[6–10] Nevertheless, the accumulated knowledge about OIT also informs us of its limitations; unresponsiveness to CM, as well as other food allergens, is only sustained as long as patients keep ingesting the allergenic foods. When stopped, the unresponsiveness can be lost in a relatively short period, despite the relentless efforts worldwide to increase the likelihood of acquiring sustained unresponsiveness (SU), making it still challenging to acquire SU.[11–14]

In tolerance to food allergens, the significance of the gut microbiota and fecal short-chain fatty acids (SCFAs) has been recognized in murine models[15–20] and in human cohorts of non-IgE-mediated CMA in infants[21] and peanut allergy in adults.[22] However, little is known about the relationship of fecal water-soluble metabolites (WSMs), except for SCFAs, to tolerance to food allergens, despite reports of the immune-modulating WSMs derived from the gut microbiota, e.g., vitamins[23] and amino acids.[24,25] Additionally, no study has reported a relationship among OIT, gut environmental factors, and the chance of acquiring SU in school-age children with IgE-mediated CMA.

To address these knowledge gaps, we analyzed data from an ancillary cohort study of a multicenter randomized, parallel-group, delayed-start design study on school-age children with IgE-mediated CMA who underwent OIT to evaluate the relationship of clinical and gut environmental factors to the acquisition of SU.

Methods

Study design and participants

By analyzing data from a multicenter randomized, parallel-group, delayed-start design study of 3-month-OIT for children with IgE-mediated CMA, i.e., the primary study, we conducted this ancillary cohort study (Fig. 1). The randomized study was carried out in two different groups. The only difference between the two groups in this ancillary cohort study was the later start of OIT in the Delayed-start group. The primary endpoint of the randomized study was the proportion of the participants with exceeding the target dose (≥44.4 ml of CM) in a double-blind placebo-controlled food challenge (DBPCFC) after 3 months. After the randomized study for 3 months, participants in the Early-start group continued to undergo an additional 10-month-OIT, and those in the delayed-start group underwent 13-month-OIT with the same protocol as the Early-start group. The sample size of the randomized study (initially n = 20 per group) was determined to detect a 0.1 % difference of the outcome between the two groups at a 1:1 allocation with a 90 % power and a significance level of 0.05; the study achieved the primary endpoint at the interim analysis of 10 participants in each group and was terminated when 16 participants in each group were recruited. Therefore, in this ancillary cohort study, we merged the data from early-start and delayed-start groups (n = 32). This study was performed according to The Declaration of Helsinki Principles, and the Ethical Review Boards of all the participant hospitals (Aichi Children's Health and Medical Center, Chiba Children's Hospital, Chiba University Hospital, Dokkyo Medical University Hospital, Gunma University Hospital, Osaka Prefectural Medical Center for Respiratory and Allergic Diseases, National Mie Hospital, National Shimoshizu Hospital, and Toyama University Hospital) approved the study protocol (Approval number, 23–7) with written informed consent from both children and their guardians. The randomized study, i.e., the primary study of this ancillary cohort study, was registered through the University hospital Medical Information Network (UMIN000003943), as is our study of hen egg OIT.[26]

School-age children with IgE-mediated CMA were recruited at nine allergy centers mentioned above located in urban areas all over Japan. Eligible participants ranged from 5 to 15 years old and had the following clinical features: an immediate hypersensitivity reaction after CM ingestion, positive milk-specific IgE, and positive results in a DBPCFC with ≤10 ml of CM. We excluded participants with soy allergy, uncontrolled asthma[27] and atopic dermatitis,[28] and cardiac, renal, or hepatic diseases.

Double-blind placebo-controlled food challenge

For DBPCFC, we used the same commercially available CM as for OIT and soy milk, adding flavors to adjust the taste as a placebo beverage. At 48 h after discontinuation of anti-histamine and anti-allergic agents, 0.01 ml of CM was ingested, and subsequently the dose was increased to 0.03, 0.1, 0.3, 1, 3, 10, and 30 ml every 20 min. We judged only obvious symptoms as positive allergic reactions. To confirm whether the symptoms were triggered by the CM intake, CM and placebo were randomly used and blinded to participants and medical staff. The threshold for the DBPCFC was set at the amount ingested one step before the symptoms were evoked. We judged the DBPCFC as successful when the participant could consume 30 ml of CM without any allergic reactions, i.e., the cumulative dose threshold was 44.4 ml.

Interventions

Our OIT protocol was developed according to a previously published study.[7] OIT consisted of the Build-up phase during hospitalization for about 1 month and the Maintenance phase at home for 12 months (Fig. 1). Hospitalization was essential for the Build-up phase because of the high risk of a severe anaphylactic reaction. The initial intake was set at approximately 1/100 or less of the symptom induction threshold in the DBPCFC at M0 (Fig. 1). For each intake, the dose was increased by a maximum of 20 %, up to 3 times per day, with the goal of a total daily intake of 200 ml. The intake interval was set at 30 min or more, with all intakes to be completed within 3 h of the first one. Before patient discharge and transition to the Maintenance phase, we confirmed that allergic symptoms did not occur during exercise and bathing 30 min after CM intake. The participants continued the intake of 200 ml of CM at home every day and visited the outpatient department for regular follow-up. Clinicians were allowed to reduce the daily CM intake dose of CM at their discretion when they judged adverse reactions to be severe.

Once-daily epinastine was administered orally to all the participants during the Build-up phase to relieve allergic symptoms[29] and discontinued when the ingestion dose reached 200 ml per day during the Build-up phase. No anti-histamine agents were used during the Maintenance phase unless the participants had already been taking them before this study.

Adverse effects

All adverse effects were strictly monitored and graded by modifying the Sampson's grading system[30] as shown in Supplementary Table 1. We downgraded “rhinorrhea” and the “sensation of throat pruritus or tightness, several intermittent

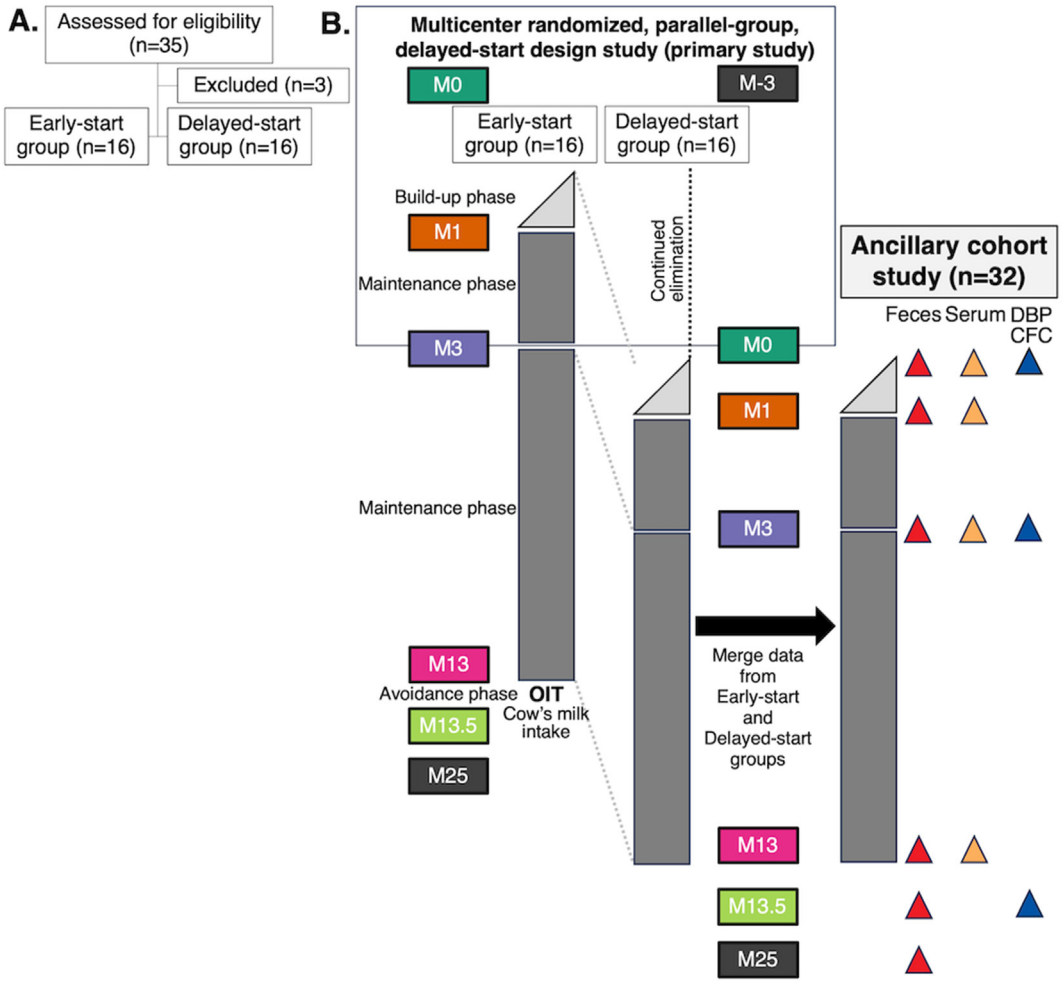

Fig. 1. Overview of study design. **(A)** Flow diagram shows the enrollment, exclusion, and randomization of participants in a multicenter randomized, parallel-group, delayed-start design study of oral immunotherapy (OIT) for school-age children with IgE-mediated cow's milk allergy, i.e., the primary study. In the randomized study, the participants were divided into the Early- and Delayed-start groups. **(B)** The participants in the Early- and Delayed-start groups underwent OIT with a three-month offset. OIT comprised of the Build-up, Maintenance, and Avoidance phases, and specimen collection and double-blind placebo-controlled food challenge (DBPCFC) were performed longitudinally during OIT. The data from the two groups were merged for the current ancillary cohort study.

coughs" according to our previous practical knowledge that these adverse symptoms did not always influence OIT intervention. For moderate adverse reactions, we discontinued the scheduled dose and immediately administered treatment for systemic symptoms. The next day, the intake was resumed at an amount one step lower than the dose that caused the adverse reaction. For severe adverse reactions, we discontinued OIT for the participant after immediate and appropriate treatment.

Data collection

During OIT, clinical data, as well as blood and fecal specimens, were collected at each facility at the time points summarized in Fig. 1. Of the clinical data, 'a history of allergic diseases' was defined as having the previous physician's diagnosis regardless of receiving the treatment during OIT, and 'concurrent treatment of allergic diseases' was defined as receiving treatment during OIT. Blood specimen collection and skin prick test (SPT) were conducted 48 h after discontinuing anti-histamine and anti-allergic agents. When antibiotics were administered, fecal specimens were obtained 2–3 weeks after cessation of the antibiotics. From the 16 who received OIT later, we collected blood and fecal specimens at entry (M−3) in addition to the start of OIT (M0; i.e., about 3 months after the entry; Supplementary Table 2). Furthermore, we collected fecal specimens 1 year after evaluation of SU (M25) (Supplementary Table 2). Since this study focused on the relationship of gut environmental factors with the acquisition of SU during the period of OIT start (M0) to SU evaluation (M13.5), data from fecal specimens collected at M−3 and M25 were not used for the analysis of the OIT effect but only used for module construction as described below. Missing specimens, i.e., specimens that were not collected, specimens from participants who discontinued OIT and specimens in which measurement errors occurred during 16S rRNA sequencing or mass spectrometry analysis are summarized in Supplementary Table 2. For blood specimens, serum specific IgG4 and IgE were quantified using a Phadia CAP System (Phadia, Uppsala, Sweden). The SPT was performed at each center with a CM allergen solution (Torii scratch extract; Torii Pharmaceutical Co., Ltd., Japan) and a Bifurcated Needle (Alo Laboratories, Inc., USA) at the same timing as blood specimen collection. For fecal specimens, the preparation and subsequent omics and bioinformatics approaches are described in Supplementary Methods.

Outcome

In this study, the primary outcome was SU defined as exceeding the target dose (≥44.4 ml) in the DBPCFC 13 months after the start of OIT followed by 2-weeks of CM elimination.[31]

Statistical analysis

Analysis used R version 4.1.3 (R Foundation, Vienna, Austria). All p values were two-tailed, with $p < 0.05$ considered statistically significant. We computed the Benjamini-Hochberg false discovery rate (FDR), which allows for the interpretation of statistical significance in the context of multiple hypothesis testing,[32] with FDR <0.05 considered statistically significant.

We first determined the change of immunological parameters by the Wilcoxon rank-sum test compared to M0. Next, to determine the change in gut microbiota and WSMs, we clustered these data into modules with weighted gene co-expression network analysis (WGCNA)[34–36] using the R "wgcna" package. This approach can reduce the dimensionality of the data and facilitate understanding of the interrelationship between gut microbiota and fecal WSMs. WGCNA was initially developed to interpret high-throughput gene expression data[33] but has since been applied to 16S rRNA gene-based metagenome[34] and metabolome[35] data sets. Data from fecal specimens collected at M−3 and M25 were only used in constructing modules by WGCNA to compensate for the small sample size and to stabilize the result of clustering. We removed the rare gut microbiota or fecal WSMs observed in less than 20 % of all specimens. For fecal WSMs, we normalized the concentration by a mean-centered approach followed by $\log_2$-transformation.[35] We then applied the relative abundance of the gut microbiota at the operational taxonomic unit (OTU) level and the concentration of fecal WSMs into the WGCNA. We identified the optimal soft-thresholding powers for clustering based on a scale-free topology criterion. We set the powers to 3 for gut microbiota, 5 for fecal WSM and 3 for all minimum cluster size. Then, we constructed the signed correlation network, i.e., connection strengths nodes with negative correlation are considered unconnected, and identified clusters with the dendrogram cut height of 0.2 and the minimum cluster size of 3. We labeled the gut microbiota modules with "Mb" and the fecal WSMs modules with "WSM" and added the number in descending order of the number of module members. We also combined the unclustered factors into a module and added the number 00 to that module. Additionally, at the end of module annotation, we added the family level to which the most OTUs belonged for Mb-modules and the subclass to which the most WSMs belonged for fecal WSM-modules. Lastly, we computed the eigenvalue, i.e., the first principal component [PC] of the module abundance matrix, and applied the value for the following analysis.

We next clarified the changes in gut environmental factors during OIT. First, to evaluate the importance of timepoint during OIT in the Mb- and WSM-module profiles among clinical factors, we determined a set of proportions of variance explained by computing Euclid distances and performing permutational multivariate analysis of variance with the "vegan" R package.[36] Second, to evaluate the change of the profiles according to the timepoint, we used the Euclidean distances for visualizing the principal coordinates analysis (PCoA) plots. Third, to interpret characteristics in gut environmental factors between timepoints, we created heatmaps by the median module eigenvalues using the "pheatmap" R package.[37] The between-timepoint differences in the eigenvalues were tested by the Kruskal–Wallis test. To cluster modules and timepoints with similar characteristics, we also created dendrograms by their Euclidean distances using hierarchical clustering and the Ward method using the "pheatmap" R package.[37] At last, to examine the differences in alpha diversity between timepoints, we computed Chao1 (i.e., evenness), Simpson (i.e., richness), and Shannon (i.e., evenness and richness) indices for gut microbiome data by using the "vegan" R package[36] and the number of WSM for fecal WSM data and compared the differences by using the Wilcoxon's rank-sum test with M0 as the reference.

We also examined the association of clinical and gut environmental factors at M0 with acquiring SU by constructing unadjusted logistic regression models. Of the logistic regression models, the models with sparse outcome data were corrected by Firth's method using the R package "logistf"[38] to reduce a bias of maximum likelihood estimate. Because of the small sample size, we did not construct multivariable models. For the factors that were measured longitudinally and with statistical significance in the models at M0 (SU-associated factors, $p < 0.05$), we visualized the probability of acquiring SU using locally estimated scatterplot smoothed curves and determined the association of these factors at M1, M3, M13, and M13.5 with acquiring SU. Additionally, considering the effect of antibiotics use during OIT, we confirmed the consistency in the associations of the factors with SU regardless of excluding the data after antibiotics use. Furthermore, we visualized and tested the change during OIT by the Wilcoxon's rank-sum test compared to M0. Additionally, for the SU-associated gut environmental modules, we identified the important gut microbiota or fecal WSMs by visualizing scatter plot using the module membership (FDR of the Spearman's correlation analysis between the relative abundance and the module eigenvalue) and the association of the abundance with SU (p value estimated by logistic regression model). In this scatter plot, the members that locate more to the upper right are more important for acquiring SU in the module.

Finally, to examine the relationship of these SU-associated factors to other gut environmental modules, we computed the partial Spearman's rho adjusted by CM daily intake using the R package "ppcor"[39] to mitigate the effect of CM intake *per se* and visualized the significant relationship by heatmap using absolute coefficients above 0.25 and FDR below 0.05 as criteria. We also visualized the relationship by correlation network using the R package "igraph"[40] and, to determine the important modules in this network, computed betweenness centrality using the edge number that satisfied the criteria.

Results

Study design and study population

An overview of this ancillary study from July 2011 to October 2013 is depicted in Figure 1. Thirty-five children were recruited, and 3 of them were excluded from the study because their threshold of baseline DBPCFC was above the inclusion criterion ($\leq$10 ml; Fig. 2A). The demographic data of the 32 participants are summarized in Table 1, and there was no difference between the early- and delayed-start groups ($p \geq 0.05$; Supplementary Table 3).

Improvement of clinical parameters during OIT and a low probability of acquiring SU

Of the 32 participants, two discontinued the OIT due to severe adverse events during the Build-up phase, and one participant withdrew from the OIT one month after the Build-up phase. An overview of observed harmful events during OIT is summarized in Supplementary Table 4. In particular, 44 % experienced grade 3–5 gastrointestinal adverse events (i.e., single or recurrent vomiting or diarrhea) during the build-up phase, while 9 % had the symptoms during the Maintenance phase. Of the remaining 29 participants, 21 passed DBPCFC at M3, and we judged that they had acquired desensitization to CM (Fig. 2A). After DBPCFC at M3, one participant discontinued after six months of the Maintenance phase due to recurring adverse events. The immunological parameters,

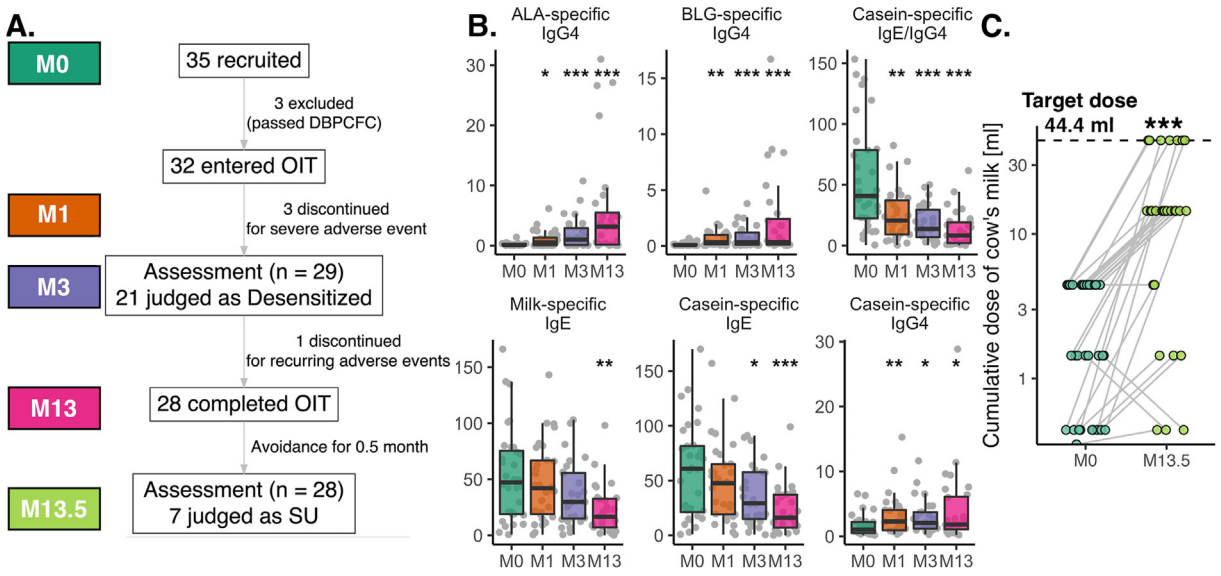

Fig. 2. Cow's milk oral immunotherapy and the outcome. **(A)** Flow diagram of the clinical study-enrollment and the result of a double-blind placebo-controlled food challenge (DBPCFC) for the assessment of desensitization and sustained unresponsiveness (SU). **(B)** Change of levels of serum immunoglobulin (IgE, kU_A/L; IgG4, mgA/L; IgG4/IgE, kU_A/mgA) during oral immunotherapy (OIT). Boxplots show the median with interquartile range. **(C)** Change of the cumulative dose of cow's milk at DBPCFC. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (the Wilcoxon rank sum test, compared to M0).

including milk- and casein-specific IgE, casein-, alpha-lactalbumin- and beta-lactoglobulin- specific IgG4, and the casein-specific IgE/IgG4 ratio, significantly improved from M0 to M13 (Fig. 2B). After the Maintenance phase for 12 months, the participants discontinued CM intake for 2 weeks (the Avoidance phase; Fig. 1) and underwent DBPCFC at M13.5. Despite their significantly improved immunological parameters at M13 (Fig. 2B) and the increased threshold for the cumulative dose of CM (Fig. 2C), only seven out of the 28 participants exceeded the cumulative target dose for SU (Fig. 2A).

Table 1
Patient characteristics.

Variables	n = 32
Demographics	
Male sex, number (percentage)	26 (81)
Age (years), median (IQR)	7 (6–9)
History of allergic diseases	
Atopic dermatitis	30 (94)
Allergic conjunctivitis	11 (34)
Allergic rhinitis	18 (56)
Asthma	27 (84)
Concurrent treatment of allergic diseases	
Atopic dermatitis	17 (53)
Allergic conjunctivitis	2 (6)
Allergic rhinitis	4 (12)
Asthma	24 (75)
Severity of CMA	
Complete elimination of before OIT	24 (75)
Threshold dose at DBPCFC (ml)	3.0 (1.0–10.0)
Epinephrine use at DBPCFC	6 (19)
Immunological parameters, median (IQR)	
Milk-specific IgE (kU_A/L)	47.2 (23.6–75.3)
Casein-specific IgE (kU_A/L)	67.0 (21.9–90.2)
Casein-specific IgG4 (mgA/L)	1.0 (0.5–2.1)
ALA-specific IgG4 (mgA/L)	0.1 (0.1–0.3)
BLG-specific IgG4 (mgA/L)	0.1 (0.1–0.2)
Casein-specific IgE/IgG4 (kU_A/mgA)	42.6 (21.4–81.9)
SPT wheal size to milk (mm)	12.6 (10.0–15.0)

IQR, interquartile range; CMA, cow's milk allergy; OIT, oral immunotherapy; DBPCFC, double-blind placebo-controlled food challenge; SPT, skin prick test; ALA, alpha-lactalbumin; BLG, beta-lactoglobulin.

Temporary changes in gut environment factors during OIT

For analysis of gut environmental factors, after removing those observed in less than 20 % of all specimens, the remaining 203 out of 3580 OTUs and 185 out of 315 WSMs were clustered into 29 Mb-modules (Supplementary Table 5) and 12 WSM-modules (Supplementary Table 6). Regarding major SCFAs, acetate, propionate, and butyrate were assembled into WSM-03, -01, and −00 modules, respectively. Of clinical factors, the profiles of Mb- and WSM-module were most significantly explained by timepoints during OIT (Fig. 3A). Indeed, profiles of Mb- and WSM-modules showed some degree of differences between timepoints on the PCoA plot (Fig. 3B, C); 7 Mb-modules, e.g., Mb-03: *Bacteroidaceae* and Mb-09: *Lachnospiraceae* (Fig. 3D) and 3 WSM-modules, e.g., WSM-10: Fatty Acids and Conjugates (Fig. 3E), were significantly different (FDR <0.05). Compared to the gut environmental module profiles at M0, the profiles were different from those at M1 while became similar to those at M13 and M13.5 (Fig. 3D, E). Likewise, alpha diversity indices in Mb and WSM, except for Chao1 index of Mb, significantly differed at the beginning of OIT while returned closer to the baseline levels at the end of OIT (Supplementary Fig. 1, 2).

Association of clinical and gut environmental factors with acquiring SU

Of the clinical factors at M0, concurrent treatment of atopic dermatitis or asthma was significantly associated with a lower chance of acquiring SU (atopic dermatitis; OR, 0.09; 95 % CI, 0.00–0.67; $p = 0.041$; asthma; OR, 0.16; 95 % CI, 0.02–0.87; $p = 0.034$; Table 2). Also, higher levels of milk- and casein-specific IgE were significantly associated with a lower chance of acquiring SU (milk-specific IgE; OR for each 10 kU_A/L increment, 0.67; 95 % CI, 0.41–0.93; $p = 0.046$; casein-specific IgE; OR, 0.66; 95 % CI, 0.42–0.90; $p = 0.027$; Table 2 and Fig. 4A). Likewise, of the gut environmental factors at M0, higher Mb-24: *Bifidobacteriaceae* was associated with a higher chance of acquiring SU (OR for each 0.01 eigenvalue increment, 1.40; 95 % CI, 1.10–2.03; $p = 0.024$;

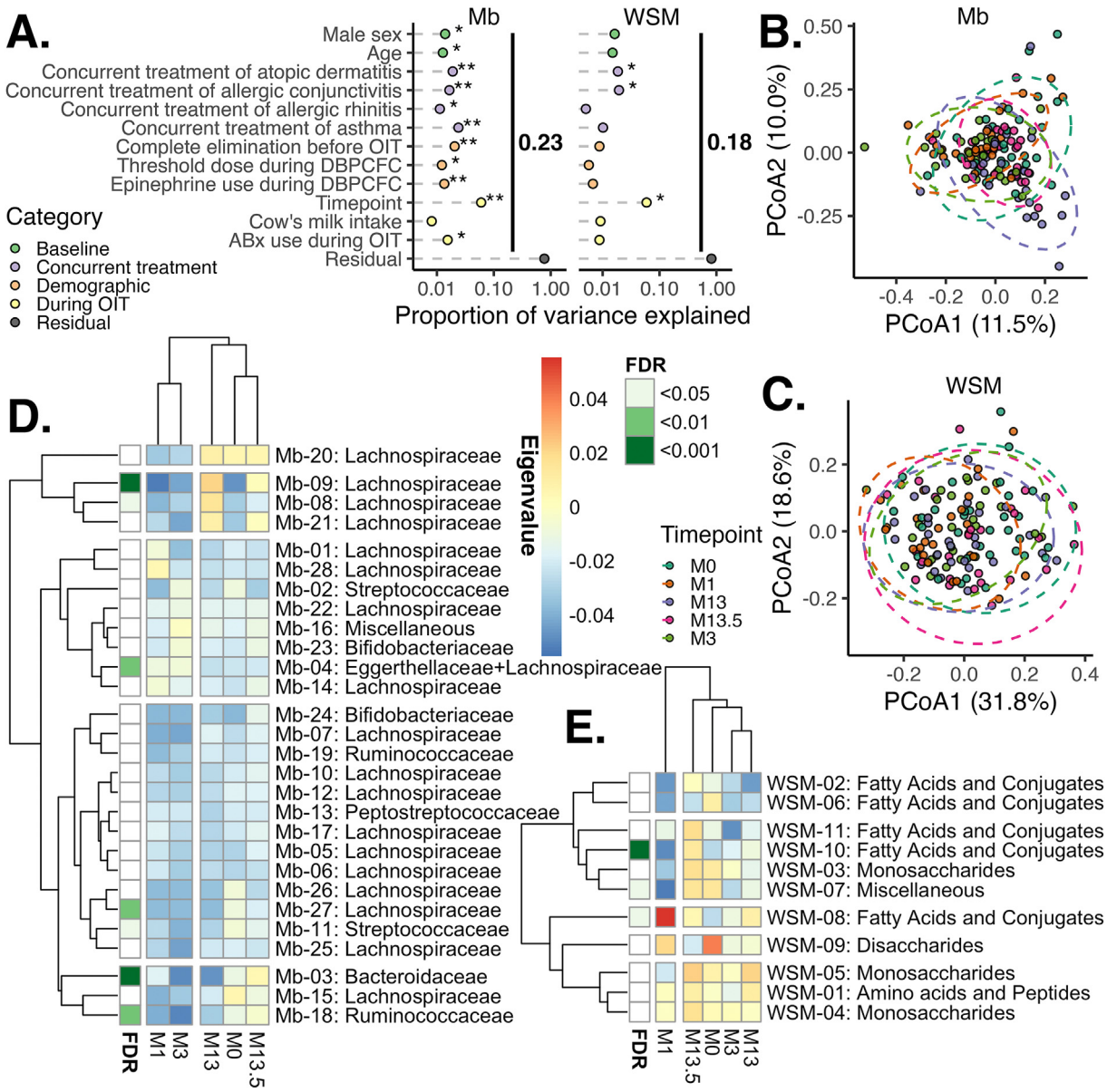

Fig. 3. Changes in the gut microbiota and fecal water-soluble metabolites modules during oral immunotherapy. Lollipop plots **(A)** show the proportion of variance of gut microbiota (Mb) or water-soluble metabolite (WSM) module eigenvalue explained by clinical factors (* FDR <0.05; ** FDR <0.01; permutational multivariate analysis of variance). The cumulative proportions of variance explained by clinical factors are represented by the numbers in the plots. Principal Coordinates Analysis (PCoA) plots by using Euclidian distances visualize changes in eigenvalues of **(B)** gut microbiota (Mb) and **(C)** fecal water-soluble metabolite (WSM) modules among timepoints during oral immunotherapy (OIT). Dashed-line ellipses represent 95 % confidence intervals. Heatmaps summarize the median eigenvalues of Mb **(D)** and WSM **(E)** modules, according to timepoints during OIT. The between-timepoint differences in the eigenvalues were tested by the Kruskal–Wallis test. The similarities of the eigenvalues were represented by dendrograms using hierarchical clustering with Euclidean distances and the Ward method.

FDR = 0.70; Table 2, Fig. 4A, and Supplementary Table 7). Additionally, these three factors at M3, M13, and M13.5 were also associated with subsequent SU acquisition (Supplementary Table 8). Furthermore, these associations of the three factors at M3, M13, and M13.5 remained significant after removing post-antibiotics use data for 3 participants, except for milk-specific IgE at M3 (Supplementary Table 9). In the Mb-24: *Bifidobacteriaceae* module, all the members were assigned to the *Bifidobacterium* genus (Supplementary Fig. 3). Among these three longitudinally SU-associated factors, the changes in the SU and non-SU groups during OIT were different (Fig. 4B). Milk-specific IgE showed decreasing trends in both the SU (median at M0, 21.2 kU_A/L; median at M13, 7.6 kU_A/L; $p = 0.053$) and non-SU groups (median at M0, 62.8 kU_A/L; median at M13, 25.3 kU_A/L; $p = 0.008$). Likewise, casein-specific IgE showed decreasing trends in both the SU (median at M0, 17.8 kU_A/L; median at M13, 7.5 kU_A/L; $p = 0.097$) and non-SU groups (median at M0, 72.8 kU_A/L; median at M13, 30.4 kU_A/L; $p = 0.003$). By contrast, Mb-24: *Bifidobacterium* showed an increasing trend only in the SU group (median at M0, 0.01; median at M13, 0.15; $p = 0.096$) and not in the non-SU group (median at M0, -0.04; median at M13, -0.04; $p = 0.25$).

Table 2
Clinical and gut environmental factors at baseline associated with the subsequent sustained unresponsiveness by oral immunotherapy.

Variables	Odds ratio[†] (95 % CI)	*p* value[†]
Demographics		
Male sex	2.10 (0.24–14.51)	0.46
Age (years)	1.02 (0.67–1.52)	0.93
History of allergic diseases		
Atopic dermatitis[‡]	1.60 (0.11–229.75)	0.76
Allergic conjunctivitis	0.25 (0.01–1.78)	0.23
Allergic rhinitis	0.50 (0.08–2.74)	0.42
Asthma	0.34 (0.04–3.10)	0.30
Concurrent treatment of allergic diseases		
Atopic dermatitis	0.09 (0.00–0.67)	0.041
Allergic conjunctivitis[‡]	0.63 (0.00–8.92)	0.76
Allergic rhinitis[‡]	0.32 (0.00–3.59)	0.40
Asthma	0.16 (0.02–0.87)	0.034
Severity of CMA		
Complete elimination before OIT	0.79 (0.13–6.50)	0.81
Threshold dose at DBPCFC (ml)	1.1 (1.0–1.3)	0.15
Epinephrine use at DBPCFC	0.20 (0.00–2.08)	0.21
Immunological parameters		
Milk-specific IgE (kU_A/L)	0.67 (0.41–0.93)[§]	0.046
Casein-specific IgE (kU_A/L)	0.66 (0.42–0.90)[§]	0.027
Casein-specific IgG4 (mgA/L)	0.99 (0.93–1.04)[‖]	0.76
ALA-specific IgG4 (mgA/L)	1.02 (0.67–1.20)[‖]	0.45
BLG-specific IgG4 (mgA/L)	0.87 (0.37–1.20)[‖]	0.66
Casein-specific IgE/IgG4 (kU_A/mgA)	0.93 (0.73–1.13)[§]	0.52
SPT wheal size to milk (mm)	0.77 (0.55–0.99)	0.081
Alpha diversity		
Chao1 index in Mb	1.00 (1.00–1.01)	0.50
Simpson index in Mb	11.78 (0.77–325.17)	0.098
Shannon index in Mb	2.60 (0.33–31.72)[‖]	0.40
Number of observed WSM	0.95 (0.87–1.03)	0.20
Mb and WSM modules[††]		
Mb-24: Bifidobacteriaceae	1.40 (1.10–2.03)[‡‡]	0.024

CMA, cow's milk allergy; OIT, oral immunotherapy; DBPCFC, double-blind placebo-controlled food challenge; SPT, skin prick test; ALA, alpha-lactalbumin; BLG, beta-lactoglobulin, Mb, gut microbiota; WSM, fecal water-soluble metabolite.

[†] Odds ratios of sustained unresponsiveness are estimated by a logistic regression model.

[‡] The logistic regression models were corrected by Firth's method to reduce a bias of maximum likelihood estimate for a low proportion of the outcome.

[§] Values are estimated for every 10 kU_A/L increase in IgE level or 10 kU_A/mgA in IgE/IgG4 level.

[‖] Values are estimated for every 0.1 mgA/L increase in IgG4 level or 0.1 increase in Shannon index.

[††] Only the module with statistical significance is shown.

[‡‡] Values are estimated for every 0.01 increase in module eigenvalue.

Correlation network among longitudinally SU-associated factors and other gut environmental modules

Next, we determined the relationships of the three longitudinally SU-associated factors, i.e., milk- and casein-specific IgE and Mb-24: *Bifidobacteriaceae*, with the other Mb- and WSM-modules. Milk- and casein-specific IgEs were significantly and negatively correlated with 5 Mb-modules, i.e., 02: *Streptococcaceae* and 09, 15, 17, and 20: *Lachnospiraceae*, and 3 WSM-modules. i.e., 04 and 05: Monosaccharides and 10: Fatty acids and Conjugates, while Mb-24: *Bifidobacteriaceae* was significantly and positively correlated with only Mb-17: *Lachnospiraceae* (Fig. 5A). We visualized the SU-associated network (Fig. 5B) and determined the importance, i.e., centrality, in the network (Supplementary Table 10). The highest centrality was observed in Mb-09: *Lachnospiraceae* among the Mb-modules (centrality, 1.25) and WSM-04: Monosaccharides among the WSM-modules (centrality, 1.33). Of the 8 OTUs in the Mb-09: *Lachnospiraceae*, 4 OTUs were assigned as *Fusicatenibacter saccharivorans* which was also identified in 3 of the 5 OTUs in Mb-20: *Lachnospiraceae* (Supplementary Table 5). In the WSM-04: Monosaccharides, 5 monosaccharides - galactose, fucose, N-Acetylglucosamine, N-Acetylneuraminic acid, and rhamnose, and 1 disaccharide, lactose, were identified (Supplementary Table 6).

Discussion

In the current prospective and longitudinal study of OIT for school-age children with IgE-mediated CMA, we found that OIT improved the immunological parameters, however the probability of SU acquisition was low. We also identified the changes in gut microbiota and fecal WSMs during OIT. Additionally, of the clinical and gut environmental factors, we identified the longitudinal association of lower levels of milk- and casein-specific IgE and a higher eigenvalue of the *Bifidobacterium*-dominant module with a higher chance of achieving SU. Furthermore, we identified the relationship between these factors associated with SU and other gut environmental modules. To the best of our knowledge, this is the first investigation that has identified changes in gut environmental factors and their association with SU acquisition in OIT for school-age children with IgE-mediated CMA.

Although, for food allergy, OIT is one of the effective treatments, OIT frequently fails to lead to SU, especially in CMA. Indeed, of the 32 participants in our study, only seven (22 %) acquired 2-week-SU (Fig. 2A, B)—a similar proportion to that previously reported.[13,14] In tolerance to food allergens, growing evidence suggests the importance of the gut environment. The results of our study using the gut microbiome and fecal metabolome data are in agreement with studies that have evaluated the relationship between the gut environment and tolerance to food allergens. For example, in experiments in mice, previous literature using a mouse model of sensitization with peanut extract has shown that high-fiber feeding reshaped gut microbiota composition, increased fecal acetate and butyrate concentrations and improved oral tolerance.[17] Another study using a mouse model of sensitization with whey has shown that OIT alleviated acute allergic symptoms, with fructo-oligosaccharides enhancing this effect[18] and that the combination of OIT and fructo-oligosaccharides increased cecal butyrate and propionate.[20] In a human cohort, previous research in infants with non-IgE-mediated CMA has shown that, after dietary management by extensively hydrolyzed casein formula (EHCF), infants acquiring tolerant had a higher relative abundance of *Oscillospilla* and a higher concentration of butyrate.[21] Thus, a better understanding of the gut environment in OIT for CMA may inform potential strategies to facilitate SU to CM, e.g., the combined use of biotics.

Although the exact mechanisms underlying the observed findings warrant further investigation, the immunological and gut environmental factors significantly shifted during OIT. However, in contrast to immunological factors that were improved during the entire period (Fig. 2C), gut environmental profiles at the baseline were different from the profiles at the beginning of OIT yet became similar to the profiles at the end of OIT (Fig. 2). These substantial shifts at the beginning could be explained by, in addition to the effects of OIT itself, the more frequent adverse events, e.g., diarrhea, compared to the end of OIT (Supplementary Table 4). Additionally, most alpha diversity indices of gut environmental factors were also decreased only at the beginning of OIT (Supplementary Fig. 1, 2). By contrast, among sparse literature, an observational study in adults with peanut allergy has shown that, compared to the baseline, their gut microbiota after 52 weeks of OIT showed higher alpha diversity.[22] The apparent discrepancy may be attributable to the difference in the target population, study design, food allergen, and any combination of these factors. Indeed, regarding the effect of CM intake on gut microbiota, a randomized controlled study among overweight adult men has reported that alpha diversity was

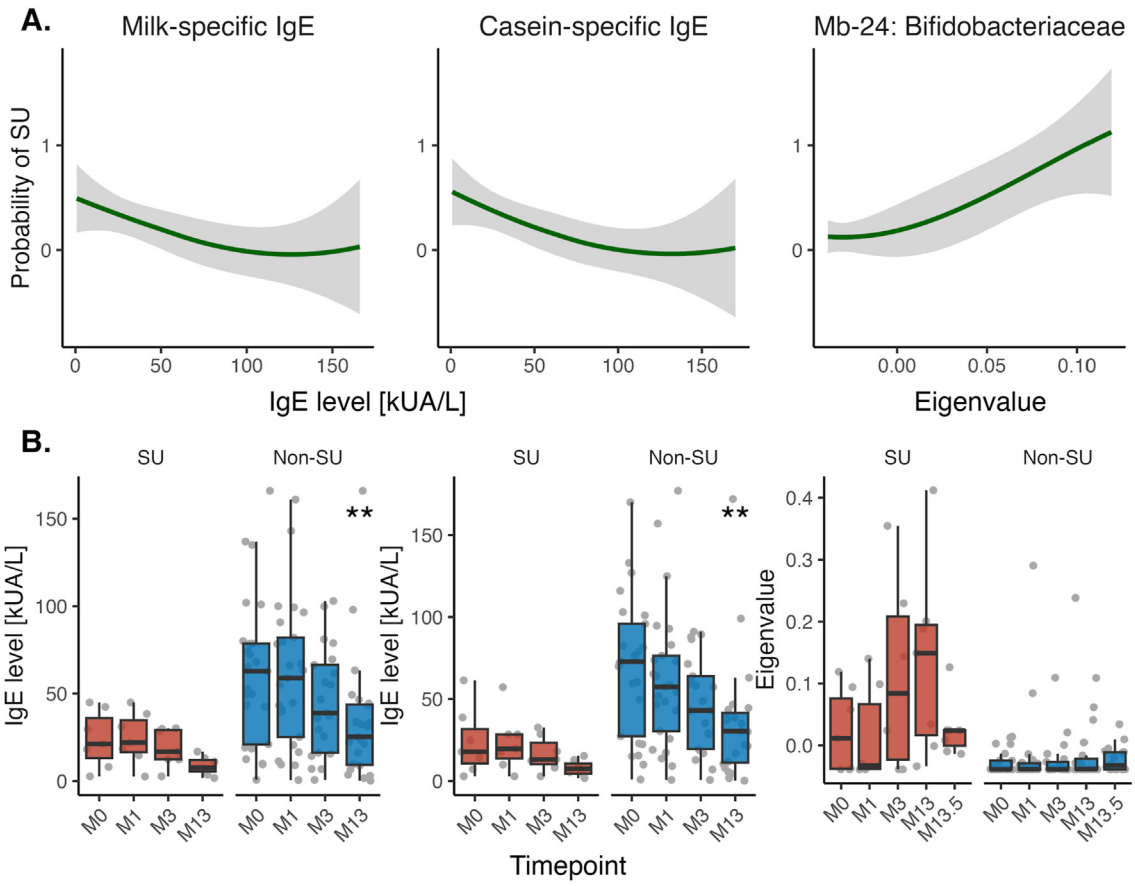

Fig. 4. Association of specific IgE and gut microbiota module with the acquisition of sustained unresponsiveness. **(A)** The locally estimated scatterplot smoothed curves (green fitted lines) with the 95 % confidence interval (light gray areas) represent the relationships of milk and casein-specific IgE levels and "Mb-24: *Bifidobacterium*" module eigenvalue with the acquisition of sustained unresponsiveness (SU). **(B)** The boxplots (median with interquartile range) illustrate the corresponding changes during oral immunotherapy in the SU and Non-SU groups.

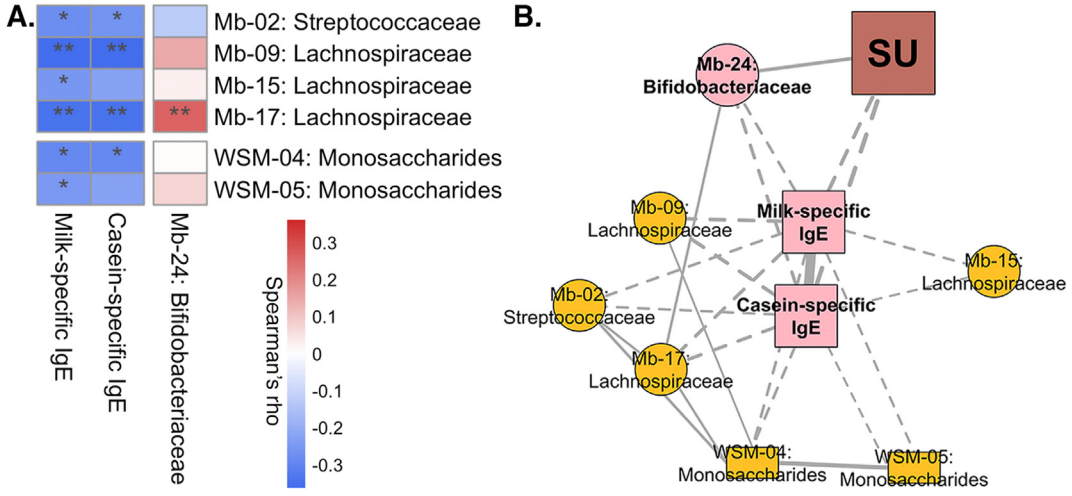

Fig. 5. Correlation network among milk- and casein-specific IgE and gut environmental factors. **(A)** The heatmap shows the correlation between factors that were significantly associated with the sustained unresponsiveness (SU) acquisition and gut environmental modules by partial Spearman's correlation analysis adjusted by daily cow's milk intake dose. **(B)** The network shows the correlation among SU (brown vertex), three factors significantly associated with SU (pink vertices), and gut environmental modules significantly correlated with the three factors (yellow vertices). Edges that satisfied the following criteria are depicted: |partial Spearman's rho adjusted by daily cow's milk intake dose >0.25 and false discovery rate <0.05. Solid and dashed lines represent positive and negative partial Spearman's rho.
Mb, gut microbiota; WSM, fecal water-soluble metabolite.

decreased after 3 months of CM intake.[41] These data collectively suggest the intricate and elusive characteristics of changes in gut environmental factors during CM-OIT.

Additionally, the current study identified the clinical factors that were associated with SU acquisition. For example, concurrent treatment of atopic dermatitis or asthma was significantly

associated with a lower chance of subsequent SU acquisition by OIT (Table 2), even in this study that excluded children with uncontrolled atopic dermatitis or asthma. Additionally, higher levels of milk- and casein-specific IgE during OIT were significantly associated with a lower chance of SU acquisition (Fig. 4A, Table 2, and Supplementary Table 7). Indeed, a guideline for allergen immunotherapy has suggested the associations of these clinical factors with higher risks of severe adverse events.[42] For example, a study for children who underwent CM-OIT has reported that not resolved atopic dermatitis and a higher level of casein-specific IgE were associated with higher risks of anaphylactic adverse events.[43] Additionally, another study of OIT using microwave heated CM has reported that a higher proportion of severe asthma was observed in children who failed to acquire SU.[13] Furthermore, previous research has also shown the relationship of higher milk- and casein-specific IgE levels with a lower likelihood of developing tolerance during their natural history[2,44] and acquiring SU[13,45] Taken together, the severity of CMA and comorbidities at baseline are important factors for acquiring SU.

The current study also identified the gut environmental factors that were associated with SU acquisition. For example, Mb-24: *Bifidobacterium* was associated with acquiring SU during OIT (Fig. 3A, B and Supplementary Table 6, 7). Consistently, previous studies have reported a lower relative abundance of *Bifidobacterium* in children with CMA compared to healthy controls[46,47] and *Bifidobacterium*'s allergy-suppressive effect to mice[48] and infants with CMA.[49] Considering the limited correlation of Mb-24: *Bifidobacterium* with WSM-modules (Fig. 5A, B), a direct mechanism, e.g., extracellular vesicles—derived protein of *Bifidobacterium*,[50] rather than one mediated by fecal WSMs may be more likely involved in this SU acquisition. Additionally, Mb-24: *Bifidobacterium* showed non-significant temporary change during OIT in the overall population (Fig. 3D) but a higher increase in the SU group than in the Non-SU groups (Fig. 4B). Consistently, recent evidence suggests that differences in changes in gut microbiota during treatment are associated with an outcome; for example, an observational study has reported that, in adults who underwent a treatment using thermal spring water for atopic dermatitis, the differences of change in gut microbiota compositions were associated with the decrease in disease severity.[51] Hence, the present findings collectively indicate the importance of gut environmental factors during OIT for acquiring SU.

The current study also found that these three SU-associated factors were correlated with other gut environmental modules and, in the correlation network, Mb-09: *Lachnospiraceae* and WSM-04: Monosaccharides were the important modules (Fig. 4 and Supplementary Table 8). These modules contain many components that potentially act in gut protection and sugar metabolism. For example, of the eight OTUs comprising Mb-09: *Lachnospiraceae*, four were OTUs close to *F. saccharivorans* (Supplementary Table 5). *F. saccharivorans* ameliorates intestinal inflammation[52] and produces various monosaccharides by its galactosidase and glucosidase activities.[53] Additionally, of the 11 WSMs comprising WSM-04: Monosaccharides, five were monosaccharides, e.g., galactose, fucose, N-Acetylglucosamine, N-Acetylneuraminic acid, and rhamnose, and one was a disaccharide lactose (Supplementary Table 6). These sugars are components of mucins — heavily glycosylated proteins[54,55] that serve as a gut microbiota energy source. These data collectively suggest the importance of intestinal mucosal immunity, including the mucus barrier, in oral tolerance to food allergens.[56]

The probability of SU acquisition by CM-OIT is much lower than that of hen egg and peanut OIT,[57–59] probably due to the tendency for more severe adverse reactions with CM. To overcome the difficulty in acquiring SU with CM, modified protocols have been tested. For example, OIT using microwave heated CM[13] and omalizumab, a mAb that inhibits the binding of IgE to FcεRI,[14] did not increase the chance of acquiring SU. However, previous literature in infants with IgE- and non-IgE-mediated CMA has shown that dietary intervention with an EHCF supplemented with the probiotic *Lactobacillus rhamnosus* GG increased the chance of acquiring tolerance compared to EHCF without *L. rhamnosus* GG[60,61] and altered the gut environment.[21,46] The current study builds on these earlier reports and suggests the efficacy of OIT supplementation with biotics for acquiring SU in school-age children with IgE-mediated CMA.

There are several limitations to our study. First, it was a prospective study with a small sample size. Therefore, in determining the association of gut environmental factors with acquiring SU, we did not perform adjustment for confounding factors, and the FDRs did not reach statistical significance. Second, the number of dropouts was relatively high due to severe adverse events by OIT, which may bring potential selection bias. Third, we set the duration of OIT at 13 months and the duration of OIT discontinuation at 2 weeks, referring to the protocols in place at the time,[27] but these were shorter than those in recent reports.[13,14] Fourth, this study lacked some clinical data that potentially influenced gut environmental factors such as the presence of household pets and intake of fermented food or prebiotics. Fifth, in the analysis of gut microbiome data, 16S rRNA gene amplicon sequencing was performed, which limits the microbial resolution in annotation compared to whole genome sequencing. Nevertheless, we believe that this limitation was compensated for by analyzing the fecal WSMs that reflect gut microbial function. Lastly, while our findings are clinically and biologically plausible, the findings require additional validation by an independent cohort and mechanistic studies.

Based on data from a prospective multicenter trial of OIT in school-age children with CMA in Japan, we found that immunological parameters improved during OIT, yet the acquisition of SU was not straightforward. We also found that, in terms of gut environmental factors, substantial shifts during OIT were limited to the early periods of therapy. Our data also clarified that lower levels of milk- and casein-specific IgE and a higher eigenvalue of a *Bifidobacterium*-dominant module were associated with a high chance of acquiring SU. Additionally, these SU-associated factors were related with enteroprotective and sugar-related gut environmental modules. These findings offer an evidence base for understanding the mechanism of OIT in terms of the gut environment. Furthermore, our data should advance investigations into adjuvant treatment for OIT, e.g., modulating gut environmental factors by biotics, to improve the outcome for children with CMA.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.alit.2023.10.001.

Conflict of interest

TF is an auditor of the Japanese Society of Pediatric Allergy and Clinical Immunology and receives consulting fees from BML and honoraria from Thermo Fisher Scientific. NS is an auditor of the Japanese Society of Pediatric Allergy and Clinical Immunology and receives honoraria from Miyarisan Pharmaceutical. HO receives honoraria from Miyarisan Pharmaceutical, Kyowa Kirin. Fujifilm, Boehringer Ingelheim Japan, Mitsubishi Tanabe Pharma, Astellas Pharma and Sanwa Kagaku Kenkyusho. The rest of the authors have no conflict of interest.

Authors' contributions

RS and NI carried out the main statistical analysis, drafted the initial manuscript. MN, TI, HY, and TF reviewed and revised the initial manuscript. YN and TK carried out the bioinformatic analyses of the metagenomic and metabolomic data, reviewed and revised the initial manuscript. MH and WS carried out the bioinformatic analyses of the metagenomic data, reviewed and revised the initial manuscript. HO and NS conceptualized the study, obtained funding, supervised the statistical analysis, reviewed and revised the initial manuscript. All the authors approved the final manuscript as submitted.

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