Early-life gut microbiome composition and milk allergy resolution

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Background: Gut microbiota may play a role in the natural history of cow's milk allergy.
Objective: We sought to examine the association between early-life gut microbiota and the resolution of cow's milk allergy.
Methods: We studied 226 children with milk allergy who were enrolled at infancy in the Consortium of Food Allergy observational study of food allergy. Fecal samples were collected at age 3 to 16 months, and the children were followed longitudinally with clinical evaluation, milk-specific IgE levels, and milk skin prick test performed at enrollment, 6 months, 12 months, and yearly thereafter up until age 8 years. Gut microbiome was profiled by 16s rRNA sequencing and microbiome analyses performed using Quantitative Insights into Microbial Ecology (QIIME), Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt), and Statistical Analysis of Metagenomic Profiles (STAMP).
Results: Milk allergy resolved by age 8 years in 128 (56.6%) of the 226 children. Gut microbiome composition at age 3 to 6 months was associated with milk allergy resolution by age 8 years (PERMANOVA $P = .047$), with enrichment of *Clostridia* and *Firmicutes* in the infant gut microbiome of subjects whose milk allergy resolved. Metagenome functional prediction supported decreased fatty acid metabolism in the gut microbiome of subjects whose milk allergy resolved ($\eta^2 = 0.43$; ANOVA $P = .034$).
Conclusions: Early infancy is a window during which gut microbiota may shape food allergy outcomes in childhood. Bacterial taxa within *Clostridia* and *Firmicutes* could be studied as probiotic candidates for milk allergy therapy. (J Allergy Clin Immunol 2016;138:1122-30.)

Key words: *Cow's milk allergy, microbiome, microbiota,* Clostridia, Firmicutes, Bacteroidetes, *metagenome, fatty acid, food allergy, 16s rRNA sequencing*

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Cow's milk allergy is the most common food allergy in young children, affecting 2% to 3%.[1,2] Individuals with milk allergy are at risk for allergic reactions and also face challenges in finding suitable replacements for the nutritional content that milk-based products provide.[3] A child may have to live with the limitations imposed by milk allergy for several years, as recent studies have shown that milk allergy often continues into later childhood

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Abbreviations used
CoFAR: Consortium of Food Allergy Research
LDA: Linear discriminant analysis
LefSe: Linear discriminant analysis effect size
OTU: Operational taxonomic unit
PCoA: Principal coordinate analysis
sIgE: Specific IgE
SPT: Skin prick test

and adulthood.[1,4] Parents of milk-allergic children often ask allergists to gauge whether their child's milk allergy will resolve.

We hypothesized that gut microbiota play a role in the natural history of milk allergy, as the etiology of food allergy is thought to involve deviation from the default state of mucosal immune tolerance that may be driven by diet, commensal microbiota, and interactions between them.[5,6] Variations in infant gut flora have been associated with allergy skin prick test (SPT) response,[7] specific IgE (sIgE) levels,[8] atopic dermatitis,[8,9] and food allergy status.[10] Much of this previous work has relied on culture methods, which allow for examination of species specifically targeted and cultured, but exclude the large majority of bacterial organisms that cannot be cultured. These excluded organisms may play key roles in the natural history of milk allergy.

In this study, we used high-throughput sequencing to comprehensively characterize the gut microbiota of 226 children aged 3 to 16 months with cow's milk allergy. We followed these children up to age 8 years and examined for associations between early-life gut microbiota diversity, composition, and milk allergy resolution.

METHODS

Study protocols were approved by the institutional review boards of the participating institutions.

Study design and subjects

The subjects of this study are a subset of a larger observational cohort study by the Consortium of Food Allergy Research (CoFAR) of 512 participants with milk allergy, egg allergy, and/or moderate-to-severe atopic dermatitis but without known peanut allergy.[1,11] Participants were recruited at age 3 to 15 months from 5 study centers across the United States and were followed over time. The study sites included the Icahn School of Medicine at Mount Sinai, New York, New York; Duke University School of Medical Center, Durham, North Carolina; Johns Hopkins University School of Medicine, Baltimore, Maryland; National Jewish Health, Denver, Colorado; and Arkansas Children's Hospital, Little Rock, Arkansas. The goal of the CoFAR observational study was to identify factors associated with the development of peanut allergy in a high-risk cohort. Examination of the natural histories of milk and egg allergy was a secondary objective.

We collected stool from 234 of the 244 CoFAR observational study participants who had milk allergy at study entry. Stool samples were collected at or near the time of enrollment. Samples were collected during a study visit or by the parent at home using a stool collection kit provided by CoFAR. For this study, we excluded from the analysis 3 subjects who submitted stool samples after age 17 months, yielding 231 stool samples from 231 participants for DNA isolation.

DNA isolation and sequencing

DNA was isolated using the MoBio Power Soil DNA Isolation kit (Carlsbad, Calif). The V4 region of the 16*S rRNA* gene was amplified with barcoded primers and 16S rRNA sequencing was performed on the Illumina MiSeq platform using 2 × 250 bp paired-end read. Stool samples from 5 participants yielded less than 2000 sequences/sample (2.2% of 231 samples sequenced) and these subjects were removed from the analysis, yielding a final sample size of 226 subjects for analysis. In total, 5,478,379 sequences were assigned to 16S rRNA gene sequences for the 226 samples, with a median of 23,682 sequences per sample.

Outcomes

Data were prospectively collected from study enrollment up through a visit at approximately age 8 years. Participants were considered to have milk allergy if they had either (1) positive physician-supervised oral food challenge or a convincing reaction and sensitization to milk by milk sIgE level of 0.35 kU_A/L or more and/or SPT wheal of more than 3 mm, or (2) a flare of atopic dermatitis associated with milk ingestion along with a milk sIgE level of more than 5 kU_A/L. At entry, dietary, medical, and social histories were obtained by questionnaires administered to the parents of participants. Milk sIgE levels measurement and milk SPT were carried out at entry and periodically thereafter as clinically indicated. Atopic dermatitis severity was graded on the basis of criteria previously described.[1] Participants were evaluated in person at enrollment, 6 months, 12 months, and yearly thereafter with additional telephone follow-up between each visit. Milk allergy was considered persistent if the subject had milk allergy according to the above definitions at the last documented encounter.

We used logistic regression implemented in R 3.2.2 (R Foundation for Statistical Computing, Vienna, Austria) to test for the associations between early-life exposures that shaped the infant gut microbiome (mode of delivery, breast-feeding, solid food intake, antibiotic use) and milk allergy resolution by age 8 years or milk allergen sensitization.

Microbiome analyses

On the basis of observations from previous studies[12,13] of marked shifts in the gut microbiome composition in early life associated with developmental stages (eg, predominant nutrition from breastmilk and formula until age 6 months, predominant nutrition from solid food and transition to whole milk or milk alternatives around 12 months), we *a priori* stratified the participants into the following 3 groups for microbiome analysis: participants whose stool samples were obtained at (1) age 3 to 6 months, (2) age 7 to 12 months, and (3) age 13 to 16 months. The members of the investigative team who conducted the microbiome and bioinformatic analyses had no role in acquiring or assessing the clinical results of the cohort study.

Unless noted otherwise, we performed all analyses using Quantitative Insights into Microbial Ecology (QIIME) 1.8.0, an open-source bioinformatics pipeline for performing analysis of microbiome sequence data. Operational taxonomic units (OTUs), defined as taxonomic units based on DNA sequences that share high identity,[14] were constructed using a more than 97% similarity threshold. Within the 3 age groups for analysis, alpha diversity (the richness of a sample in terms of the diversity of OTUs observed in it) was estimated using Faith's phylogenetic diversity.[15] Beta diversity (distance between samples based on differences in OTUs present in each sample) was measured using unweighted UniFrac.[16] Principal coordinate analysis (PCoA) was used to visualize clustering patterns between samples based on beta diversity distances. Linear discriminant analysis effect size (LEfSe),[17] a method for biomarker discovery, was used to determine taxa that best characterize each population, ie, bacterial groups associated with milk allergy resolution or persistence. LEfSe scores measure the consistency of differences in relative abundance between taxa in the groups analyzed (ie, resolution vs persistence), with a higher score indicating higher consistency. Association between microbiome composition and covariates was tested using PERMANOVA, a nonparametric test similar to ANOVA but that does not require the data to be normally distributed. Significance of PERMANOVA tests was determined using 999 permutations with adjustment for multiple testing.

Prediction of metagenome functional content

We used Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) 1.0.0[18] to predict metagenome function from the 16*S rRNA* data. Bray-Curtis distances were used to determine similarity