SUPPLEMENTARY MATERIAL

Supplementary Methods

Inclusion criteria

All eligible infants were between 0 and 8 months of age. **AI** were recruited at the Allergy Service of *Hospital Universitario Infantil Niño Jesús* and *Hospital General Universitario Gregorio Marañón* in Madrid (Spain), including infants with a clinical history consistent with IgE-mediated food allergy and positive skin prick test (SPT) to cow's milk α-lactalbumin, β-lactoglobulin and/or casein (Diater®, Madrid, Spain) and/or prick by prick test to pasteurized milk. Infants with a history compatible with CMA and a negative SPT on their first visit were included if the test was positive after three months. All SPT were performed using standardized techniques and interpretation according to international guidelines [1].

Eligible **CI** were infants without any symptom related to either CMA or any severe pathology concomitant to allergy and were recruited at five health centres in Madrid. The exclusion criteria were hydrolyzed formula intake for more than 2 weeks at the time of CMA diagnosis and antibiotics intake in the 3 months prior to study recruitment (also applicable to their mothers and grandmothers).

All participating centers applied the same recruitment protocols and questionnaires. Information on personal data and potential risk factors for allergy in infants such as age, gender, feeding regime, delivery mode and antibiotics use at birth for infants; and age, diet, and smoking and allergy status of mothers and grandmothers were collected through questionnaires.

Sample Collection and Processing

Each participant received a sample collection kit consisting of a small disposable cooler, a hermetic bag, a stool collection sterile container with a spoon for easier collection, a cold-pack, collection paper, a pair of gloves, labels and an easy-to-read guideline, developed by our group, that patients could follow at home (Figure S1). Subjects were

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asked to record the sample collection date, to not contaminate the sample with urine or toilet paper and to store the samples in the home freezer at -20°C before taking them to the laboratory. A total of 148 faecal samples were collected at the laboratory and stored at -80°C before being processed for DNA extraction.

Epidemiological variables and analysis

Demographic, clinical and lifestyle variables from all subjects involved in the study were analysed. Delivery mode, the use of antibiotics at birth, having older siblings and pets were neither reasons for inclusion nor exclusion in the study. These variables were collected in the comprehensive questionnaires as secondary variables, being cow's milk allergy the primary one. Descriptive statistics (mean and standard deviations for continuous variables, percentages for categorical variables, and p-values) were used to describe the epidemiological variables. The two groups were compared in their proportions using the chi-squared test. Descriptive statistics of the epidemiological variables of the three generations according to the case-control status of infants were computed using the compareGroups R package [2]. The association of each epidemiological variable with the case-control status of infants was tested using univariate logistic regressions. A multivariate logistic regression including the variables showing significant associations at the univariate analyses was also performed. All the statistical analyses were performed in R.

16S rRNA Gene Sequencing

The 16S rRNA gene sequencing was performed in samples from infants between 4 and 6 months including 11 **AG**; 32 **NA_G**; 18 **AM**; 27 **NA_M**; 19 **AI** and 7 **CI** (n=114 out of 148, 77%). This selection allowed to standardize even more the AI and CI groups and avoid bias due to the age of the infants and solid food introduction.

DNA extraction

DNA was extracted from 200 mg of faeces using the QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions plus an additional membrane disruption step using glass beads [39]. The extracted DNA was quantified by Qubit® 2.0 Fluorometer following the dsDNA HS Assay Kit protocol. Those samples with a concentration higher than 0.2 µg/µL were selected for sequencing (n=115).

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16S rRNA gene amplification and sequencing

The V3-V4 regions of the 16S rRNA gene were amplified and sequenced using the MiSeq platform from Illumina, as described in the manual for "16S Metagenomic Sequencing Library Preparation" of the MiSeq platform (Illumina, San Diego, California, EEUU).

Bioinformatics and statistical analyses

16S rRNA sequences were denoised and processed with DADA2 v1.11 in order to define ASVs [3]. In addition, DADA2 and the command removeBimeraDenovo were used to remove chimeras. Taxonomy was assigned using the DADA2 implementation of the RDP classifier [4], using the formatted RDP training set 16 release 11.5 from the DADA2 website.

Shannon's α-diversity index was estimated using the package vegan v2.5-3 and the R 3.4.0 software. Richness was defined as the number of ASVs identified in a given sample. To analyse global differences in the microbiota, the Bray-Curtis distance between pairs of samples was calculated using the vegan package. Subsequently, Principal Coordinate Analysis was applied on the generated Bray-Curtis distance matrix. In order to analyse if two groups of samples differ in their microbiota structure, the permutational multivariate analysis of variance (PERMANOVA) test was applied using the Bray-Curtis distance matrix. ANCOM II, a statistical approach specifically designed for microbiome analysis [5], was applied to identify specific taxonomic differences between groups of samples. This approach accounts for compositional constraints to reduce false discoveries in detecting differentially abundant taxa at an ecosystem level, while maintaining high statistical power. The analysis was applied at the different taxonomic levels (i.e. Phylum, Class, Order, Family, Genus and ASVs). ASVs taxonomy was assigned using BLASTN tool [6] against the expected sequences from each community, using a cutoff of 97% identity. To adjust for multiple hypothesis testing, we used the FDR approach by Benjamini and Hochberg implemented in the fdr.R package [7]. Statistical analyses were performed with those groups with sufficient sample size ($n \geq 3$). Results were considered significant when *p*-values were lower than 0.05. In case of multiple hypothesis testing, results were considered significant when q-values were lower than 0.1.

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Supplementary Results

Gut microbiome profile differences over three generations

The faecal microbiome composition was different between adults and infants (PERMANOVA p=0.017). At the phylum level, the adult microbiota was mainly constituted by Firmicutes (60%), Bacteroidetes (15%), Actinobacteria (7%), Verrucomicrobia (4%) and Proteobacteria (3%), whereas in infants, the relative abundance of both Actinobacteria and Proteobacteria phyla was higher (ANCOMII p=7.4e−06 for Actinobacteria and p=9.8e−14 for Proteobacteria), representing 30% and 15% of the infant gut microbiota composition. On the contrary, the relative abundance of Firmicutes was lower in infants (30% *vs*. 60%) (ANCOMII p= 1.2e−07) (Figure S3A). At the family level, the predominant families in adult microbiota were *Ruminococcaceae* (25%), *Lachnospiraceae* (23%), *Bacteroidaceae* (10%), *Coriobacteriaceae* (5%), *Bifidobacteriaceae* (2-4%), *Verrucomicrobiaceae* (2-4%) and *Rikenellaceae* (2-4%), while in the case of infants the most abundant families were *Bifidobacteriaceae* (25-30%), *Enterobacteriaceae* (15-20%) and *Lachnospiraceae* (5%) (Figure S3B). Alpha diversity (Shannon's diversity index) and richness were analysed, showing a statistically significant increase in adults compared to infants (p<0.001) (Figure S4).

Gut microbiota differences associated with CMA and type of feeding in infants

Aiming to assess if the infant's allergy was associated with a different microbiota composition, the 15 most abundant families (relative abundance > 0.1%) identified using 16S rRNA gene sequencing were compared between **AI** and **CI**. The **AI** phenotype showed an increased relative abundance of *Bifidobacteriaceae, Clostridiaceae, Verrucomicrobiaceae, Lactobacillaceae* and *Streptococaceae* families, while *Ruminococcaceae, Coriobacteriaceae, Bacteroidaceae* and *Veillonellaceae* families were decreased compared to **CI.** However, these differences were not significant (Figure S3B). On the other hand, **AI** had decreased within-sample bacterial diversity compared to **CI** (p<0.05). Moreover, multivariate analysis using PERMANOVA showed that there were statistical compositional differences at bacterial family level in the gut microbiota between **AI** and **CI** (Adonis p=0.025). However, the overall microbiome composition of their mothers did not significantly differ. At ASV level, significant differences between

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the relative abundances for *Veillonella parvula, Veillonella dispar, Streptococcus lutetiensis* and *Enterococcus casseliflavus* were detected between **AI** and **CI.**

As can be observed in Figure S5, the relative abundance of the bacterial families changes according to the feeding regime, observing an increase of *Bacteroidaceae* family in formula milk group and the *Lachnospiraceae* family increased in hydrolysate and breast milk group compared with the other feeding groups. Moreover, *Bifidobacteriaceae* and *Veillonelaceae* families were decreased in formula milk and hydrolysate group, respectively.

Gut microbiota composition differences between AI and CI mothers

PERMANOVA multivariate analysis was performed to identify statistical differences between mothers of allergic infants compared to mothers of control infants. Principal coordinate analysis (PCoA) and Adonis *p*-value did not show significant differences (Figure S6).

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SUPPLEMENTARY FIGURES

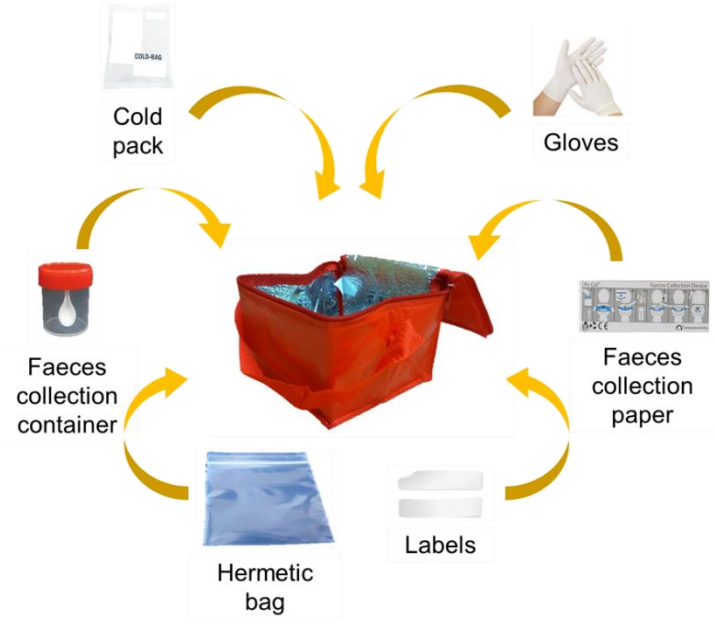

Figure S1. Faecal sample collection kit. The kit contained a small disposable cooler, a hermetic bag, a stool collection container with a spoon, a cold pack, collection paper, a pair of gloves, labels and an easy to read guideline.

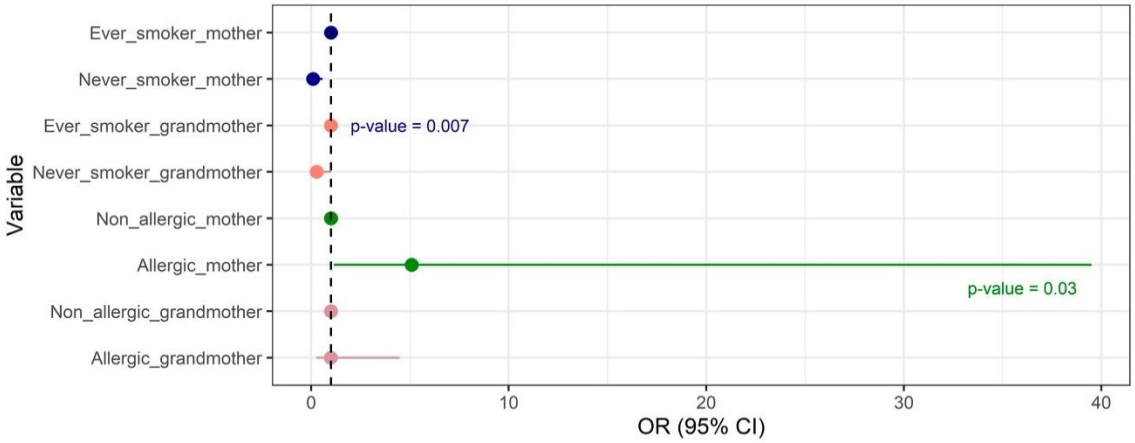

Figure S2. Forest plot for the odds ratio estimates of the associations between infant's allergy and their mothers and grandmother's allergy and smoking habits obtained in the univariate logistic regressions.

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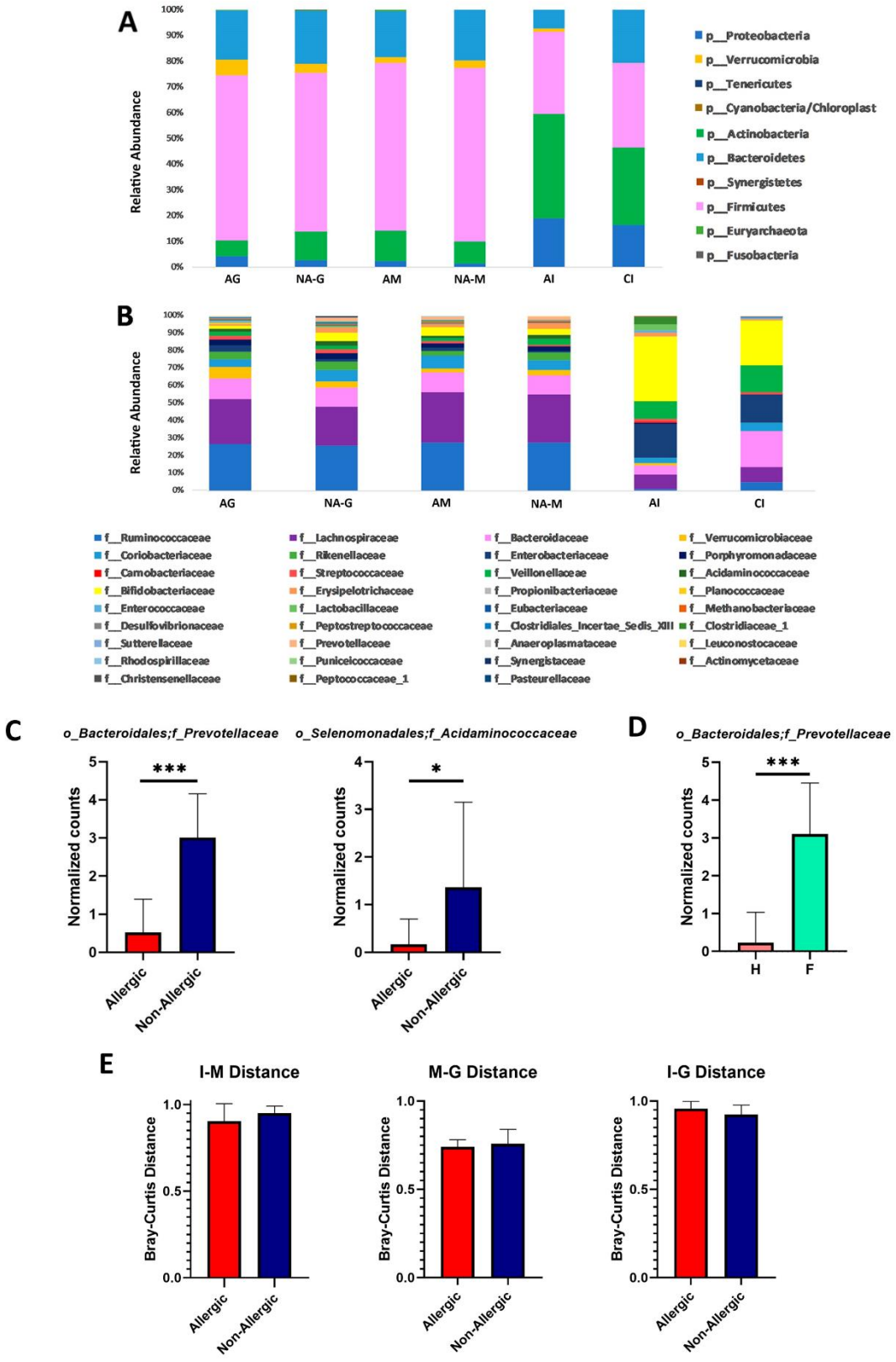

Figure S3. Gut microbiota composition of CMA infants vs control infants, their mothers and grandmothers. A, Bacterial phyla composition over the three generations. **B**, Bacterial

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family composition (top 35 most abundant families). **C**, Bacterial families with a significantly different abundance between AI and CI using ANCOMII test. **D**, Bacterial families with a significantly different abundance between groups using ANCOMII test. **E**, Beta diversity analysis based on Bray-Curtis distance between members of the same family. T-test was performed for statistical analyses using GraphPad Prism. (q<0.1). * p<0.05, ** p<0.01, ***p< 0.001. **AG**: allergic grandmothers; **NA-G**: non-allergic grandmothers; **AM**: allergic mothers; **NA-M**: non-allergic mothers; **CI**: control infants; **AI**: allergic infants; **H**: hydrolysate fed infants; **F**: formula milk fed infants.

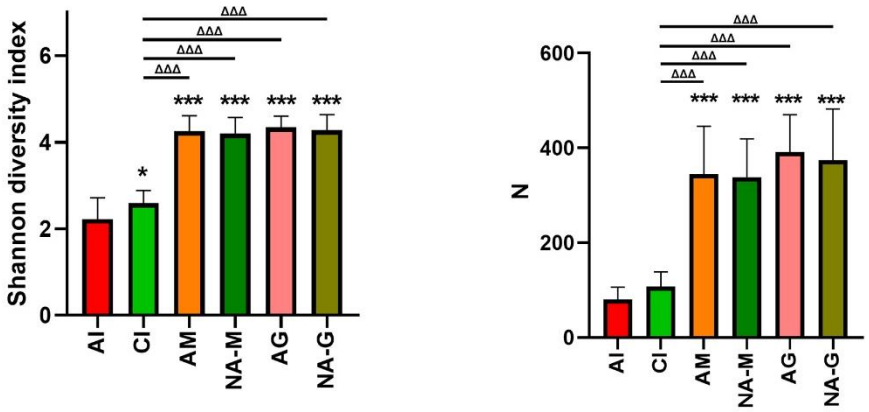

Figure S4. Gut microbiota composition over three generations. Alpha diversity differences among groups (Shannon's diversity index). Richness (N) differences among groups. T-test was performed for statistical analyses using GraphPad Prism. * indicates statistical differences between AI and the rest of groups, Δ indicates statistical differences between CI and the rest of groups * p<0.05, ***/ΔΔΔ p< 0.001.

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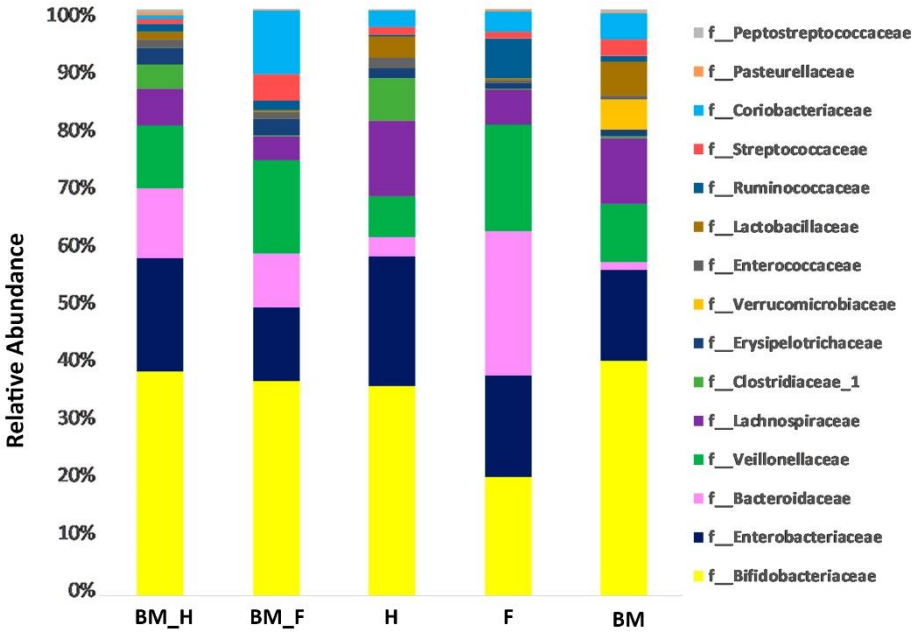

Figure S5. Distinct gut microbiota composition associated to infants' diet. Bacterial family composition in BM_H, BM_F, BM, H and F groups. Breast milk (BM), breast milk together with hydrolysate (BM_H), breast milk together with formula milk (BM_F), hydrolysate (H) and formula milk (F).

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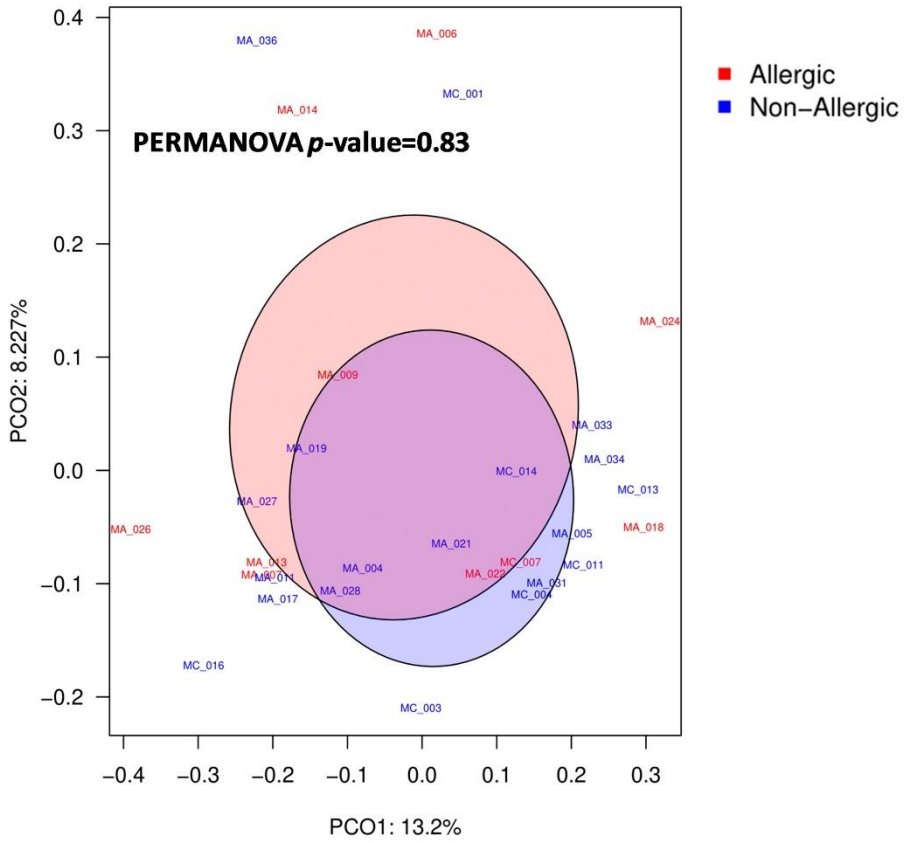

Figure S6. Comparison of the gut microbiota composition between mothers of AI and CI. Principal coordinate analysis showing clustering of individual gut microbiota composition as well as PERMANOVA multivariate analysis (PERMANOVA *p*-value).

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	Control *N=16*	Case *N=34*	p.overall
Age (months)	5.00 (1.71)	4.93 (1.47)	0.883
Gender:			0.893
Female	10 (62.5%)	19 (55.9%)	
Male	6 (37.5%)	15 (44.1%)	
Type of birth:			1.000
Vaginal	13 (81.2%)	28 (82.4%)	
Cesarea	3 (18.8%)	6 (17.6%)	
Antibiotics:			1.000
No	14 (87.5%)	28 (82.4%)	
Yes	2 (12.5%)	6 (17.6%)	
Detailed.Feeding:			<0.001
Formula	7 (43.8%)	0 (0.00%)	
Breast milk	2 (12.5%)	7 (20.6%)	
Hydrolysate	0 (0.00%)	12 (35.3%)	
Breast milk + Formula	7 (43.8%)	3 (8.82%)	
Breast milk + Hydrolysate	0 (0.00%)	12 (35.3%)	
Older sibling:			1.000
No	9 (56.2%)	20 (58.8%)	
Yes	7 (43.8%)	14 (41.2%)	
Pet:			0.508
No	13 (81.2%)	24 (70.6%)	
Yes	3 (18.8%)	10 (29.4%)	
Mother's age	33.8 (4.45)	34.3 (3.98)	0.715
Allergy (mother):			0.060
No	14 (87.5%)	19 (55.9%)	
Yes	2 (12.5%)	15 (44.1%)	
Smoking habits (mother):			0.019
Never	15 (93.8%)	19 (55.9%)	
Ever	1 (6.25%)	15 (44.1%)	
Grandmother's age	65.4 (5.28)	63.4 (6.08)	0.263
Allergy (grandmother):			1.000
No	12 (75.0%)	24 (70.6%)	
Yes	4 (25.0%)	8 (23.5%)	
'Missing'	0 (0.00%)	2 (5.88%)	
Smoking habits (grandmother):			0.077
Never	11 (68.8%)	12 (35.3%)	
Ever	5 (31.2%)	20 (58.8%)	
'Missing'	0 (0.00%)	2 (5.88%)	

Table S1. Study population characteristics. Epidemiological characteristics of the participants in the study. “Ever”, within the variable smoking habits, refers to the condition of having been a regular smoker at some point in their life. Even if they were not smokers at the time of collection.

Variable	OR	CI.low	CI.high	p.value
No	Ref			
Allergy.motherYes	4.43	0.81	35.51	0.1076
Never	Ref			
Smoking.motherEver	9.8	1.53	194.1	0.0422
Never	Ref			
Smoking.grandmotherEver	1.94	0.42	8.94	0.3864

Table S2. Multivariate analysis including the smoking status of mothers, grandmothers and allergy status of the mothers. **OR**: Odds Ratio; **CI**: Control Infants.

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