

Gut microbiota from infant with cow's milk allergy promotes clinical and immune features of atopy in a murine model

To the Editor,

Numerous clinical and epidemiological studies suggest an association between abnormal development of the gut microbiota in early life and development of clinical manifestations related to allergy including cow's milk allergy (CMA). Despite advances, at present there is no consensus on a clear signature of a CMA microbiota due to variations in allergic phenotypes and methods used. Moreover, it remains unclear whether the observed microbial alterations are a cause or a consequence of allergy. Here, we report the effects of fecal microbiota transfer (FMT) of a healthy control (HC) and CMA infant in a gnotobiotic murine model of CMA. Detailed methods are provided in Appendix S1.

Infants with CMA ($n = 5$), under the care of Great Ormond Street Hospital, London, UK, were recruited alongside healthy controls ($n = 6$) from the community (Tables S1 and S2) (REC 14/LO/0364). Stool samples from the infants with CMA analyzed by 16S rRNA-gene sequencing showed significant increased bacterial diversity, decreased abundances of *Bifidobacterium* spp., and increased abundances of *Lachnospiraceae* spp., and one of its genera, that is, *Eisenbergiella* (FDR < 0.05), was compared with HC (Figure 1A-C). These specific gut microbiota signatures corroborate previous findings comparing CMA infants with breastfed HC.^{1,2} Significant increased levels of bacterial-derived short chain fatty acids (butyrate, iso-valerate, and iso-butyrate) were observed in infants with CMA compared with HC (Figure S1). No significant differences in stool pH, acetate, D-lactate, L-lactate, sIgA, calprotectin, and eosinophil-derived neurotoxin levels were recorded (Figure S1). All these parameters were supplemented into a principal component analysis (PCA) of microbial compositions that revealed distinct patterns between CMA and HC (Figure 1D).

We selected 2 infants, 1 with CMA and the other with HC (infants 3 and 4), matched for age (9 and 10 months old), gender (female), and delivery mode (cesarean section) for FMT into three-week-old germ-free mice (C3H/HeN). Following 12 days of microbiota establishment, mice received, once a week for 5 weeks, whole whey protein (WP) and cholera toxin (CT) (sensitized mice; S) or CT only (nonsensitized control; NS) (Figure S2A) (REC CEEA34. AJWD.062.12). Despite an adaptation to the murine host, the main microbial and metabolic signatures of CMA and HC were sustained over the course of the experiment (Figure 1E,F, S2 and S3), validating our model of choice. No differences in microbiota compositions were observed between S and NS mice receiving the same fecal transfer.

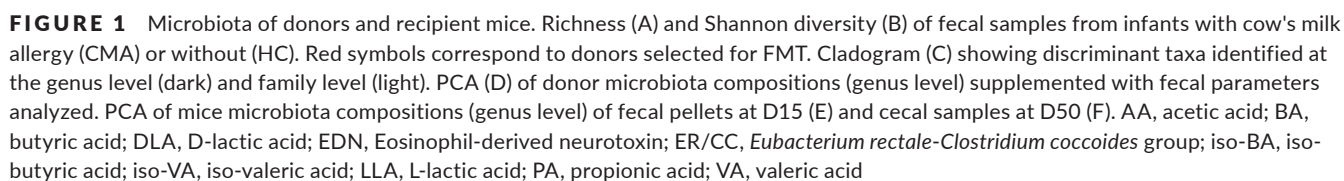
Following oral allergen sensitization, CMA microbiota was associated with diarrhea-related symptoms (Figure 2A), with higher fecal scores (reflecting softer to diarrheic stools/ anal inflammation) that

persisted at least 24 hours for the CMA-S group compared with HC-S group ($P < 0.001$). No significant differences in fecal scores were recorded between HC-S and HC-NS, which may indicate a protective effect of healthy microbiota upon allergen exposure as we and others observed previously.^{3,4} In addition, clinical scores (scratching/ puffiness/ loss of mobility) after oral challenge with β -lactoglobulin (BLG) were significantly higher in CMA-S group versus CMA-NS and HC-S groups (Figure 2B). Minimal differences in mouse mast cell protease 1 (mMCP-1) and allergen-specific sensitization markers were observed between the two fecal transfers (Figure 2C-E), which contrasted with the increases observed in total IgE levels and total IgG1/IgG2a ratio in CMA-S and CMA-NS groups compared with HC-S and HC-NS groups, respectively (Figure 2F,G). The latter observations were consistent with increased *gata3* mRNA expression in the colon (Figure 2H), which is a marker of Th2 lymphocytes. Interestingly, despite non-significant, colonic *fcγRIII* mRNA expression was increased in CMA-S mice (Figure 2H, $P = 0.07$). FCγRIII is an IgG1 binding/activating receptor, and its increased expression may imply a pathway linked to IgG1 and basophils and the potential development of anaphylaxis.⁵

Literature indicates that germ-free mice have increased total IgE, which can be normalized upon colonization with commensal microbiota until 8 weeks of age.⁶ We found that only colonization with HC-associated microbiota led to low total IgE levels similar to that of nonsensitized control. This may be due to the observed enrichment of protective bacteria including bifidobacteria and *Anaerostipes* spp. (Figure S2),⁷ the latter of which corroborates the findings by Feehley et al⁴ using a similar mice model. In contrast, CMA microbiota was associated with higher total IgE, a phenomenon that is linked to poor long-term outcome in atopic dermatitis⁸ or an increased risk of developing other allergic manifestations.⁹

Our findings confirm a Th2-type immune orientation following FMT with CMA microbiota. This Th2 profile was associated with minimal differences in mMCP-1 levels or allergen-specific immunoglobulin levels between the CMA-S and HC-S groups, raising the hypothesis that non-IgE mediated immunity might be at play. Indeed, patients with non-IgE-dependent food-allergy predominantly present gastro-intestinal tract symptoms. The diarrhea-like symptoms and signs of colonic inflammation in mice with the CMA-associated microbiota support this hypothesis.

We noted an increase in colonic *foxp3* mRNA gene expression in CMA-S group compared with HC-S group. Foxp3 has been associated with the production of Th2 cytokines in several cell lines, including Foxp3⁺Gata3⁺ cells, as well as with regulatory T-cells (Treg). If it is associated with Treg cells, the increased *foxp3* expression may reflect a regulatory mechanism in response to the enhanced Th2



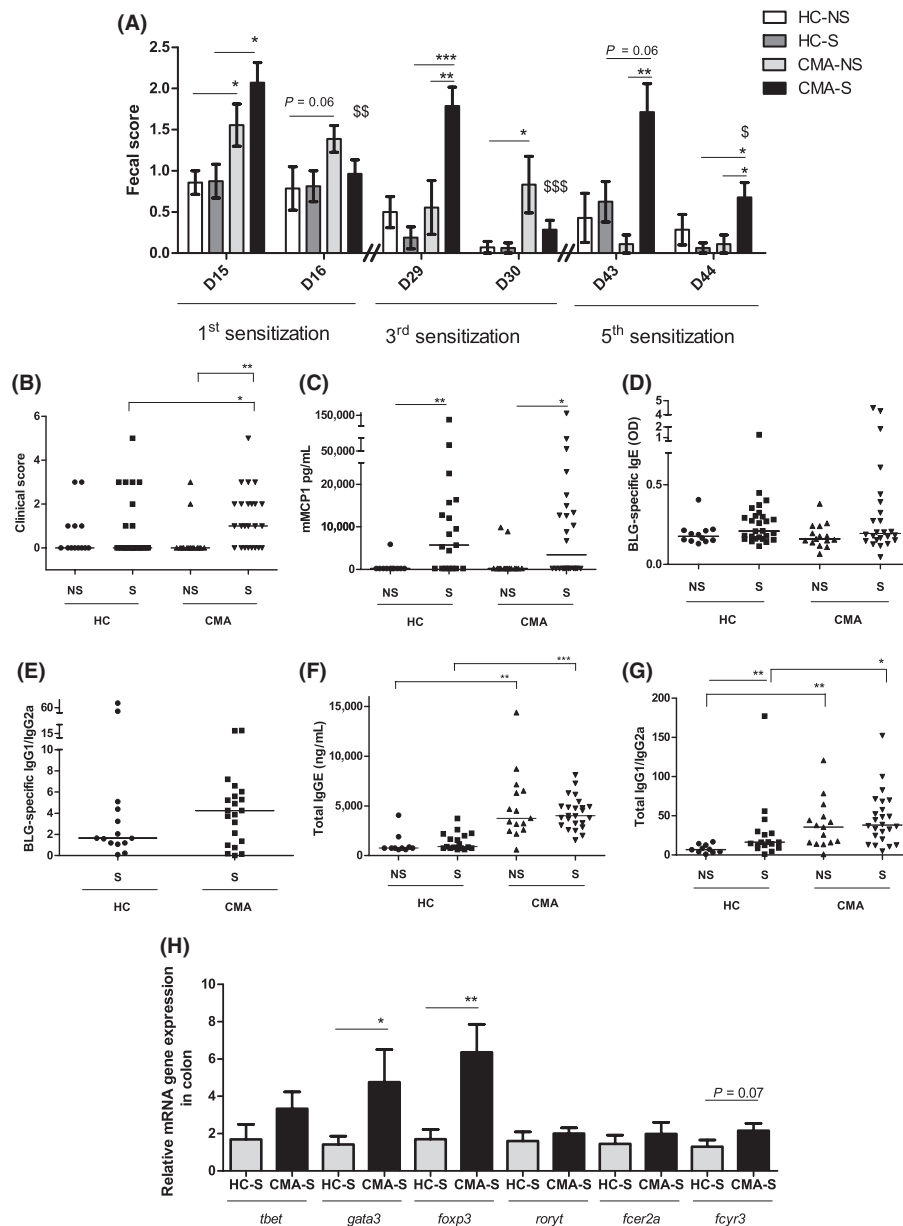


FIGURE 2 Mice model results (A) Fecal scores: Pellets were collected at D15 & D16, D29 & D30, and D43 & D44. (B) Clinical score, (C) concentration of mMCP-1, (D) BLG-specific IgE, (E) BLG-specific IgG1/IgG2a ratio, (F) total IgE, and (G) total IgG1/IgG2a ratio. (H) Gene mRNA expression in colon of sensitized groups. mRNA expression is relative to HC-S mice. *P*-values were calculated using Mann-Whitney test (**P* < 0.05, ***P* < 0.001, and ****P* < 0.0001)

profile of the CMA-S group. Another explanation of the increased *foxp3* expression could be linked to the increase in *Lachnospiraceae* spp. and the associated increase in butyrate (Figure S3), which has been implicated in the induction of Foxp3 Treg cells.¹⁰ However, in our study, this was not associated with protection against allergic sensitization.

In summary, we demonstrate for the first time that infant microbiota with a low bifidobacteria/*Lachnospiraceae* ratio orients the murine immune system toward a Th2 atopic profile with enhanced clinical symptoms of allergy, showing the pivotal role of the microbiota in allergy. Our study, however, has two limitations: (a) the concomitant medication and elimination diet in infants with CMA, which

are the both factors known to influence the gut microbiota, but are also inherent to the medical condition, and (b) the use of only two representative microbiotas to avoid antagonistic effects that might arise by mixing different microbiotas. Despite these limitations, our model system is a valuable tool providing novel insights into the role of gut microbiota in CMA.

CONFLICTS OF INTEREST

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

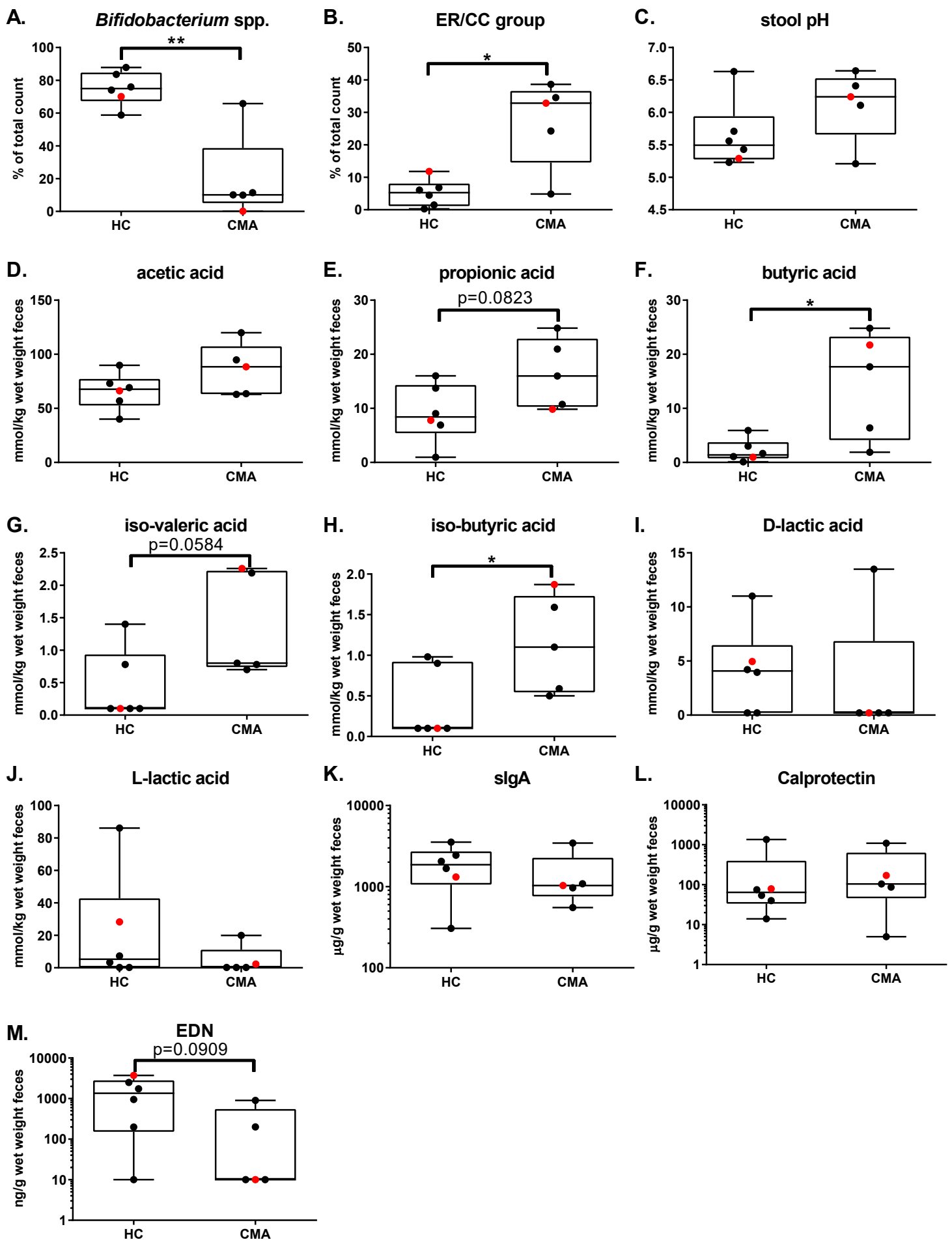


Figure S1

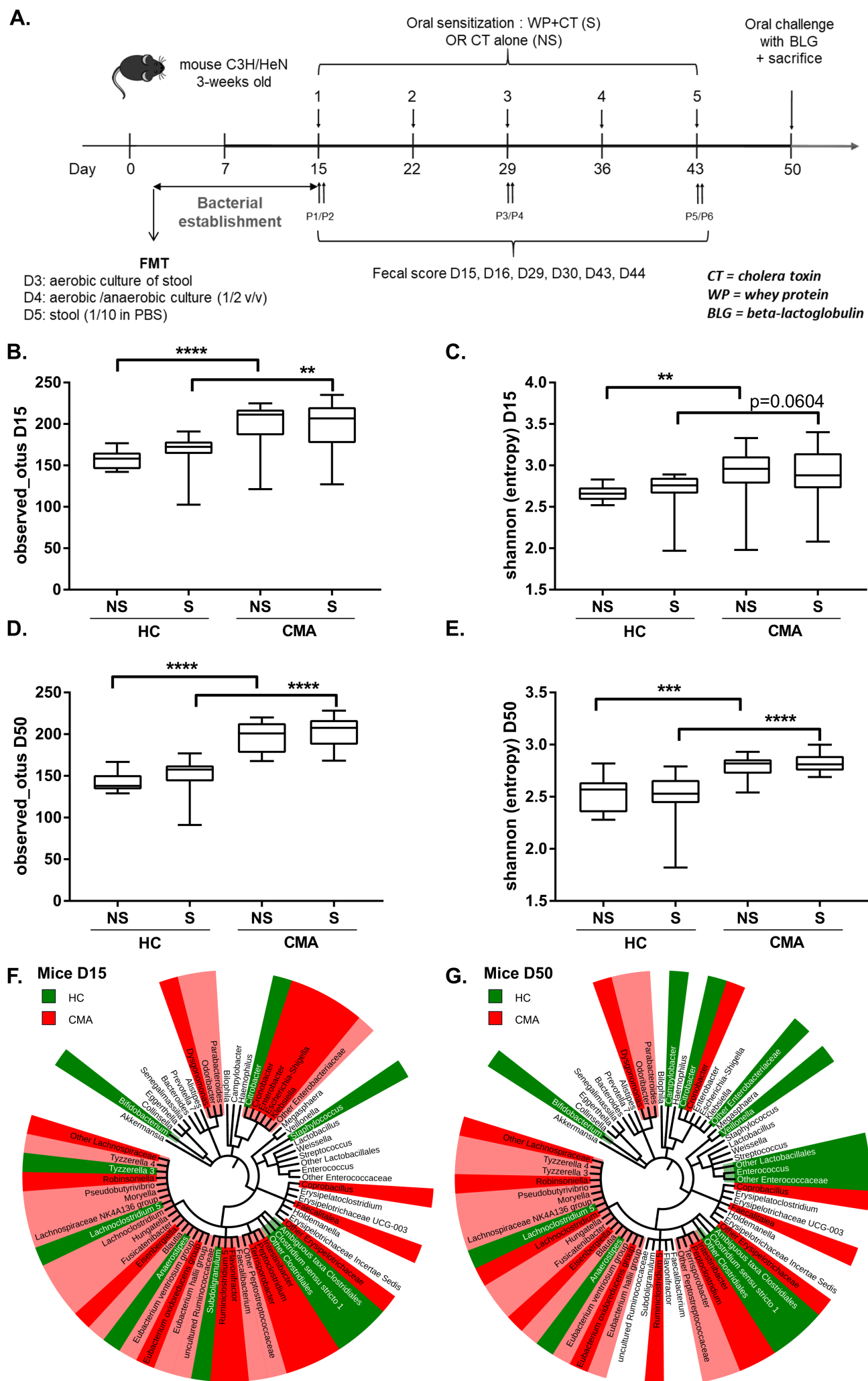


Figure S2

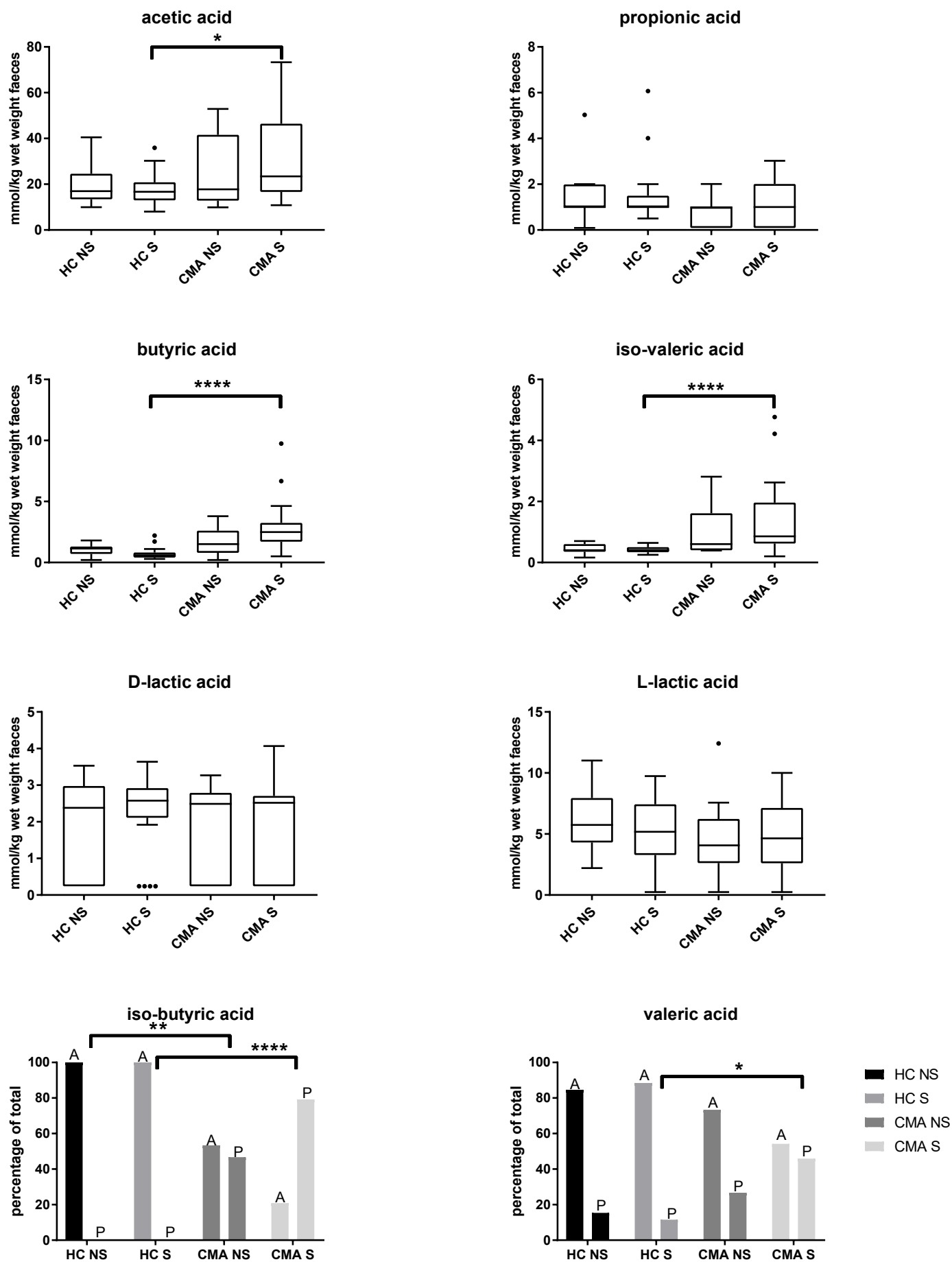


Figure S3

Supporting Information

Supplementary materials and methods

Subjects, allergic workup

Allergic infants under the care of Great Ormond Street Hospital, London, UK, were recruited alongside healthy infants from the community (REC No 14/LO/0364). The CMA was diagnosed based on clinical presentation (immediate reaction with urticaria) and this was confirmed subsequently following oral challenge. Only term, healthy infants who were fully or partially breastfed, with no family history of atopy, no exposure to antibiotics (in the antenatal or postnatal period), nor any other medications were recruited for comparison with the allergic cohort. Stool from 1 nappy were separated into three containers (1 container with 5 ml 10% glycerol in 0.9% NaCl), then stored and transported at 4°C until storage at -80°C within 24 hours.

Colonization of germ-free mice

The protocol was approved by the Regional Council of Ethics for animal experimentation (Île-de-France, Paris Descartes, CEEA34.AJWD.062.12).

Germ-free C3H/HeN mice (Anaxem, INRA, Jouy-en-Josas, France) were housed in sterile isolators in the animal care facilities of CRP2-UMS 3612 CNRS-US25 INSERM-IRD at the Faculté de Pharmacie de Paris, Université Paris Descartes, France. Mice were allowed *ad libitum* intake of autoclaved water and pellet AIN93G based chow sterilized by γ -irradiation at 50 kGy (ssnifspezialdiäten, Soest, Germany). They received the two selected fecal microbiotas by oral gavage at weaning age (21±3 days of life) producing 2 groups, i.e. healthy infant microbiota-associated mice (HC group) and CMA infant microbiota-associated mice (CMA group). This transfer was performed on 3 consecutive days as described hereafter. Stool was cultured in Tryptone-Glucose-Yeast-Hemin medium at 37°C for 24h under aerobic conditions or 48h under anaerobic conditions (MACS anaerobic cabinet; AES-Chemunex, Bruz, France; N₂/H₂/CO₂; 80:10:10). On day 3 (D3), 100µl of the aerobic culture was administered to mice; on D4, 100µl mix of anaerobic/aerobic culture (2:1, v/v) was

administered and on D5, the mice received 100µl of stool diluted in PBS at 1:10 (figure S2A). This sequential inoculation allowed the establishment of extremely oxygen sensitive bacteria (1).

Oral sensitization and immune challenge

Each group was divided into 2 subgroups: non-sensitized group included 5 to 6 mice and sensitized groups included 12 to 15 mice. The first subgroup (S group) was sensitized with whey proteins (WP, lacprodan 80, Arla, Lyon, France; 15mg per mouse) and adjuvant cholera toxin (CT) (List Biological Laboratories, Campbell, CA; 10µg mouse⁻¹) in PBS. The second subgroup received only CT in PBS as a control (non-sensitized group, NS). Sensitizations were performed by oral gavage once a week for 5 weeks (figure S2A). One week after the last sensitization (D50), all mice received an oral challenge with 60mg of β-lactoglobulin (BLG, Sigma Aldrich, Saint-Quentin-Fallavier, France). Data from 2 independent experiments are shown.

Evaluation of allergic response

Clinical scoring was performed on D50, 30 min after the BLG challenge, by two investigators blinded to the sensitization protocol and the mouse groups for 15 min. As previously described (2), allergic symptoms were evaluated based on three criteria: scratching behavior, loss of mobility, and puffiness (including bristled fur, edema around nose and eyes, laborious breathing). Scratching was defined as the number of scratching episodes per 15-min interval as follows: 1-3 episodes=0, 4-5 episodes=1, and >6 episodes =2. Loss of mobility was graded in terms of the duration of absence of any movement as follows: <10 min=0; >10 min = 1, during the 15 min =2. Puffiness was graded as none=0 and puffiness=2. The clinical score was defined as the sum of the three individual scores, and therefore, ranged from 0 to 6.

Sampling

Fecal pellets were collected after the 1st, 3rd and 5th sensitization for two consecutive days and scored as follows: normal=0, wet=1, glairy and very soft=2, diarrhea=3, no feces or anal inflammation = 1. The fecal score was defined as the sum of the two scores (consistency and inflammation) and therefore, ranged from 0 to 4.

On D50, blood was recovered in K3-EDTA tubes, centrifuged immediately, and plasma was stored at -80°C for immunoglobulin and mast cell protease-1 (mMCP-1) measurements. Spleens and mesenteric lymph nodes (MLN) were used for lymphocyte cultures followed by cytokine dosages. Two-cm of duodenum, jejunum, ileum and colon were collected and stored in RNA-later (Sigma-Aldrich, France) for relative expression gene measurement, and cecum contents stored in dry tube. Samples were stored at -80°C until analysis.

Gut microbiota and other parameters in infant and mice samples

The gut microbiota composition and diversity were determined by 16S rRNA-gene sequencing, except for infant nr.10 due to insufficient fecal specimen to perform DNA extraction. DNA was extracted from fecal and cecal samples using a phenol-chloroform based method combined with bead-beating as described previously (3). Infant and mice microbiota compositions were determined by sequencing and bioinformatic analysis as described in detail before (4). Briefly, extracted DNA samples were profiled by sequencing the PCR-amplified V3-V4 regions of the 16S rRNA gene on an Illumina MiSeq instrument (San Diego, USA). Illumina reads were preprocessed, quality filtered, merged and analyzed with an adapted version of the 'Quantitative Insights Into Microbial Ecology' (QIIME) v1.9.0 pipeline (5). Sequences were clustered into Operational Taxonomic Units (OTUs) based on 97% sequence identity using VSEARCHv2.4.1 with exclusion of chimeric sequences identified against the RDP gold database (6,7). Taxonomic assignment was performed using the RDP classifier (8) against the SILVA123 database (9). Singleton OTUs, OTUs with eukaryotic assignments, and OTUs with a low relative abundance up to 0.005% were excluded from further downstream analysis. The species diversity (α -diversity) metrics for richness (observed OTUs) and the Shannon index for diversity (10) were calculated using the R-

package phyloseq (11) with correction for the differences in sequencing depths by rarefaction.

The 16S rRNA-based sequencing of infant stools was complemented by fecal pH-analysis plus the quantification of *Bifidobacterium* spp. and *Eubacterium rectale/Clostridium coccoides* group (ER/CC) by fluorescent *in situ* hybridization (FISH) (4). Additionally, the immune markers eosinophil-derived neurotoxin (EDN) (12), calprotectin (13), and secretory IgA (14) were determined as previously described. Bacterial metabolic activity in both infant and mice samples was assessed through the analysis of short-chain fatty acids (SCFA) (i.e., acetate, propionate, butyrate, iso-butyrate, valerate, and iso-valerate) and lactic acids (D- and L-lactate) as described previously (3).

Measurement of plasma mouse mast cell protease-1 and sensitization markers

Plasma mMCP-1, total IgE, IgG1 and IgG2a were measured by ELISA according to manufacturer's recommendation (Ready-SET-Go!, Ebioscience, San Diego, USA). Measurements of BLG-specific IgE levels were performed by capturing with rat anti-mouse IgE (Pharmingen, BD Biosciences, Le Pont-de-Claix, France) antibody and by detecting with biotinylated BLG (Pierce, Rockford, USA) and streptavidin-horseradish peroxidase (HRP) (Clinisciences, Nanterre, France) as previously described (2). Data were expressed in terms of OD at 450nm. Levels of anti-BLG IgG1 and IgG2a were determined using BLG as the capture antigen, and goat anti-mouse IgG1 and IgG2a-HRP (Southern Biotech, Birmingham, USA) were labeled as detection antibodies as previously described (15).

Relative expression of genes in the colon

Total RNA was isolated from 2-cm segment of colon, using an RNeasy Plus universal kit (Qiagen, Courtaboeuf, France). Extracted RNA was treated with DNase I and first-strand cDNA was synthesized using Invitrogen reagents (Thermo Scientific, Illkirch, France) (15).

Quantitative real-time PCR (qRT-PCR) was performed on an ABI Prism 7900HT sequence detection system (Applied Biosystems, Thermo Scientific, Illkirch, France). QuantiTect SYBR green and QuantiTect primer assays (Qiagen) were used to quantify *fcγR3* and *fcεr2a*

expression. TaqMan gene expression assays with TaqMan universal master mix II (Applied Biosystems) were used to quantify *foxp3*, *t-bet*, *gata3* and *roryT*. Measurement were performed in duplicate, and gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method (16), where *CT* is the threshold cycle, with the *tata* box (TaqMan) assay as reference gene.

Statistical analysis

For statistical analysis and visualization of microbiota community data, Graphpad Prism software version 7.00 for Windows (La Jolla California USA) was adopted for comparisons of species diversity and Canoco 5 software was used for Principal Component Analysis (PCA) (17) with Aitchison log-ratio transformation of bacterial genus compositional data (18). Differential abundance analysis was performed at the bacterial family and genus level using the R-package ANCOM (19) and the iTOL version 4 for visualization of the discriminant taxa in cladograms (20). Corrections for multiple comparisons were controlled using the Benjamini-Hochberg false discovery rate (FDR) method with significance below 0.05 (21). Other fecal and ceecal parameters, mice clinical response data and immune data were analyzed with Graphpad Prism software applying Mann-Whitney test for two-group comparisons and Kruskal-Wallis to compare more groups, with Dunn's correction for multiple testing. A *P* value of less than 0.05 was considered significant.

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Table S1: Patients characteristics. a M=Male, F=Female; b HC=healthy control, All =allergic; c NVD = normal vaginal delivery, LSCS = lower segment caesarean section, EI= Elective; d Previous medicine including antibiotics. In bold character, selected donor infant for fecal microbiota transfer to the murine model.

N°	Sex ^a	Age collection (month)	Status ^b	Delivery ^c	Family History atopy	Maternal Antibiotics	Previous medicine ^d	Current medicines	% Breast feeding	Feed	When started feed	Age weaned
1	M	7	HC	NVD	No	No	No	No	100	NA	NA	5.5
2	F	5.5	HC	NVD	No	No	No	No	mostly (1 bottle/day)	Aptamil	birth	5
3	F	9	HC	LSCS	No	No	No	No	2x/day	Aptamil	1 week	5.5
5	F	7	HC	NVD	No	No	No	No	100	Breast Feed	birth	5
6	M	9	HC	NVD	No	No	No	No	100	NA	NA	4.5
8	M	9	HC	EI LSCS	No	No	No	No	100	Breast Feed	NA	6
4	F	10	All	LSCS	Yes	No	Ranitidine, Lansoprazole	Montelukast, Atrovent, Salbutamol, Abidec, Sytron	100 (3 months)	Nutramigen AA	4.5	4.5
7	F	16	All	EI LSCS	Yes	No	Infacol, Colic ease	No	100	Neocate	5 weeks	6
9	F	16	All	EI LSCS	Yes	No	Paracetamol	No	100	Neocate	5 months	6
10	F	7	All	NVD	Yes	No	Paracetamol	No	100	Neocate	6	6
11	F	12	All	NVD	Yes	No	Gaviscon, Ranitidine, Omeprazole	Ketotifen	100	Neocate	4 months	6

Table S2: Characteristics of CMA infants. CMPA=cow's milk protein allergy; GOR= gastroesophageal reflux. The CMA was diagnosed based on clinical presentation (immediate reaction with urticaria) and this was confirmed subsequently following oral challenge.

	Patient 4*	Patient 7	Patient 9	Patient 10	Patient 11
Age (months)	10	16	16	7	12
Sex	Female	Female	Female	Female	Female
Clinical manifestations	Urticarial rash, vomiting/GOR	Immediate vomiting, rash, abdominal pain, blood per rectum	Urticarial rash, vomiting, faltering growth, loose stools (with blood and mucus)	Urticarial rash, vomiting	Immediate swelling and rash, vomiting/GOR, faltering growth
Personal atopic past history	CMPA, wheat, egg, soya, reactive airway disease	CMPA, egg, wheat, soya, seafood, beef, eczema	CMPA, egg, wheat, soya, shellfish, nut, eczema, chesty	CMPA	CMPA, egg, wheat, soya, rice, eczema, constipation
Familial atopic past history	Yes	Yes	Yes	Yes	Yes
Final diagnosis	Multiple Food Protein Allergies	Food protein-induced enteropathy syndrome	Multiple Food Protein Allergies	CMPA	Multiple Food Protein Allergies
Elimination Diet	Nutramigen AA®	Neocate®	Neocate®	Neocate®	Neocate®

*Post-exclusive breast feeding, infant #4 developed urticarial rash and classic CMA symptoms when challenged with dairy on repeat occasions; with symptom resolution on exclusion. Infant 4 was stable (amino acid-based formula with dairy/egg/wheat/soya exclusion) when FMT sample was obtained.

Figure S1: Fecal markers analyzed in infant donor samples. Box-whisker plots with FISH quantified levels of *Bifidobacterium* spp. (A), and *Eubacterium rectale* – *Clostridium coccoides* (ER/CC) group (B), and stool pH (C). Concentrations of fecal SCFAs, except for valeric acid due to majority of samples had undetectable levels, and lactic acids are summarized (D-J), as well as the concentration of secretory IgA (sIgA) (K), Calprotectin (L) and eosinophil-derived neurotoxin (EDN). Final selected donors for fecal transfer to mice model are highlighted in red. Statistics comparing healthy control (HC) with cow's milk allergic (CMA) infants were performed using Mann-Whitney test. * $p < 0.05$, ** $p < 0.001$.

Figure S2: Microbiota establishment in murine model of CMA (A.). Richness and Shannon diversity analysed in fecal pellets collected at D15 (B and C) and in cecum content collected at D50 (D and E). Cladogram with discriminant taxa identified in mice at D15 (F) and at D50 (G). Taxa that were differentially abundant when comparing HC-mice with CMA-mice are highlighted at the genus (darkest colour) and family level (lighter colour). Green colours indicate an increase of relative abundances in HC, and red colours indicate increased relative abundances in CMA.

Figure S3: Levels of SCFAs and lactic acids in cecal content mice (D50). Box-whisker plots with the concentrations of acetate (A), propionate (B), butyrate (C), iso-valeric acid (D), D-lactic acid (E) and L-lactic acid (F). The detection of iso-butyrate (G) and valerate (H) are presented as percentage of samples with detectable levels (P) and undetectable levels (A). Statistics comparing the HC-groups with the CMA-groups were performed using Kruskal-Wallis test with Dunn's correction for multiple comparisons and applying fisher's exact test for presence (P) -absence (A) data. * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$, **** $p < 0.00001$.