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ORIGINAL ARTICLE

Gastroenterology

The intestinal microbiome of infants with cow's milk-induced FPIES is enriched in taxa and genes of enterobacteria

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Abstract

Objectives: Food protein-induced enterocolitis syndrome (FPIES) is a severe type of non-IgE (immunoglobulin E)-mediated (NIM) food allergy, with cow's milk (CM) being the most common offending food. The relationship between the gut microbiota and its metabolites with the inflammatory process in infants with CM FPIES is unknown, although evidence suggests a microbial dysbiosis in NIM patients. This study was performed to contribute to the knowledge of the interaction between the gut microbiota and its derived metabolites with the local immune system in feces of infants with CM FPIES at diagnosis.

Methods: Twelve infants with CM FPIES and a matched healthy control group were recruited and the gut microbiota was investigated by 16S amplicon and shotgun sequencing. Fatty acids (FAs) were measured by gas chromatography, while immune factors were determined by enzyme-linked immunosorbent assay and Luminex technology.

Results: A specific pattern of microbiota in the gut of CM FPIES patients was found, characterized by a high abundance of enterobacteria. Also, an intense excretion of FAs in the feces of these infants was observed. Furthermore, correlations were found between fecal bifidobacteria and immune factors.

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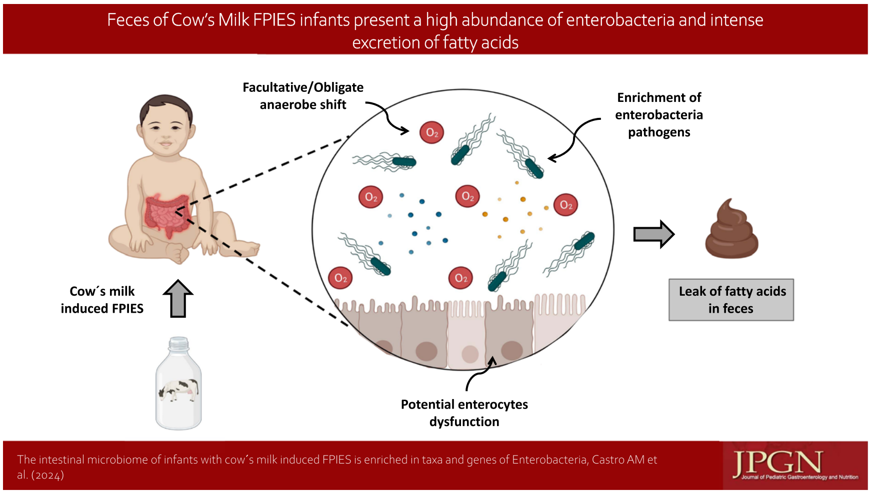
Conclusion: These fecal determinations may be useful to gain insight into the pathophysiology of this syndrome and should be taken in consideration for future studies of FPIES patients.



KEYWORDS
dysbiosis, food hypersensitivity, food protein-induced enterocolitis syndrome, SCFAs

1 | INTRODUCTION

Food protein-induced enterocolitis syndrome (FPIES) is a rare form of non-IgE (immunoglobulin E)-mediated (NIM) food allergy affecting less than 1% of infants and children.[1] In FPIES, the ingestion of a triggering food elicits a reaction that typically appears from 1 to 4 h after that intake. There are geographical differences regarding the offending food, but cow's milk (CM) is the main food responsible for FPIES throughout the world.[2] CM FPIES may have two different clinical presentations: acute and chronic. Although FPIES is a NIM form of CM protein allergy (CMPA), sometimes positive specific IgE to CM proteins could be detected at diagnosis or at follow-up (atypical FPIES). Diagnosis is established based on clinical criteria,[3] and only in doubtful cases, an oral food challenge might be performed in a hospital setting, as severe reactions might appear, even with small doses of CM.

The pathophysiology of the syndrome is not fully understood, but involvement of the immune and neuroendocrine systems, has been hypothesized.[4] Several inflammatory mediators have been studied in peripheral blood and in feces of children with FPIES,[5,6] but no diagnostic marker has been discovered to date. Besides, it is well known that the interaction between gut microbiota and the immune system is a key factor in allergy and tolerance development.[7] Data on the relationship between gut microbiota and FPIES are scarce.[8] The objective of this study was to analyze the gut microbiome and its relationship with intestinal immune mediators in infants with CM FPIES.

What is Known

- Food protein-induced enterocolitis syndrome (FPIES) is a severe type of non-IgE-mediated food allergy (NIM-FA), with cow's milk (CM) being the most common offending food.
- FPIES pathology in not fully understood, but gut dysbiosis has been considered a possible pathogenic factor involved.

What is New

- Infants with CM FPIES patients show aspecific pattern ofgut microbiota characterised by a high abundance of enterobacteria.
- Moreover, these infants show an intense excretion of fatty acids in the stools.

2 | METHODS

2.1 | Subject recruitment and sample collection

The study sample included a group of 12 patients with CM FPIES recruited at the time of diagnosis at different health centers in Spain (Table S1). International consensus guidelines for the diagnosis and management of FPIES were used for patient inclusion.[3] Infants with acute FPIES presented with vomiting

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in the first 4 h after the ingestion of CM, without associated skin or respiratory symptoms, and with at least three of the minor criteria indicated in the guidelines. Those with chronic FPIES presented with different gastrointestinal symptoms that improved or resolved with the elimination of CM proteins from the diet, and presented an acute FPIES reaction when the CM was reintroduced. A control group of 14 five-month-old infants was recruited by pediatricians in primary care centers. To be eligible for inclusion, participants should not have taken any antibiotics or probiotics in the 2 weeks before the start of the study. Clinical data were collected from the patient's medical history anonymously by the treating pediatrician. Fresh stool samples were collected at the time of diagnosis, in a special container (GutAlive®; MicroViable Therapeutics) that allows the transport of the samples in anaerobic conditions at room temperature.[9] Samples were processed in a biosafety cabinet within the first 24 h after deposition. The study was conducted in accordance with applicable local and legal regulations and following internationally accepted ethical standards. In addition, signed individual informed consent were obtained from all families participating in the study. The study was approved by the "Comité de Ética de la Investigación del Principado de Asturias" (Reference number 343/19).

2.2 | Intestinal microbiota analysis

Fecal samples (1 g) were homogenized in 9 mL of phosphate-buffered saline solution (PBS) and used for DNA extraction according to the DNA extraction protocol Q[10] using the QIAamp DNA Stool Mini Kit (Qiagen) with some modifications. These modifications were mainly in the lysis steps using a Fastprep FP24 homogenizer (Biomedicals). In this case, the cycles lasted 45 s, leaving the samples on ice for 5 min between each treatment. Quantification of the extracted DNA was performed using the Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific).

2.3 | Analysis of 16S ribosomal RNA gene amplicons sequencing

The 16S recombinant DNA (rDNA) was amplified from the DNA of the samples according to Milani et al.[11] and 250 bp paired-end sequences were obtained using an Illumina MiSeq System (Illumina) at the spin-off of the University of Parma Genprobio srl. Sequences were processed using the Quantitative Insights Into Microbial Ecology software suite and assigned to the lowest possible taxonomic rank considered, using the SILVA database v. 132 as reference. The raw sequences data were deposited in the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/sra) under bioproject code PRJNA1036152 (sequence library IDs SAMN38115097–SAMN38115122).

2.4 | Analysis of shotgun metagenomics sequencing

Total DNA extracted was submitted to an external sequencing service (www.BaseClear.com). Paired-end sequence reads (2×150 bp) showing an average minimum of 25 million reads per sample were generated using an Illumina NovaSeq system. Contaminant low-quality reads were removed using Kneaddata (v0.7.4) and Trimmomatic (v0.39) software. An assembly-free analysis of decontaminated reads was carried out following MetaPhlAn 3.0 (v3.0.4) and HUMAnN 3.0 (v3.0.0) pipelines to perform taxonomic and functional analysis, respectively.[12,13] Then, several statistical methods implemented on R (v.4.2.3) were computed to analyze the results. Composition barplots were calculated and generated using "Phyloseq" and "Microbiome" R packages.[14,15] To investigate statistically significant differences ($p < 0.05$ and corrected p values considering a false discovery rate [FDR] of 0.25 [$q < 0.25$]) between controls and FPIES patients at all taxonomic levels, MaAsLin2[16] and several microbiota-specific statistical methods (ANCOM, LEfSe, and DESeq2) implemented in "microbiomeMarker" R package were computed.[17–20] The raw sequences data were deposited in the SRA of the NCBI under bioproject code PRJNA1037775 (sequence library IDs SAMN38197428–SAMN38197447).

2.5 | Intestinal inflamation markers determination

Fecal calprotectin (FC) was analyzed using the CALPROLAB™ Kit (Calpro) following the instructions for making an enzyme-linked immunosorbent assay. The final absorbance (450 nm) data were measured in a Modulus Microplate Photometer (Turner BioSystems).

The profile of cytokines, chemokines, and growth factors excreted in the fecal water was analyzed using the Bio-Plex Pro Human Cytokine 27-plex Assay Kit (Bio-Rad Laboratories Inc.) in a Bio-plex 200 system instrument (Bio-Rad). For that, 1 g of fecal samples was suspended in 9 mL of PBS. After homogenization, the samples were centrifuged (13,000 rpm, 15 min, 4°C) and the supernatants were collected and stored frozen (–20°C) until analysis. Determinations in fecal samples were performed in duplicate according to the manufacturer's protocol and calibration curves (five parameter logistic) were constructed for each analyte using duplicate values

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for each known concentration and the Bio-Plex Manager 6.2 software (Bio-Rad).

2.6 | Quantification of fatty acids (FAs) in feces

For the determination of FAs, a 1:2 dilution of feces (1 g) in PBS was used. 0.1 mL of this dilution was supplemented with 50 µL of 2-ethyl butyric acid (Sigma-Aldrich), as internal standard (1.05 mg/mL in methanol) and acidified with 50 µL of 20% formic acid (vol/vol). The acidic solution was then extracted with 450 µL of methanol and centrifuged for 10 min at 16,200*g*. The supernatants were stored at −20°C until analysis in a gas chromatography (GC) apparatus composed of a 6890 GC injection module (Agilent Technologies) with a HP-FFAP (30 m × 0.250 mm × 0.25 µm) column (Agilent Technologies). The chromatographic system was equipped with a flame ionization detector. Data acquisition and processing were performed using ChemStation Agilent software (Agilent Technologies). All samples were analyzed in duplicate and FAs were quantified as previously described.[21]

2.7 | Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics v.28.0.1 (IBM) and RStudio software version 4.3.0 (R Foundation for Statistical Computing). Normality was checked using the Kolmogorov–Smirnov test. The nonparametric Mann–Whitney *U* test was used to examine differences in continuous variables of general characteristic and $p < 0.05$ were considered significant. A statistical significance of $p < 0.05$ was also considered for both 16S and metagenomic sequencing analyses. In addition, statistical results from shotgun metagenomic analysis were adjusted considering a FDR of 0.25 ($q < 0.25$). α-Diversity estimators and general statistical analysis of microbiota composition were calculated using the Microbiome R package.[15] Additionally, β-diversity analysis of microbial communities was conducted in accordance with the Bray–Curtis dissimilarity method,[22] implemented in "Phyloseq" R package,[14] with the adonis2 function (vegan), employing permutational multivariate analysis of variance (PERMANOVA). The Origin Pro 2021 software (OriginLab) was also used for graph design, and GraphPad Prism 9 (GraphPad Software Inc.) to calculate the curve of 4-point sigmoidal fit used in the determination of FC. Statistical correlations ($p < 0.05$) between microbial composition, FAs, and immunity factors were calculated using base R functions and expressed as Pearson correlation coefficients.

3 | RESULTS

3.1 | Participant characteristics and clinical data

Information about the age at diagnosis, gender, mode of delivery, and type of feeding before diagnosis was recorded (Table S1). None of the studied infants were born preterm (taking into account prematurity at less than 37 weeks' gestation). No significant differences were found between patients and controls with respect to gender, mode of delivery, and type of feeding. While the mean age of the patients was 4 ± 2 months, the mean age of the controls was 5 ± 1 months ($p = 0.04$).

3.2 | Microbiome

First, we sequenced amplicons of 16S rDNA from fecal samples of the 12 FPIES recruited patients and the 14 age-matched controls. Analysis of α-diversity estimated by Shannon and Simpson indexes revealed no statistically significant differences ($p > 0.05$) between patients and controls (data not shown), however β-diversity analysis performed by the Bray–Curtis dissimilarity method showed statistically significant differences ($p < 0.05$) between both groups when PERMANOVA was applied (Figure S1). Additionally, analysis at the compositional level revealed that patients had a lower abundance of Actinomycetota members than controls ($p = 0.05$). Within this phylum, sequences assigned to the family Bifidobacteriaceae stood out, being less abundant in infants with FPIES (Table S2). On the other hand, representatives of the family *Enterobacteriaceae* were found in higher percentages in these patients compared to controls although the differences were not statistically significant ($p = 0.08$).

To further investigate the observed differences in the gut microbiome at both taxonomic and functional levels, we performed shotgun metagenomics sequencing on samples from half of the infants with FPIES (six samples) and the control group (14 samples). The results were consistent with those observed by 16S sequencing and showed differences between both groups at phylum, family, and genus level. Actinomycetota (Figure 1A), Bifidobacteriaceae (Figure 1B) and *Bifidobacterium* (Figure 1C) were significantly more abundant in the control group compared to FPIES patients ($p < 0.05$ and $q < 0.25$). In contrast, Pseudomonadota (Figure 1A), *Enterobacteriaceae* (Figure 1B), *Klebsiella*, and *Escherichia* (Figure 1C) showed significantly higher ($p < 0.05$ and $q < 0.25$) abundances in patients compared to control infants.

Regarding the functional analysis of metagenomes, gene count for the control group and FPIES

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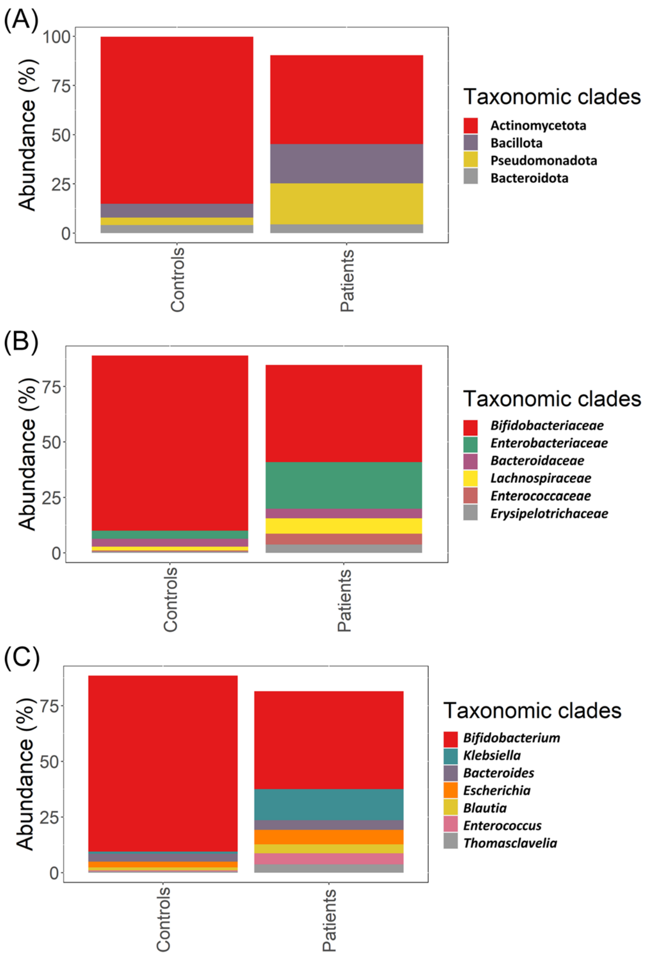

FIGURE 1 Taxonomic clades identified at phylum (A), family (B), and genus (C) level in the fecal microbiota of controls and patients. Data are expressed as abundance percentages (%).

patients was 17,829 ± 5775 and 17,086 ± 2547, respectively. The statistical analysis of functional profiles showed microbial gene families and metabolic pathways with highest abundances (Table 1) in metagenomes from the control group, corresponding with *Bifidobacterium bifidum* and *Bifidobacterium longum* species. On the other hand, patients with FPIES have a large number of gene families and metabolic pathways of *Enterobacteriaceae* (709 families and 21 pathways), specifically of the species *Escherichia coli* and *Klebsiella pneumoniae*, presenting abundances significantly higher than those of the control group ($p < 0.05$ and $q < 0.25$). These pathways comprise a wide variety of functions involved in carbohydrate, aminoacid and ribonucleotide metabolism (Table S3). Of note that among the carbohydrate degradation pathways those of D-fructuronate and D-galactarate were predominant in FPIES infants, whereas those of sucrose degradation were more abundant in controls.

TABLE 1 Number of microbial gene families and metabolic pathways showing highest abundances in controls and patients ($p < 0.05$ and $q < 0.25$).

Microbial gene families showing higher abundances in control group		Microbial gene families showing higher abundances in patients	
Taxa	**Frequency**	**Taxa**	**Frequency**
Bifidobacterium bifidum	2447	*E. coli*	689
Bifidobacterium longum	255	*Klebsiella pneumoniae*	20
Escherichia coli	7	*B. longum*	3
Eggerthella lenta	2	*Enterococcus faecalis*	2
Total	2711	Total	714
Microbial metabolic pathways showing higher abundances in control group		**Microbial metabolic pathways showing higher abundances in patients**	
Taxa	**Frequency**	**Taxa**	**Frequency**
B. bifidum	48	*E. coli*	11
E. lenta	7	*K. pneumoniae*	10
E. coli	1	Total	21
Total	56		

Note: These gene families and metabolic pathways are summarized by bacterial species.

3.3 | Immunological and inflammatory markers

Regarding the levels of FC, higher values were observed in patients: 292.88 µg/g (72.31–825.93) feces than in controls 92.83 µg/g (47.01–267.69) feces, although the differences were not statistically significant ($p = 0.06$) (Figure S2). Of the 27 immune factors examined, including cytokines, chemokines, and growth factors excreted in fecal waters, only five mediators were detected in half of the subjects ($n > 13$) or in half of one of the two groups ($n > 6$ for patients and $n > 7$ for controls) (Table S4). These analytes included interleukins (ILs) 4 and 10, the IL-1 receptor antagonist protein (IL-1ra), the cytokine interferon gamma inducible protein-10 (IP-10), and the platelet-derived growth factor BB (PDGF-bb). The levels of IL1-ra, IP-10, and PDGF-bb which presented a significant reduction in the patient group compared to the healthy group ($p = 0.01$) are showed in Figure 2. For the other immune compounds quantified in feces there were no statistical differences between the infants of both groups.

3.4 | Fecal FAs

The concentration of fecal FAs, and specifically acetic acid, was higher in patients ($p < 0.001$) (Figure 3A). It was also observed that the excretion of FAs derived from

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protein degradation, branched chain FAs (BCFAs) that includes isovaleric and isobutyric acids, were significantly higher in patients compared to controls ($p = 0.04$) (Figure 3B).

3.5 | Association between metagenomes and fecal parameters

Correlation analysis was performed to investigate possible associations between the abundance of gut microorganisms and the other fecal biochemical parameters measured. As shown in Figure S3, moderate positive associations were found between bifidobacteria and the levels of the factors IL-1ra, IP-10, and PDGF-bb (Pearson correlation coefficient values of 0.5). In contrast, these immune compounds were negatively correlated with *E. coli*. In addition, correlation analysis revealed strong positive associations between *Enterobacteriaceae* and *Klebsiella* abundance and propionic acid (Pearson correlation coefficient values of 0.8), while Actinomycetota showed negative correlations with propionic and butyric acids (Figure S3). No significant associations were found for acetic acid levels.

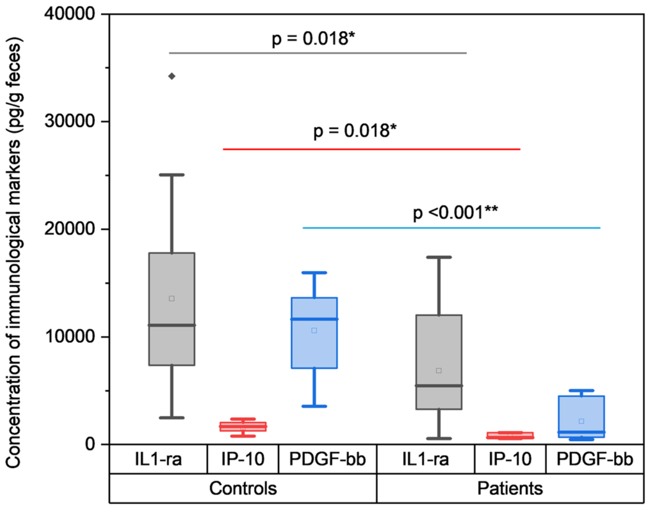

FIGURE 2 Relevant immunological markers in feces. Data are represented in a box-and-whisker plot. Mann–Whitney *U* test were used to evaluate differences in concentrations among groups. Significant differences for $^*p < 0.05$ and $^{**}p < 0.01$. IL-1ra, interleukin-1 receptor antagonist protein; IP-10, interferon gamma inducible protein-10; PDGF-bb, platelet-derived growth factor BB.

4 | DISCUSSION

The incompletely understood pathophysiology of FPIES, the lack of diagnostic biomarkers, and the poor knowledge of its natural history have recently made this disease an important target for research.[23] There are reports suggesting that microbiota may be involved in the pathogenesis of this syndrome.[24] However, studies are really scarce and the gut microbiota of this type of NIM food allergy remains poorly characterized with few authors using omics techniques to explore it.[25] Recently, differences in gut microbiota composition in patients with pediatric FPIES respect to healthy control infants have been reported by sequencing of 16S rDNA amplicons[8] though the studied cases were not induced by CM proteins. In fact, to our knowledge, there are no previous research papers on shotgun sequencing of the gut microbiome in cases of CM FPIES. In our study, we used both 16S rDNA amplicons and in depth shotgun sequencing. By both techniques, a different microbiota structure between

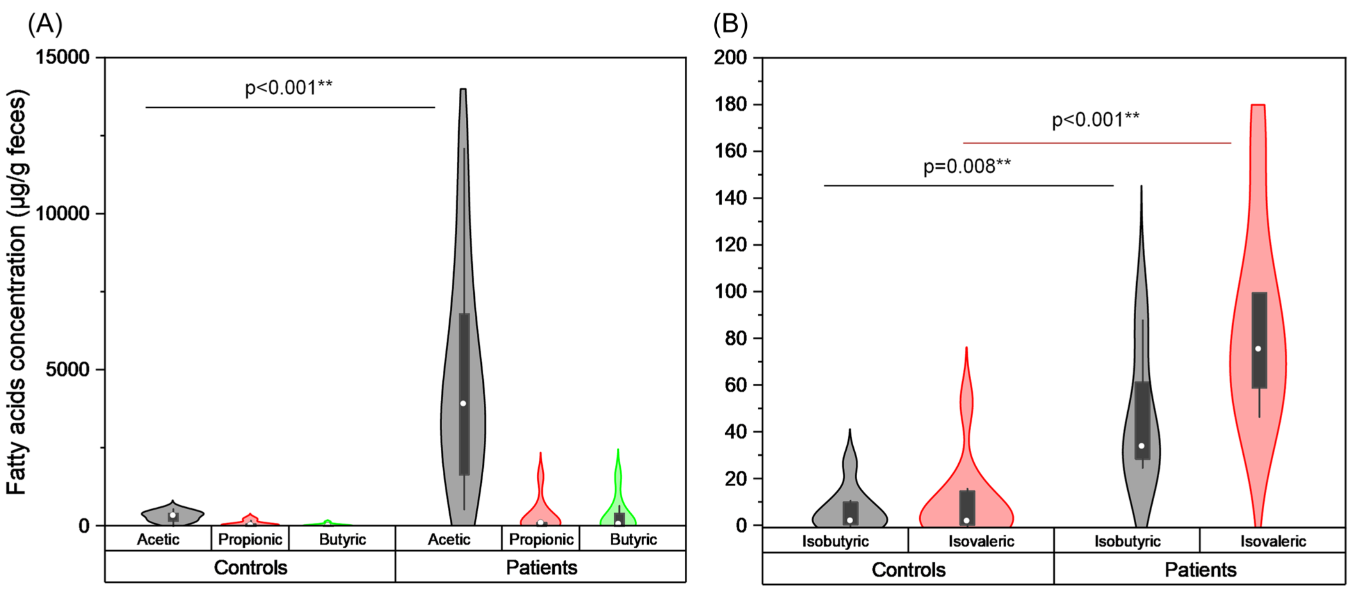

FIGURE 3 Fatty acids in fecal samples. Box-and-whisker plot depicting concentrations (in µg/g) of major short chain fatty acids (A) and branched chain fatty acids (B) in fecal samples of the two groups of study. Mann–Whitney *U* test was used. Significance was set at $p < 0.05$.

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patients and controls was observed, with differences in the distribution of bacterial taxa. Mainly, it was noted a depletion in bifidobacteria in CM FPIES compared to controls, and an enrichment of enterobacteria, in particular the enteric pathogens *Klebsiella* and *E. coli*, in the gut microbiome of these patients. These are facultative anaerobic microorganisms belonging to the phylum of Pseudomonadota (earlier known as Proteobacteria) potentially harmful to the colonocytes.[26] Indeed, a dysbiotic expansion of this phylum in the gut has been proposed as a potential diagnostic microbial signature of epithelial dysfunction.[27] An increased abundance of Proteobacteria has been observed in humans with severe intestinal inflammation, including patients with inflammatory bowel disease,[28] necrotizing enterocolitis,[29] or even FPIES, which is consistent with our findings.[30]

In addition, in our work the fecal excretion of FAs produced by the gut microbiota was quantified. Short-chain fatty acids (SCFAs), mainly acetic, propionic, and butyric acid are produced as result of bacterial fermentation in the colon, and they have been associated with gut health and exert beneficial effects on the host at various levels.[31,32] Gut microbiota composition and fecal butyrate levels in children affected by NIM-CMPA were evaluated by Berni Canani et al.[33] These authors observed that these children had a significantly lower fecal butyrate concentration compared to healthy controls. In our previous work with NIM-CMPA cases, we did not find such differences for the main SCFAs between controls and patients, however, the BCFAs were significantly higher in patients.[34] In all these studies, the average age of the infants was higher and they were not FPIES patients. Apart from these, we have not found any other work on this type of food allergy in which the levels of butyrate or other SCFAs were measured directly in feces. In the present work, the excretion of total FAs and BCFAs was indeed increased. In particular, acetic, which is the most abundant SCFAs in the colon,[32] was 10 times higher in FPIES respect to controls. It has been described that colonocytes rapidly absorb an estimated 95%–99% of microbial produced FAs, while the remaining 5% are secreted in the feces.[31] We hypothesized that the amount detected in feces in patients could not represent that produced by the microbiota in the lumen, but rather a result of colonocyte dysfunction associated to the shift in the obligate to facultative anaerobic ratio observed in these infants with FPIES.[27] The high levels detected in feces of these FAs could be due to a reduced absorption and an alteration of colonocyte metabolism according to the "oxygen hypothesis."[35] Future studies are needed to confirm and understand this phenomenon. Ideally, the determination of SCFAs in serum might give some clues about circulating levels and reduced absorption, although it is difficult to obtain this type of samples in young infants, almost all from controls. Another point that can give some consistence to our theory is the fact that we did not observe any association between fecal excretion of acetic acid (higher in patients) and abundance of bifidobacteria (higher in controls), which are the main bacterial producer of this compound in the infant gut.[32] Nevertheless, for propionic acid, we found a correlation with abundance of enterobacteria, for which a propanediol pathway for the conversion of deoxy-sugars to propionate has been described,[32,36] and accordingly, metabolic genes related to fermentation to propionate (MetaCyc Pathway; PWY-7013) were revealed from the metagenomic functional analysis to be associated to enterobacteria in some of these patients (data not shown).

In our study, we combined metagenomic sequencing with the quantification of fecal immune factors looking for correlations, since the involvement of the immune system is poorly understood, and gastrointestinal immune events may be relevant in this syndrome where the gastrointestinal tract is the main system affected. In this sense, identification of new fecal biomarkers will be optimal to improve diagnosis and management of CM FPIES occurring in small babies, especially for the noninvasive collection requirements.[37] Among fecal biomarkers, the measurement of FC is considered useful for intestinal mucosal inflammation. Reference values are clearly defined in adults, however no clear cut-offs have been convincingly established in children,[38] with the studied cases of NIM-CMPA reporting no differences respect to healthy infants.[34,39] In our study of CM FPIES, no statistically significant differences were found, probably due to the dispersion of the data, but a tendency to a higher concentration was observed in patients with a mean value double than in controls and above 250 µg/g. For other cytokines and local immune mediators, we observed a significant reduction in the fecal concentrations in patients. In particular, PDGF-bb, a platelet-derived factor involved in vascular remodeling and angiogenesis, IP-10, and the IL1-ra. This suggests to us that their low levels in the feces of CM FPIES infants could be related to a disrupted colonic homeostasis.

5 | CONCLUSION

In support of our theory, a positive correlation between these immune factors and the levels of the beneficial bifidobacteria was observed in our study. It is clear that several cellular elements and cytokines may be involved,[23] although the exact molecular mechanism and interactions with commensal bacteria remain to be elucidated. By using different approaches, the experimental data obtained revealed potentially pathogenic taxa capable of damaging intestinal colonocytes and disrupting intestinal immune homeostasis. This should be taken into consideration for future studies, ideally with a high number of patients, to confirm the findings. We are conscious that the reduced sample size is a limitation, but this is the first study

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exclusively in CM FPIES cases. Although diet is a factor that can influence the microbiota, and the FPIES patients in our work were on different types of feeding (breastfeeding, mixed, or bottle feeding), we did not find statistical differences in dietary habits with respect to the age-matched control group of infants. However, it is true that for further studies with large cohorts of patients dietary differences in feeding should be taken into account.

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CONFLICT OF INTEREST STATEMENT

Susana Delgado and Abelardo Margolles are Scientific Founder and Member of the Scientific Advisory Board of MicroViable Therapeutics S.L. The remaining authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Sequencing files and metadata were deposited in the Sequence Read Archive of the National Center for Biotechnology Information under bioprojects codes PRJNA1036152 for amplicons sequences and PRJNA1037775 for shotgun sequences.

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