Gut microbial metabolomics to understand allergies in early life

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Contents

Chapter 1

General Introduction and Scope

Food allergies

In the past decades the prevalence of food allergies, especially in Western countries, has been on the rise.[1,2] The term "food allergy" encompasses various clinical conditions, sharing a common underlying mechanism: a loss of clinical and immunological tolerance to ingested food proteins.[3] Consequently, individuals with food allergies experience an exaggerated immune response to otherwise harmless food proteins.[4] Allergic reactions are mainly classified as immunoglobulin E (IgE)-mediated, non-IgE mediated, and mixed based on clinical symptoms and immunological mechanism.[5] IgE-mediated food allergies are characterized by IgE antibody production and immediate onset of symptoms following allergen ingestion.[5] In comparison, non-IgE mediated food allergies are marked by a delayed response to the allergen and involve immunological mechanisms that are not yet well-defined.[5] Symptoms of food allergy range from mild skin, respiratory, gastrointestinal symptoms to life-threatening anaphylaxis.[3]

Often allergic disease follows a temporal progression from atopic dermatitis and food allergy in infancy to allergic asthma and allergic rhinitis in childhood, also known as “atopic march”.[6,7] Notably, early life atopic dermatitis is also a well-established risk factor for food allergies later in life.[3] Immune tolerance to the food allergen is often, but not always, acquired with time leading to outgrowth of the food allergy.[5] For instance, hen egg and cow’s milk allergies are often outgrown in childhood, whereas nut allergies can persist into adulthood.[5]

Despite the increasing knowledge on food allergies treatment and prevention, allergen avoidance remains the most common management strategy.[3] Considering the reduction of quality of life of allergic individuals and the associated financial burden,[8] the development of prevention and treatment strategies is urgently needed.

Multiple hypotheses have been suggested to explain the increase in allergies in Western countries with a leading hypothesis proposing that reduced microbial exposure in the developed world has disturbed the once beneficial commensal human-microbe relationship leading to a loss of certain immunoregulatory pathways.[9] In other words, this theory suggests that reduced bacterial exposure results in an ill-trained immune system, which is incapable of recognizing a friend from a foe, resulting in food sensitization and thus allergies.

The gut microbiome and early life

Our guts harbor to the greatest number of humoral immune cells in our bodies[10] and are home to densely populated microbial communities which have coevolved with us in a symbiotic relationship.[11] We, as hosts, provide the gut microbiome (GM) with a hospitable habitat and nutrients and in turn the GM aids nutrient absorption, produces vitamins, protects against pathogenic microorganisms and more.[11] The colonization of infants’ gut starts at birth and is very rapid in the first 3 years of life, especially in the first year[12,13] (**Figure**

1). Prior the introduction of solid food, the GM of healthy breastfed infants is dominated by *Bifidobacterium* species, including *B. breve*, *B. longum*, *B. bifidum*.[14] Their prevalence is a result of their excellent capability to (co-)ferment human milk oligosaccharides (HMOs) abundant in breastmilk.[13] Following solid food introduction, the microbiome starts diversifying and eventually closely resembles that of an adult at 3 years of age[12,13] (**Figure 1**).

The microbiome composition in early life is highly dependent on multiple factors, including birth mode, feeding mode, solid food introduction, and antibiotic usage[13,15–17] (**Figure 1**). For instance, at birth the GM of vaginally delivered infants resembles that of mother's vagina, whereas that of the infants delivered via C-section – the environment and mother's skin.[18] Meanwhile, the consumption of infant formula promotes the colonization of microbial taxa commonly found in adults.[19]

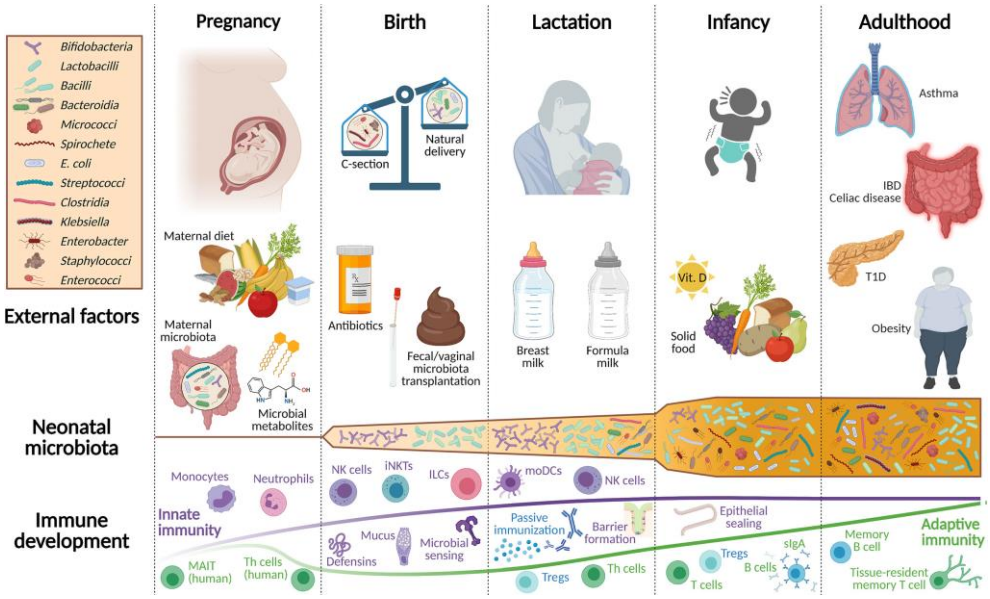

Figure 1. *Overview of factors shaping the development of the GM and mucosal immune system through pregnancy, birth, lactation, infancy, and adulthood. Abbreviations: IBD, inflammatory bowel disease; sIgA, secretory immunoglobulin A; ILCs, innate lymphoid cells; iNKTs, invariant natural killer T cells; MAIT, mucosal-associated invariant T cells; moDCs, monocyte-derived dendritic cells; NK, natural killer cells; Th cells, T helper cells; Treg, regulatory T cells; T1D, type 1 diabetes. Image derived from[15]*

The development of the immune system starts prior to birth and is influenced by the maternal diet[15] as well as by gut microbial-derived metabolites which reach the fetus via the placental barrier.[20] At birth, simultaneous to the rapid temporal development and maturation of the infants GM, the immune system of the infant also develops substantially[21] (**Figure 1**). Considering their simultaneous development and high density in the gut, it is not surprising that the host's immune system and GM have coevolved to influence each other.

Consequently, the proper development and maturation of the GM in early life is essential for optimal immune system development.[15,22,23] Among others, bifidobacterial species *B. breve*, *B. longum*, *B. bifidum* have well-recognized beneficial effects for the immune system development in early life.[24] Meanwhile, disturbances in the GM composition in early life, also known as dysbiosis, and reduction in bifidobacteria in particular, have been linked to multitude immune-mediated diseases, including food allergies.[25,26] Because of that, the first three years of life are often viewed as a critical period during which GM disturbances can lead to adverse health outcome and disease.[27–29] Alternatively, the period can be viewed as a window of opportunity in which the dynamic nature of the GM and its ability to adapt to changes can be advantageous and be used to treat and prevent diseases, including allergies.[27–29]

Infant diet and bifidogenic interventions

Human milk is considered the golden standard for infant nutrition.[30] In addition to the macromolecules (carbohydrates, protein, fat), human milk also contains minerals, vitamins, hormones, immune cells, cytokines, and other bioactive components such as the above-mentioned HMOs.[31] Exclusive breastfeeding is highly recommended for the first six months of life, followed by breastfeeding alongside solid food consumption until age of two or beyond.[30] The alternative feeding mode in the cases when infants cannot receive breastmilk is formula-feeding. Infant formulas commonly use cow's milk or soymilk as a base and are often supplemented with minerals, vitamins, nucleotides, and fatty acids in the attempt to resemble human breastmilk as much as possible and meet all nutritional needs of the infant.[31] When infants cannot tolerate standard formula, specialized formulas are prescribed instead. In those formulas the milk proteins are either partially or extensively hydrolyzed to reduce their allergenicity.[32] When the infant cannot tolerate the partly and extensively hydrolyzed formula, an amino acid-based formula is assigned instead.[31]

In contrast to breastmilk, cow's milk and soymilk used in infant formula do not contain HMOs, which are essential for the growth and proliferation of bifidobacteria in infants' guts.[31] Due to the importance of bifidobacteria for the immune system development and maturation, infant formulas are sometimes supplemented with *Bifidobacterium* probiotic or bifidogenic (bifidobacteria-enhancing) prebiotics and synbiotics.[33,34] Probiotics are microorganisms with known beneficial effect on health; prebiotics are non-digestible food ingredients that promote the growth of beneficial microorganisms; while synbiotics are a blend of probiotics and prebiotics.[31] *Bifidobacterium* probiotics include but are not limited to *B. breve*, *B. longum*, *B. bifidum*, whereas prebiotics include non-digestible carbohydrates such as galactooligosaccharides (GOS), fructooligosaccharides (FOS), and inulin.[33,34] The latter two are linear fructose chains ending in a glucose unit that vary in chain length: 3–5 units in short-chain FOS, 6–10 in long-chain, and up to 200 in inulin.[35]

Microbiome analysis

To study the link between the GM and disease, the microbiome composition and/or function could be analyzed in fecal samples. In the past, microbiome research was limited by traditional culturing, which was biased toward aerobic microorganisms, limiting their ability to capture the predominantly anaerobic gut microbiota.[36] Fluorescence *in situ* hybridization (FISH) is a culture-independent molecular technique that enables quantification of bacterial taxa such as *Bifidobacterium* spp.[37] This technique targets the 16S rRNA gene, which is ubiquitously found in bacteria.[36] This gene consists of constant regions shared by all bacteria, and hypervariable regions which facilitate phylogenetic differentiation at the genus or species level.[36] Next-generation sequencing technologies revolutionized GM research by offering high-resolution large-scale analysis of microbial communities.[38] 16S rRNA gene sequencing, which as the name suggests also utilizes the 16S rRNA gene, has been considered the golden standard in compositional microbiome analysis for many years.[13] The technique provides relative abundance data, typically at genus level.[38] Alternatively, a metagenomic approach, also known as shotgun metagenomics, allows for the examination of the entire microbial genome.[13,39] This methodology offers much richer data, providing insights into the genetic functional potential of the GM.[13,39] Although shotgun metagenomics enables taxonomic resolution down to the species and strain level, its higher cost and computational demands make 16S rRNA sequencing the more widely used approach.[38]

Metabolomics

Functional insights into host–GM interactions can be derived through metabolomic analysis.[40] Metabolomics is the scientific field that focused on the comprehensive analysis of metabolites - small molecules (≤ 1500 Da) involved in metabolic processes within biological systems.[41] By producing a wide range of metabolites, the GM impacts gut physiology in both beneficial and harmful ways.[40] Studying these microbial metabolites provides direct insight into host-microbiota interactions.[40] Different biological matrixes (feces, urine, plasma) could be subjected to metabolomic analysis in the field of GM research depending on the research question. Plasma is used to study the circulating metabolome, capturing microbial metabolites that have been absorbed from the gut into the blood and may affect the immune system.[42] However, plasma is an invasive matrix, difficult to obtain from infants. Fecal samples are widely used in microbiome and metabolomic research, particularly in infant studies, due to their non-invasive nature, and their ability to reflect gut microbial composition and activity.[43] Fecal metabolomics provides detailed insights into the metabolic interactions between the host, diet, and gut microbiota.[44]

Metabolomics measurements are typically acquired using either nuclear magnetic resonance (NMR) or mass spectrometry (MS).[40] Even though superior to MS in terms of absolute quantification capability, NMR's low sensitivity limits its application only to the

most abundant analytes.[40] The high sensitivity, broad dynamic range, and high throughput make MS ideal for GM research.[40] Typically, MS is coupled to a chromatographic separation technique to decrease the sample complexity and enhance the detectability and identification capabilities of MS. Gas chromatography (GC) is suitable for the analysis of volatiles and of non-volatiles after suitable derivatization.[40] Meanwhile, liquid chromatography (LC) is suitable for the analysis of a wide range of polar (Hydrophilic Interaction LC - HILIC) and apolar metabolites (reverse phase LC).[40,45]

Translational gut-on-a-chip

In vitro gut-on-a-chip models are an alternative approach to study host–microbiome interactions mechanistically in a controlled manner, with some platforms enabling high-throughput experimentation.[46] In recent years, there has been a significant shift from traditional 2D in vitro models, where microbes are cultured under static conditions, to advanced 3D models like gut-on-a-chip, which more accurately replicate human gut physiology.[46,47] Even though no model can capture the full complexity of the human gut, a diverse range of gut-on-a-chip models have been reported reflecting different aspects of gut physiology.[46,47] The selection of models is driven not only by technological progress but also by the specific research question, as some can be adequately addressed with simple models.[46,47] Design aspects worth mentioning are the choice of epithelial cell lines, e.g., Caco-2 monolayer; presence and choice of immune and vascular cells; the addition of mucus; choice of microbiota, e.g., pathogens, probiotic species; the inclusion of flow to mimic in vivo fluid flow and shear stress; and oxygen gradient.[46,47] The latter is necessary when coculturing aerobic epithelial cells and anaerobic gut microbiome taxa.[46,47] The flexibility in the choice of model components has enables studies on disease mechanisms and therapeutics, including probiotic interventions.[47,48]

Outline and scope of the thesis

The early life gut microbiome (GM) is a dynamic and rapidly evolving ecosystem that plays a crucial role in immune system development and has been associated with allergic disease. Despite increasing recognition of importance of the GM, the complex interplay between the GM, environmental and dietary factors, and the allergic disease remains poorly understood. To unravel the intricate host–microbiome interactions, there is a growing shift toward metabolomic studies, which offer functional insights beyond taxonomic profiling. The research is based on the hypothesis that the GM in early life influences the development and resolution of allergies via the production of metabolites and that metabolomics can be used to study the function of the GM. The aim of this research is to study the links between allergy and intestinal health, the GM, and external factors by exploring the metabolome in longitudinal clinical studies and in vitro gut-on-a-chip models.

Chapter 2 aims to provide a systematic review on the role of the GM in the most common food allergy in early life: IgE-mediated cow's milk allergy (CMA). To offer a comprehensive overview of the current knowledge in the field, this review focusses on the microbiome,

transcriptome, proteome, metabolome, and immune response data from studies in children (≤12 years of age) and from animal models. Case-control and intervention studies are included to detail both disease-associated alterations and the impact of microbiome-based interventions. As most studies focus on microbiome compositional analysis, functional insights into the host-microbiome interplay remain scarce. The limited coverage of the available metabolomics studies hampers mechanistic understanding, highlighting the need for more comprehensive metabolomics and integration with other omics analyses.

Addressing the limited metabolomic scope in the existing literature (**Chapter 2**), in **Chapter 3** and **Chapter 4** the aim is to study the role of a broad range of host and microbial metabolites, including aromatic amino acid metabolites, bile acids, and short chain fatty acids in early-life allergy. In **Chapter 3** infants, exclusively breastfed for at least 16 weeks and at risk of developing allergies are followed during the first year of their lives, the period when "atopic march" typically starts, and microbial colonization is most rapid. This study aims to (1) explore the fecal metabolome and microbiome association with allergy development in the first year of life, and (2) evaluate the impact of age, delivery mode, and feeding practices, i.e. breastfeeding, formula feeding, and complementary feeding on the fecal metabolome and microbiome during this critical period.

IgE-mediated CMA is typically managed through elimination diets, including the use of amino acid-based formulas (AAF) in formula-fed infants. Given the growing evidence of the beneficial role of *Bifidobacterium* spp. for the immune development and their association with allergy, as shown in **Chapter 2** and **Chapter 3**, bifidogenic supplementation of AAF has emerged as a promising strategy in the management of IgE-mediated CMA. **Chapter 4** aims to study the links between IgE-mediated CMA, the GM, and bifidogenic synbiotic supplementation. For this, samples of the PRESTO clinical trial are analyzed. This trial follows infants diagnosed with IgE-mediated CMA who receive either standard AAF or AAF supplemented with synbiotic blend of probiotic *Bifidobacterium breve* M-16 V and prebiotic inulin and oligofructose. This study aims to (1) assess the changes in the fecal metabolome associated with the acquisition of tolerance to cow's milk protein, and (2) to evaluate the impact of bifidogenic synbiotic supplementation on the fecal metabolome.

To investigate how allergy onset, tolerance acquisition and synbiotic supplementation modulate the immune responses and intestinal barrier function, physiologically relevant in vitro experimental models are essential. Gut-on-a-chip systems provide a promising platform to replicate key aspects of intestinal physiology. However, to ensure robust and reproducible results, optimization of experimental conditions, particularly the selection of appropriate cell culture media, is crucial. The goal of **Chapter 5** is to examine how exposure to proinflammatory cytokines impacts intestinal barrier integrity and the secretion of signaling lipids under serum-containing and serum-free medium conditions. Using Caco-2 tubules in a membrane-free microfluidic organ-on-a-chip platform, barrier integrity is examined simultaneously using transepithelial electrical resistance (TEER), DRAQ7 staining, and actin cytoskeletal analysis. Meanwhile, lipid mediators are profiled using targeted LC -

tandem MS method across apical and basolateral compartments of the tubule to study the impact of the culture medium on the inflammatory responses and lipid mediator profiles in response to proinflammatory cytokine exposure.

Finally, **Chapter 6** offers a general conclusion of the studies described in this thesis. Perspectives and recommendations on further research are also discussed.

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Chapter 2

Current insights into cow's milk allergy in children: microbiome, metabolome and immune response – a systematic review

Based on:

Current insights into cow's milk allergy in children: microbiome, metabolome and immune response – a systematic review

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Abstract

Background: The increasing prevalence of IgE-mediated cow's milk allergy (CMA) in childhood is a worldwide health concern. There is a growing awareness that the gut microbiome (GM) might play an important role in CMA development. Therefore, treatment with probiotics and prebiotics has gained popularity. This systematic review provides an overview on the alterations of the GM, metabolome and immune response in CMA-children and animal models, including post-treatment modifications.

Method: MEDLINE, PubMed, Scopus and Web of Science were searched for studies on the GM in CMA-diagnosed children, published before March 1, 2023.

Results and conclusions: A total of 21 articles (13 on children, 8 on animal models) were included. The studies suggest that the GM, characterized by an enrichment of the Clostridia class and reductions in the Lactobacillales order and *Bifidobacterium* genus, is associated with CMA in early life. Additionally, reduced levels of short chain fatty acids (SCFAs) and altered amino acid metabolism were reported in CMA-children. Commonly used probiotic strains belong to the *Bifidobacterium* and *Lactobacillus* genera. However, only *Bifidobacterium* levels were consistently upregulated after intervention, while alterations of other bacteria taxa remain inconclusive. These interventions appear to contribute to the restoration of SCFAs and amino acid metabolism balance. Mouse models indicate that these interventions tend to restore the T_h2/T_h1 balance, increase the T_{reg} response, and/or silence the overall pro- and anti-inflammatory cytokine response. Overall, this systematic review highlights the need for multi-omics related research in CMA-children to gain a mechanistic understanding of this disease and to develop effective treatments and preventive strategies.

Keywords

infant, cow's milk allergy, gut microbiota, metabolomics, synbiotics, mouse model, immune response

1. Introduction

One of the most common food allergies in early childhood is cow's milk allergy (CMA).[1,2] Allergic reactions can be IgE-mediated, non-IgE-mediated, or a mix of both.[3] Multiple studies have shown that among the children diagnosed with CMA those with IgE-mediated reactions to cow's milk tend to have persistent symptoms more often and acquire tolerance slower than those with non-IgE-mediated reactions.[4–7] At present, infants diagnosed with CMA are placed on an elimination diet consisting of an extensively hydrolyzed formula (EHF) or, if symptoms persist, an amino-acid formula (AAF).[8] Because of the increasing evidence linking food allergies with alterations in gut microbial composition,[9,10] modifying the gut microbiome (GM) with probiotics, prebiotics or synbiotics has emerged as a promising way to prevent and treat allergies.[11] However, there is still little mechanistic understanding on how the GM influences host immune health, leading to allergies, including CMA.[12] Recent technological innovations in the field of microbiome, proteomics and metabolomics have opened new doors for research and provided opportunities to address the gap in understanding the role of GM in CMA. The objective of this systematic review is to further the understanding of the relationship between the GM and CMA, by reviewing existing studies examining microbiome, metabolome, proteome, and immune response data on IgE-mediated CMA in children and animal models.

2. Methods

This systematic review is registered in PROSPERO (CRD42021290177).

2.1 Search strategy

A search in MEDLINE, PubMed, Scopus and Web of Science was performed using the queries in **Table S1**. The search was limited to research articles published in English before March 1, 2023.

2.2 Inclusion and exclusion criteria

Human case, case-control, and intervention studies were included only if they examined children with IgE-mediated CMA aged 0-12 years. The allergy had to be medically diagnosed by either a skin prick test (SPT) or an IgE-specific test combined with a cow's milk food challenge. In studies with fecal transplantation (FT), the IgE-mediated CMA status of the donor must be confirmed by the diagnosis criteria used for human studies. For studies reporting data on groups of subjects diagnosed with different types of CMA, only the group with IgE-mediated CMA was reviewed. For animal studies, only case-control and intervention studies on models that included both sensitization and challenge steps were included. The studies were included only if they contained analytical data that examined the GM or metabolome and were excluded when they failed to meet the inclusion criteria, had unclear diagnosis, or involved antibiotic treatment.

2.3 Study selection

Titles, abstracts, and methods were screened independently by two of the authors MVS, PZ, DMH, and by a third author in case of disagreement. Subsequently, the full text of the studies marked as potentially eligible was retrieved and independently checked for eligibility by at least two of the authors MVS, PZ, DMH, and by a third author in case of disagreements or doubts.

2.4 Data extraction

For human studies, the extracted data included general study details (author, year), participant information (age, sample size), CMA diagnosis, analytical data types, data acquisition techniques, measured analytical parameters and significant results. For intervention studies, the intervention details were also extracted. If available, the age range for each group in the study was reported. When only the mean and standard deviation (sd) were available, the age was reported as mean ± sd. The results were split in two: increased and decreased variables between the compared groups. For animal intervention studies, the extracted data included general study details, model information, challenge information, intervention details, data acquisition techniques, measured analytical parameters and significant results.

3. Results

3.1 Search strategy

Our search yielded 733, 479, 512, 897 articles in respectively Scopus, PubMed, MEDLINE and Web of Science. Forty-nine studies were eligible for inclusion. **Figure 1** shows the PRISMA[13] flow diagram. Of the 49 papers, 28 were excluded after careful consideration by two authors or three in case of a disagreement or a doubt (**Table S2**).

3.2 Study findings

3.2.1 Human studies

CMA diagnosis criteria and measured parameters in human studies are summarized in **Table S3**.

3.2.1.1 Case and case-control studies

Human studies include one case and nine case-control studies (**Table 1**), among which four examined both the microbiome and metabolome,[14–17] five the microbiome,[18–22] and one the metabolome.[23] For all case-control studies, healthy controls (HC) were used except for one study[23] that considered atopic eczema/dermatitis syndrome infants as controls.

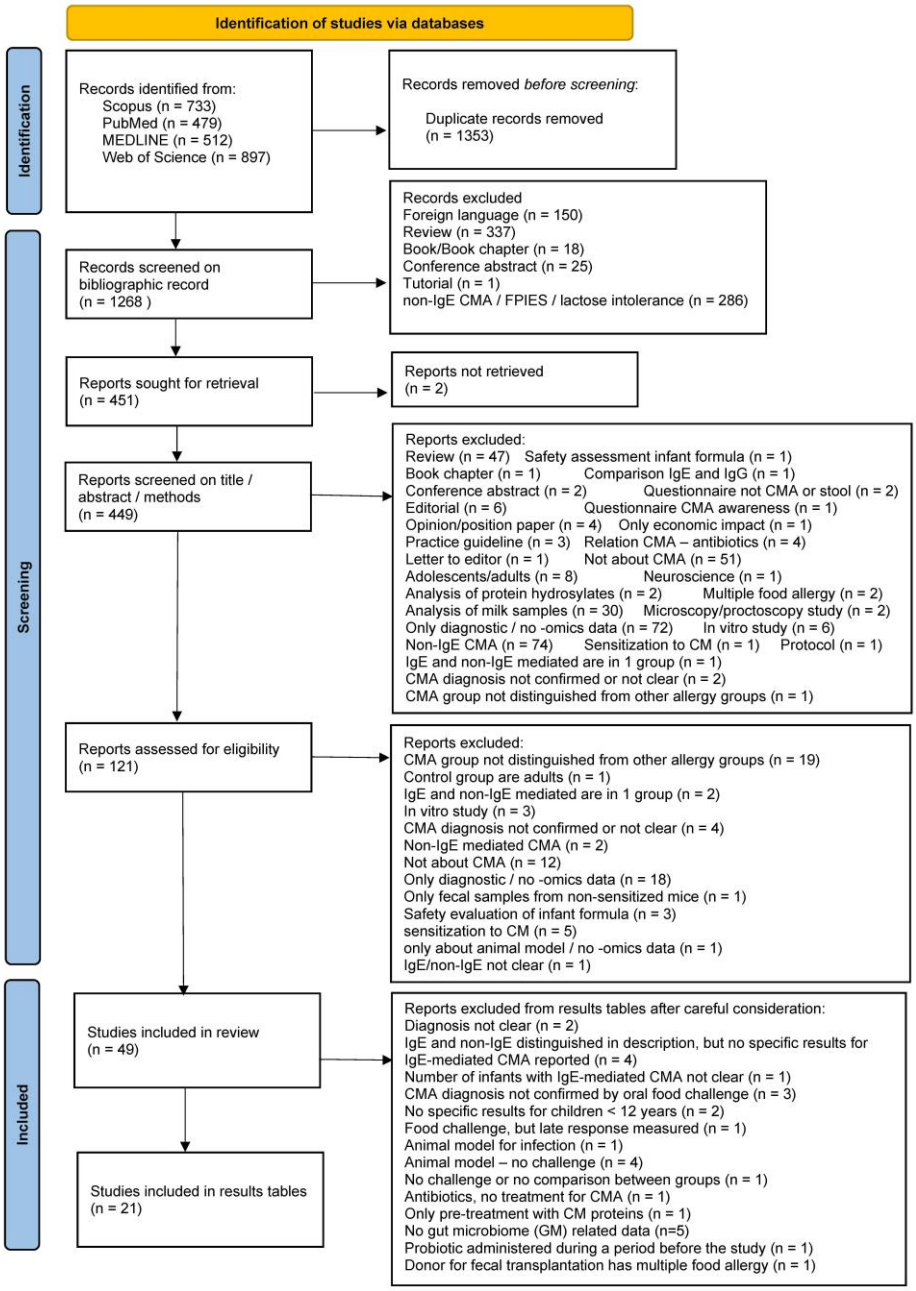

Figure 1. PRISMA flow chart for this systematic review.

GM modifications

The GM-related studies include four case-control reports,[15,19,17,20] four case-control findings in intervention studies,[14,16,18,21] and one case study.[22] Techniques applied for GM profile identification included bacteria culture[18] and 16S rRNA gene-based approaches (DGGE,[19] FISH[14,15] and gene sequencing[16,17,21,20,22]). Two studies applied specific probes to target certain bacteria groups,[14,15] and six used universal probes or primers to target the V3 region,[19] V4 region[16,22] or both.[17,20,21]

Six studies compared α- and β-diversity between CMA-group and HC, three of them noted increased[16,19] or decreased[20] Shannon α-diversity difference in the CMA-groups, and one reported β-diversity (unweighted UniFrac) difference between CMA-group and HC.[21] A single study reported a higher total bacteria count in the CMA-group.[18]

Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, and Verrucomicrobia were the primary reported GM phyla. Elevated abundances of the Firmicutes phylum were consistently observed in the CMA-groups.[14–19,21] These included: total Firmicutes;[17,21] the class Clostridia;[17] the families *Lachnospiraceae*[16] and *Ruminococcaceae*[16,17]; the genera *Clostridium*,[14,19] *Faecalibacterium*,[16] *Lactobacillus*,[18] *Ruminococcus*[16] and *Subdoligranulum*[19] and the species *Clostridium coccoides*[15] and *Clostridium celerecrescens*.[19] Conversely, certain Firmicutes phylum, including the genus *Granulicatella*[21] *and the families Streptococcaceae,*[16] *Enterococcaceae,*[16] and *Acidaminococcaceae,*[20] decreased in the CMA-groups. Additionally, enriched bacteria of the Firmicutes phylum, including the class Clostridia, were also observed in the infants who outgrew CMA.[22]

Bacteroidetes phylum members also showed varying changes in the CMA-groups.[14,17,19–21] These included increased levels of the *Flavobacteriaceae* family,[17] the *Bacteroides*,[14,19] and *Prevotella*[21] genera, along with reduced abundance of the *Prevotellaceae* family[20] and the *Parabacteroides* genus.[21] Furthermore, several bacteria from the Proteobacteria phylum, including the *Haemophilus*, *Actinobacillus* and *Klebsiella* genera,[21] and the *Escherichia coli* species,[19] increased in the CMA-groups. In contrast, total Proteobacteria,[17] the *Enterobacteriaceae* family,[16,18] and the *Escherichia* genus[16] decreased. In the Actinobacteria phylum, one study reported increased *Atopobium* cluster (genus) levels,[15] while *Bifidobacteriaceae* family members, including *Bifidobacterium* spp., consistently exhibited decreased abundance in the CMA-groups.[14,16,18,19] Additionally, the Verrucomicrobia phylum dropped in the CMA-group.[21]

Two studies reported certain bacteria only in the CMA group or the HC. The Clostridium *celerecrescens* species,[19] and the *Burkholderiaceae*, *Nannocystaceae*, *Shewanellaceae*, *Thermomonosporaceae* and *Flavobacteriaceae* families were reported only in the CMA group.[17] In contrast, the *Bifidobacterium bifidum* species[19] and the *Methylophilaceae* and *Dietziaceae* families were exclusively detected in the HC.[17]

Table1. Human case and case-control studies in infants/children. Abbreviations: see Table S1

Age years (y); months (m)	**Analytical techniques**	**Type of analytical data**	**Sample size** (CMA/control)	**Results: modifications in case versus control (case-control study), modifications in allergic versus tolerant (case study)**		**Reference**
				Increase	**Decrease**	
1-12 m	Bacterial culture (CFU)	Microbiome	46/46	**Baseline:** Total bacteria count, Anaerobic bacteria **After 6 months:** Anaerobes count, Lactobacilli count and proportion	**Baseline:** Yeast count **After 6 months:** Bifidobacteria count and proportion, Enterobacteria proportion, Yeast proportion	Thompson-Chagoyan *et al.*[18]
0.55 ± 0.20 y	GC-MS	Metabolomics	16/16	beta-hydroxybutyrate, adipate, isocitrate, homovanillate, suberate, tartarate, 3-indoleacetate, 5-hydroxyindoleacetate	Not reported	†Salmi *et al.*[23]
2-12 m	FISH-FC (16S rRNA gene specific probes); GC-FID	Microbiome, Metabolomics	46/46	*Clostridium coccoides* group, *Atopobium* cluster, butyrate, BCSFA	Not reported	Thompson-Chagoyan *et al.*[15]
6.5-10.4 m	FISH (16S rRNA gene specific probes); GC-MS; NMR	Microbiome, Metabolomics	18/18	*Bacteroides, Clostridium*, Total esters, ketones, alcohols, aldehydes; uridine, histidine, tyrosine, TMAO, arginine/histidine	Bifidobacteria, Total SCFAs (major difference: acetate and butyrate), pyruvate, lactic acid, threonine, proline	Francavilla *et al.*[14]
5-8 y	PCR-DGGE (V3 regions + 16S rRNA gene- specific primers)	Microbiome	12/12	GM α-diversity (Shannon diversity), *C.coccoides* diversity (Shannon diversity), *Bacteroides, Clostridium, Escherichia coli'* only detected in CMA group: *C. celerecrescens*	*Bifidobacterium* (*B.*) diversity (Shannon diversity), *B. adolescent*, *B. longum*, *B. catenulatum*, *and B. breve* Only detected in control group: *B. bifidum*	Guo *et al.*[19]

Table1. Continued

Age years (y); months (m)	**Analytical techniques**	**Type of analytical data**	**Sample size** (CMA/control)	**Results: modifications in case versus control (case-control study), modifications in allergic versus tolerant (case study)**		**Reference**
				Increase	**Decrease**	
1-12 m	qPCR-16S rRNA (V4 region), GC-FID	Microbiome	19/20	GM α-diversity (Shannon diversity), Gut microbiota evenness (Pielou's evenness), *Ruminococcaceae, Lachnospiraceae*, *Ruminococcus, Faecalibacterium*	*Bifidobacteriaceae, Streptococcaceae, Enterobacteriaceae, Enterococcaceae, Bifidobacterium, Escherichia*	Canani *et al.*[16]
5-8 y	PCR-16s rRNA (V3-V4 regions), HPLC-UV	Microbiome, Metabolomics	6/8	Firmicutes, Clostridia, *Ruminococcaceae, Subdoligranulum* only detected in CMA group: *Burkholderiaceae, Nannocystaceae*, *Shewanellaceae*, *Thermomonosporaceae, Flavobacteriaceae*	Proteobacteria only detected in control group: *Methylophilaceae, Dietziaceae,* Total SCFAs	Dong *et al.*[17]
10-15 m	PCR-16S-rRNA (V3-V4 regions), qRT-PCR	Microbiome	14/14	Firmicutes, *Haemophilus, Actinobacillus, Prevotella, Klebsiella*	Verrucomicrobia, *Parabacteroides, Granulicatella*	Mennini *et al.*[21]
4-6 m	16S-rRNA (V3-V4 regions)	Microbiome	16/34	Not reported	GM α-diversity (Shannon diversity), *Acidaminococcaceae, Prevotellaceae*	Mera-Berriatua *et al.*[20]
3-16 m	16S-rRNA (V4 region)	Microbiome	226/- (3-6m: 29/-)	Fecal microbiome at 3-6 month: *Bacteroidetes*, *Enterobacter* Metagenome functional enrichment of fatty acid metabolism.	Fecal microbiome at 3-6 month: Clostridia, Firmicutes.	Bunyavanich *et al.*[22]

†AEDS as basic disease for subjects in both case and control group, and the age is calculated by the pooled mean and sd from the age groups provided in the article

Metabolome modifications

Decreased total short chain fatty acid (SCFAs),[14,17] along with increased butyrate and total branched-chain short fatty acids (BCSFAs),[15] were reported in CMA-groups. Besides, lower pyruvate, lactate, threonine and proline, along with higher total esters, ketones, alcohol aldehydes, uridine, histidine, tyrosine, trimethylamine-N-oxide (TMAO) and arginine/histidine,[14] and elevated organic acids were reported in CMA-groups.[23]

Metabolome-microbiome associations

Two studies examined the association between the GM and the metabolome.[15,17] Positive correlations were found between the *Clostridium* genus and butyrate, the *Clostridium coccoides* species and BCSFAs, and the *Bacteroides* genus and propionate.[15] Isocaproate and BCSFAs were negatively related with the *Bifidobacterium* genus.[15] Additionally, lactate was found to be negatively correlated with *Bacteroides* genus[17] *and Clostridium coccoides* species,[15] but positively correlated with *Bifidobacterium* genus.[15]

3.2.1.2 Intervention studies

Eight intervention studies for CMA treatment were included (**Table 2**).[14,16,18,21,23–26] Two examined the GM and metabolome,[14,16] one the GM and immune response,[26] four the GM,[18,21,24,25] and one the metabolome.[23] The interventions varied across studies, including synbiotics,[25] prebiotics,[24] probiotics (species of the genus *Bifidobacterium*,[21,26] *Lactobacillus rhamnostbus* GG (LGG) species[16,23]) and different formula types.[14,18]

GM modifications

The GM profile was identified with bacteria culture,[18] FISH,[25] 16S rRNA gene sequencing with specific primers/probes[14,24,26] or targeting the V4[16] or V3-V4 regions.[21]

Alterations of the phylum Firmicutes in CMA-patients were described in five intervention studies, involving treatment with EHF,[18] lactose-supplemented EHF,[14] LGG,[16] species and strains from the *Bifidobacterium* genus.[21,26] These interventions raised Firmicutes phylum members, including the Turicibacterales order,[48] the *Lactobacillaceae* and *Lachnospiraceae* families[48] and the genera like *Lactobacillus*,[18,48] *Blautia*,[16,21] *Roseburia*,[16] *Coprococcus*,[16] *Anaerofustis*,[16] *Ruminococcus*,[21,26] *Turicibacter,*[26] and *Oscillospira*.[26] Conversely, some Firmicutes phylum members, including the Clostridia class,[14] *Christensenellaceae* family,[48] and genera like *Enterococcus*, *Streptococcus*,[21] *Anaerovibrio*, *Oscillibacter*, *Bilophila*, *Dorea* and *Roseburia*[26] decreased under treatments.

The interventions also affected the Proteobacteria phylum[21] and its members. The Betaproteobacteria class, the Burkholderiales order, the *Alcalligenaceae* family and the *Sutterella* genus increased in the treated group,[26] while some studies reported decreased levels of the Deltaproteobacteria class,[26] the *Enterobacteriaceae* family,[18] and the *Sutterella* genus.[21] In the Bacteroidetes phylum, studies reported the interventions increased levels of

the *Porphyromondaceae* family[26] and the *Prevotella* genus,[21,26] and reduced levels of the *Bacteroides* and *Prevotella* genera.[14] Additionally, the Actinobacteria phylum also underwent changes with interventions.[14,18,21,25,26]

The use of probiotic *Bifidobacterium* strains consistently elevated the *Bifidobacterium* genus.[21,25,26] Increased *Bifidobacterium* were also noticed after lactose-supplemented EHF diet.[14] In contrast, the Actinobacteria phylum[21] and its members, the genera *Bifidobacterium*,[18] *Atopobium,*[21] and *Actinomyces,*[21,26] were decreased by the treatments. The Verrucomicrobia phylum and its *Akkermansia* genus were found increased in the treatment group.[21]

In addition to the taxonomy changes, enhanced α-diversity (chao1, observed species),[26] reduced total bacteria,[24] and a decreased ratio of the *Eubacterium rectale*/*Clostridium coccoides* species[25] were reported after probiotics, pectin-based thickened AAF and synbiotics treatments, respectively.

Metabolome modifications

After the LGG-supplemented hydrolyzed whey formula (HWF) diet, CMA-patients showed increased kynurenate and decreased 3-indoleacetate.[23] Additionally, butyrate increased in LGG-supplemented extensively hydrolyzed casein (EHC) formula treated CMA-patients.[16] Meanwhile, lactose-supplemented EHF raised SCFAs, lactate, threonine, uridine, histidine, tyrosine, methionine, TMAO, phenylalanine, arginine/histidine and gamma–amino–butyrate/lysine, and lowered the total esters, ketones, alcohols, aldehydes and valine/isoleucine in CMA-patients.[14]

Immune response

The single intervention study reporting findings on the immune response showed that *Bifidobacterium bifidum* reduced allergy symptoms, lowered serum IgE and raised IgG_2 levels in CMA-patients.[26] The IgG_2 and IgE were respectively positively and negatively correlated with GM α-diversity (Chao1 index, observed species, community diversity index, Shannon index). The intervention decreased the pro-inflammatory cytokines TNFα, IL-1β and IL-6 and increased the anti-inflammatory cytokine IL-10 as well.[26]

CMA outcome

Four out of eight intervention studies discussed CMA tolerance or allergic symptoms improvement between treatment and control.[16,24–26] Two studies noted significant improvement in allergic symptoms after treatment,[24,26] and one reported five out of 12 infants in the treated group outgrew CMA after six months, compared to none in the control group.[16]

Table 2. Characteristics of studies that compare CMA infants/children before and after intervention (intervention study). Abbreviations: see Table S1

Age years (y); months (m)	**Analytical techniques**	Type of analytical data	**Sample size** (treatment/ control)	**Intervention detail**				**Results: modifications in treatment versus control**		**Reference**
				Duration (months)	**Comparison groups**	**Control diet** (Basic formula (BF))	**Treatment diet** (BF + intervention)	**Increase**	**Decrease**	
0.55 ± 0.20Y	GC-MS	Metabolomics	9/5	1	Treatment vs control	HWF	HWF with LGG	Kynurenate	3-indoleacetate	Salmi *et al.*[23]
2-12 m	Bacteria culture (CFU)	Microbiome	46/46	6	CMA subjects before intervention	-	EHF	*Lactobacilli*	Enterobacteria Bifidobacteria	Thompson-Chagoyan *et al.*[18]
6.5-10.4 m	FISH (16S rRNA-specific probes), GC-MS, NMR	Microbiome, Metabolomics	16/16	2	CMA subjects before intervention	-	EHF with 3.8% lactose	*Bifidobacteria,* LAB, SCFAs, lactate, threonine, uridine, histidine, tyrosine, methionine, TMAO, phenylalanine, arginine/histidine, c–amino butyrate/lysine	*Atopobium*, *Bacteroides/Prevote*, Clostridia and sulfate-reducing bacteria, total esters, ketones, alcohols, aldehydes, valine/isoleucine	Francavilla *et al.*[14]
6.2 ± 4.3m	qPCR (16S rRNA-specific primers and probes)	Microbiome	23/17	3	Treatment vs control	RAAF	TAAF	Not reported	Total bacteria count	Dupont *et al.*[24]
1-12 m	qPCR-16S rRNA (V4 region), GC-FID	Microbiome; Metabolomics	12/7	6	Treatment vs control, CMA subjects before intervention	EHC formula	EHC formula with LGG	**er vs before intervention:** *Blautia, Roseburia, Coprococcus* **Compared to control group:** *Roseburia,Anaerofustis*. Butyrate	Not observed	Canani *et al.*[16]

Table 2. Continued

Age years (y); months (m)	**Analytical techniques**	Type of analytical data	**Sample size** (treatment/ control)	**Intervention detail**				**Results: modifications in treatment versus control**		**Reference**
				Duration (months)	**Comparison groups**	**Control diet** (Basic formula (BF))	**Treatment diet** (BF + intervention)	**Increase**	**Decrease**	
0.5-12 m	ELISA qPCR (16S rRNA-specific primers)	Microbiome, Immune response	123/121	6	Treatment vs control	-	*B. bifidum* TMC3115	After 6 months: IL-10, total IgG_2, GM α-diversity (chao1 index, observed species), Bifidobacteriales, *Bifidobacterium*, *Lactobacillaceae Lactobacillus, Turicibacter, Turicibacterales, Betaproteobacteria, Sutterella, Burkholderiales, Alcalligenaceae, Porphyromondaceae, Parabacteroides,Ruminococcus,Oscillospira, Lachnospira*	After 6 months: TNFα, IL-1, IL-6, IL-10, total IgE, *Anaerovibrio, Christensenelaceae, Oscillibacter, Bilophila, Dorea Roseburia*) Desulfovibrionales, Deltaproteobacteria, *Proteobacteria, Actinomyces)*	Jing *et al.*[26]
10-15 m	PCR-16S rRNA (V3-V4 regions), qRT-PCR	Microbiome	14/14	1	CMA subjects before intervention	-	probiotic mix: *B. breve* M-16V, *B. longum subsp. longum* BB536, *B. longum subsp. Infantis* M-63	Verrucomicrobia, Proteobacteria, *Akkermansia, Prevotella, Ruminococcus, Blautia, B. longum subspecies infantis*	Actinobacteria, *Actinomyces, Enterococcus, Streptococcus, Sutterella*	Mennini *et al.*[21]
<13 m	FISH (16S rRNA s-specific probes	Microbiome	80/89	12	Treatment VS control	AAF	Synbiotics: oligofructose, inulin, *B. breve* M-16V	After 6 and 12 month: bifidobacteria	After 6 month: ER/CC	Chatchatee *et al.*[25]

Table 3. CMA intervention studies with animal models. Abbreviations: see Table S1

Groups		Platforms	Results†		Reference
Case/Intervention	**Control**		**Microbiome/Metabolome**	**CMA outcome & Immune response**	
G1: *L. rhamnosus* **G2:** *B. longum subsp. Infantis* **G3:** *L. salivarius* **G4:** *B. bifidum* **G5:** *L. gasseri* **G6:** *B. animalis subsp. lactis*	**AC**: PBS	**Immunoglobulins** ELISA **Cytokines** IA (ex-BLG) **mRNA expression** q-PCR **Microbiome** qPCR (16s rRNA-specific primers); bacteria culture	**Microbiome** Total bacteria ↓ G1, G2, G3, G4, G5 *Clostridium* cluster IVa ↑ G1, G6 *Staphylococci* abundance ↑ G1 *C. leptum*↑ G1, G6 *Prevotella*↑ G6 *C. leptum* ↓ G2, G3, G4, G5 *Prevotella*↓ G2, G3, G4, Lactobacillus ↓ G2, G3, G4, G5 *Clostridium* cluster I/II↓ G2, G3, G5 Clostridium cluster XI ↓ G2, G3, G4 *C. coccoides*↓ G2, G3, G4, G5 *Enterococcus* ↓ G2, G3, G4, G5 *Enterococcus* ↓ G1	**Allergy markers** mMCP-1 ↓ G1, G2, G3 **Immunoglobulins** BLG-sIgE ↓ G1, G2, G3 BLG-sIgG_1/sIgG_{2a}↑ G1, G2, G3, G4, G6 **Cytokines** IL-4 ↓ G1, G2, G3, G4 (spleen, MLN) IFN-γ ↑ G1, G2, G6 (spleen) IFN-γ ↓ G3, G4 (spleen) IFN-γ ↑ G6 (MLN) IL-10 ↑ G1, G2, G6 (spleen) IL-10 ↑ G1, G5, G6 (MLN) **mRNA expression** IL-4 ↓ G2 IL-10, GATA3, RORγT ↓ G2, G3 FOXP3 ↑ G2, G3 IL-17a ↑ G1, G2, G3	Neau *et al.*[31]
G1 pWH **G2/G3:** pWH + short(G2)/long (G3) scGOS/lcFOS (9:1) **G4/G5:** pWH + short (G4)/long (G5) scGOS/lcFOS (9:1) + pAOS	**TC:** W **AC:** PBS	**Microbiota** PCR (16S rRNA V3-V4 regions) **Immunoglobulins** ELISA	**Microbiome** *Prevotella* ↑ G3, G4, G5 vs G1 *Lactobacillus* ↓ G5 vs G1	**Allergy markers** mMCP-1 ↓ G1, G5 vs AC TSLP ↓ G1 vs AC AASR ↓ TC, G1, G2, G4, G5 vs AC SAS & body-T ↓ TC, G2 vs AC	Kleinjans *et al.*[32]

Table 3. Continued

Groups		Platforms	Results†		Reference
Case/Intervention	Control		Microbiome/Metabolome	CMA outcome & Immune response	
G1: *L. rhamnosus* **G2:** *B. longum subsp. Infantis* **G3:** *L. salivarius*	**AC**: PBS	**Microbiome** PCR -16S rRNA (V3-V4 regions) **Metabolome** GC-FID, UPLC-MS/MS **Immunoglobulins** ELISA **Cytokines** IA (ex-BLG) **mRNA expression** qPCR	**Metabolome** Kynurenine, N-acetylkunurenine ↓ G1, G2, G3 **Microbiome** Richness (OTU number) ↑ G1 Beta diversity ↑ G1, G2, G3 *Prevotellaceae* ↑ G1, G2, G3 *Marinifilaceae* ↑ G1, G2 *Ruminococcaceae* ↑ G1 *Helicobacteraceae* ↓ G1 *Ruminococcaceae* ↓ G2 *Lachnospiraceae* ↓ G1, G2, G3 *Deferribacteraceae* ↓ G1, G2 *Clostridiaceae* ↓ G1 *Peptococcaceae* ↓ G1, G3 *Burkholderiaceae* ↓ G1 *Anaeroplasmataceae*↓ G2	**Cytokines** GM-CSF, IL-2, IFN-γ, IL-4 ↓ G1, G2, G3 IL12p70 and IL10 ↓ G1 IL-5 ↓ G2, G3 IL17A ↓ G1, G3 **mRNA expression** FOXP3, IL-10 ↑ for G1 and G3 TGFβ ↑ G1, G2, G3	Esber *et al.*[28]
G1: mix of W peptides (PepMix) **G2:** scFOS and lcFOS (9:1) + *B. breve* M-16V (FF/Bb) **G3:** PepMix + FF/Bb	**TC:** W **AC:** PBS	**Immunoglobulins** ELISA **Metabolites** GC-FID **Lymphocytes** FC **Cytokines** IA (ex-W)	**Metabolites** acetate, butyrate ↑ G2 butyrate ↑ G2 vs G3, TC vs AC	**Allergy markers** AASR ↓ G3, TC vs AC SAS ↓ TC vs AC **Lymphocytes** (SI-LP) T_h1/T_h2 ↑ G3, TC T_{reg}, T_h17 ↑ AC vs TC **Cytokines** (spleen) IFN-γ, IL-17A, IL-13, IL-5, IL-10 ↓ G3 vs G1 & TC vs AC IL-10 ↑ G3	Kostadinova *et al.*[33]

Table 3. Continued.

Groups		Platforms	Results†		Reference
Case/Intervention	**Control**		**Microbiome/Metabolome**	**CMA outcome & Immune response**	
G1: mix of W peptides (**PepMix)** **G2:** scFOS and lcFOS (9:1) + *B. breve* M-16V (**FF/Bb)** **G3:** PepMix + FF/Bb	**TC:** W **AC:** PBS	**Metabolites** GC-FID **Lymphocytes** FC **mRNA expression** qPCR **Immunohistochemistry**	<u>Part 1: Post-oral tolerance</u> **Metabolites** butyrate ↑ G3 cs G1 propionate ↑TC, G2, G3 vs AC Positive correlation: propionate and FOXP3+ (colon)	**Allergy markers** AASR ↓ G3, TC vs AC AASR ↑ G1, G2 vs G3 SAS ↓ TC vs AC <u>Part 1: Post-oral tolerance</u> **Lymphocytes** FOXP3+/GATA3+, T_{regs}/T_{effs} ↑ G3 vs AC, G3 vs G2, G3 vs G1 (MLN) T_{regs} ↓ G3 vs AC, G3 vs G2, TC vs AC (spleen) CD25+ ↓ G3 vs G2 **DC** (SI-LP) $CD8\alpha^{-}CD11b^{+}/CD8\alpha^{+}CD11b^{-}$, $CD11b^{+}CD103^{-}$ ↑ G3 $CD8\alpha^{+}CD11b^{-}$ ↓ G1 **mRNA expression** FOXP3/GATA3 ↑ G3 (PP) FOXP3/RORγT ↑ G3 vs AC, G3 vs G2, G3 vs G1 (PP) TGF-β ↑ G3 vs G2 (proximal SI) TGF-β ↓ G1 (colon) IL-22 ↑ G3 vs AC, G3 vs G1 (PP) IL-22 ↑ for G3 vs G1 (middle SI) IL-22 ↑ G2 vs AC & G2 vs G3 (colon) Galectin 9 ↓ TC Tbet/GATA3 ↓ G1 vs AC, G1 vs G3 (colon) <u>Part 2: Post-challenge</u> **Lymphocytes** (SI-LP) CD25+ Tcells ↑ G3 CD25+ Tcells ↑ G3 vs G2 T_{reg} ↑ G1 **mRNA expression** (PP) Tbet/GATA3 ↑ G3 IFN-γ/IL-13 ↑ G3 vs AC & G3 vs G2	Kostadinova *et al.*[34]

Table 3. Continued

Groups		Platforms	Results†		Reference
Case/Intervention	**Control**		**Microbiome/Metabolome**	**CMA outcome & Immune response**	
G1: M-C57BL/6J **G2**: M BALB/cJ **G3**: F-C57BL/6J **G4**: F-BALB/cJ	**S:** sham control (sex and strain matched to G1, G2, G3,G4 separately)	**Immunoglobulins** ELISA **Cytokines, chemokines, and acute phase proteins:** IA **Microbiota** 16S rRNA sequencing (8 regions)	**Microbiome** α-diversity ↑ G4 (Simpson and Shannon indices) α-diversity↓ G1(Simpson index) Bacteroidetes↑G3 Patescibacteria↑G3 Verrucomicrobia↓ G1 Proteobacteria↓ G1 Actinobacteria↓G3	**Allergy markers** Body-T ↓ G2 vs S, G4 vs S, G4 vs G3 SAS ↑ G2 vs S, G4 vs S, G4 vs G3 **Immunoglobulins** sIgE ↑ G2 vs S, G1 vs S, G4 vs S, G4 vs G3 $sIgG_1$ ↑ G2 vs S, G2 vs G1, G4 vs S, G4 vs G3 $sIgG_{2a}$ ↑ G2 vs S, G2 vs G1, G4 vs S, G4 vs G3 **Cytokines, chemokines, and acute phase proteins:** G1 vs S: ↑ in CCL1, CSF1, IL-13, CCL17, IL-21, FGF2, CCL12, IL-10, CCL9 G2 vs S: ↓ IL-1β, IL-13, CSF2, TNFRSF1A G4 vs S: ↑ IL-15, TNFRSF1B, ICAM-1	Smith *et al.*[30]
G1: CMA	**S**: Sham control	**Microbiome** PCR-16S rRNA (V3-V4 regions) **Immunoglobulins** ELISA **Cytokines** ELISA **mRNA expression** qPCR **Metabolome** GC-FID, RP, HILIC-MS/MS	**Microbiome** *Barnesiella*↑ *Clostridium*_XIVa↑ *Lactobacillus*↓ *Parvibacter*↓ Only observed in sham mice: *Bosea*	**Allergy markers** Body-T ↓ G1 vs S SAS ↑ G1 vs S Histamine ↑ G1 vs S mMCP-1 ↑ G1 vs S **Immunoglobulins** whey-sIgE, $sIgG_1$, $sIgG_{2a}$ ↑ G1 vs S **Cytokines** IL-6, IL-10 ↑ G1 vs S **mRNA expression** IL-8, IL-33, mTOR mRNA ↑ G1 vs S	Cao *et al.*[27]

Table 3. Continued

Groups		Platforms	Results†		Reference
Case/Intervention	**Control**		**Microbiome/Metabolome**	**CMA outcome & Immune response**	
G1: CMA-FT **G2:** *Anaerostipes caccae*-FT	**B-HC**: breast-fed HC-FT **F-HC:** formula-fed HC-FT	**Microbiome** PCR -16S rRNA (V4 region) **Immunoglobulins** ELISA **Transcriptome** RNA-seq, qPCR	**After fecal colonization before sensitization:** **Microbiome** G1 vs F-HC: *Enterococcus*↑ *Barnesiellaceae*↑ *Ruminococcus*↑*Ruminococcaceae*↑ *Coprobacillus* ↑ *Clostridiaceae* ↑ *Clostridiales* ↑ *Blautia* ↑ *Parabacteroides*↑ *Lachnospiraceae*↓ *Erysipelotrichaceae*↓ *Enterobacteriaceae*↓ *Streptococcus*↓*Enterobacteriaceae*↓ *Salmonella*↓*Anaerostipes caccae* ↓ **Transcriptome** **G1 vs F-HC:** (Mroh7, Cntn1, Slc9b2, Letm2, Acot12, Abcc2, Cyp3a59, Cyp2b10, Lrrn1, Me1, Akr1c19, Gstm1, Ces1f) ↑ (Tgfbr3, Acta1, Ror2, Slc22a13, Fbp1, Apcdd1) ↓	**Allergy markers** mMCP-1 ↑ G1, G4 vs HC mMCP-1 ↓ G2 vs G1 **Immunoglobulins** BLG-specific IgE, IgG1↑ G1 vs HC **Cytokines** IL-13, IL-4 ↑ G1 vs G2 **Transcriptome** Tgfbr3 ↓ G1 vs G2, G1 vs HC Ror2 ↓ G1, G2 vs HC Ror2, Tgfbr3 positively correlated to *Lachnospiraceae*	Feehley *et al.*[29]

3.2.2 Animal studies

The animal studies include two studies on the GM, metabolome and immune response,[27,28] four on the GM and immune response[29–32] and two on the metabolome and immune response[33,34] (**Table 3**). All animal models were on mice, details are provided in **Tables S4-S6**.

GM modifications

Three interventions,[28,31,32] two case-controls[27,30] and one FT[29] study reported GM modifications. Bacteria were identified using 16S rRNA gene-targeted primers, which targeted group/species-specific bacteria[31] or certain hypervariable regions (V3-V4,[27,28,32] V4[29] and eight other regions[30]).

In two studies comparing GM changes between CMA- and sham mice,[27,30] one observed increased Simpson α-diversity in CMA-male-C57BL/6J mice but decreased Simpson and Shannon α-diversity in CMA-female-BALB/cJ mice.[30] Regardless of the strain and gender, the β-diversity (Bray-Curtis) was significant different between the two groups.[30] Apart from the gender and strain-specific α-diversity difference, CMA-mice showed enrichment in the phyla Bacteroidetes and Patescibacteria (female-C57BL/6J) but reduction in the phyla Verrucomicrobia, Proteobacteria (male-C57BL/6J) and Actinobacteria (female-C57BL/6J).[30] Compared to mice colonized with feces from healthy children (healthy-colonized mice), a FT study reported that mice with feces from CMA children (CMA-colonized mice) had higher abundances of the Clostridiales order and the *Clostridiaceae, Ruminococcaaceae* and *Barnesiellaceae* families*,* along with lower levels of the *Lachnospiraceae, Erysipelotrichaceae* and *Enterobacteriaceae* families*.*[29] At the genus level, the CMA-mice exhibited higher *Barnesiella* and *Clostridium*_XIVa,[27] and CMA-colonized mice had enhanced *Enterococcus, Ruminococcus, Coprobacillus, Blautia* and *Parabacteroides.*[29] In contrast, the *Lactobacillus*, *Parvibacter*,[27] *Streptococcus,* and *Salmonella*[29] genera, as well as *Anaerostipes caccae* species[29] decreased in CMA and CMA-colonized mice. Additionally, the *Bosea* genus was absent in CMA-mice.[27]

Species and strains of the *Lactobacillus* and *Bifidobacterium* genera were used as probiotic in CMA-mouse models.[28,31] One study reported that five out of six probiotic strains reduced the total bacteria.[31] Another found significant differences in GM β-diversity (Bray-Curtis, UniFrac) between control and treated groups but only the *Lactobacillus rhamnosus* species increased GM richness.[28] At the family level, it was reported that *Prevotellaceae* and *Marinifilaceae* increased, whereas *Helicobacteraceae*, *Lachnospiraceae*, *Deferribacteraceae*, *Clostridiaceae*, *Peptococcaceae* and *Burkholderiaceae* decreased after taking at least one probiotic.[28] Interestingly, the *Ruminococcaceae* family increased with *Lactobacillus rhamnosus* treatment but decreased with *Bifidobacterium longum subsp. infantis* treatment.[28] Furthermore, one study found that probiotic treatments with *Lactobacillus rhamnosus* and *Bifidobacterium animalis subspecies lactis* increased the *Clostridium* cluster IVa genus and the *Clostridium leptum* species.[31] Conversely, more than

three probiotic strains decreased the *Lactobacillus*, *Clostridium* cluster I/II, *Clostridium* cluster XI, *Enterococcus* and *Prevotella* genera, as well as the *Clostridium Coccoides* and *Clostridium Leptum* species.[31] Additionally, it was reported that prebiotic administration with partially hydrolyzed whey reduced the *Lactobacillus* genus and increased the *Prevotella* genus.[32]

Metabolome modifications

Two studies examined fecal SCFAs in CMA-mice with and without synbiotic intervention.[33,34] They reported enhanced acetate,[33] butyrate[33] and propionate[34] with synbiotic diet. However, one study only observed reduced kynurenine and N-acetylkunurenine in probiotic-treated mice.[28] Additionally, a FT study compared ileal transcription signatures between CMA and healthy-colonized mice.[29] They found upregulated metabolism of monocarboxylic acid, arachidonic acid, linoleic acid and pyruvate in CMA-colonized mice, while increased carbohydrate metabolic process in healthy-colonized mice.[29]

CMA outcome and immune response

Among all animal studies only Feehley *et al.*[29] and Kostadinova *et al.*[34] correlated the immune response to the GM. Feehley *et al.*[29] reported that growth factor TGF-β receptor and ROR2 genes in CMA-colonized mice was positively correlated with *Lachnospiraceae* family.[29] Meanwhile, Kostadinova *et al.*[34] showed that propionate was positively correlated with FOXP3+ cell frequency in the colon.[34]

All intervention studies reported immune response data which relates to the treatment outcome.[28,31–34] Unlike post-sensitization,[28] pre-sensitization[31] intake of *Lactobacillus salivarius*, *Lactobacillus rhamnosus* and *Bifidobacterium longum subspecies infantis* successfully lowered the mast cells degranulation marker mucosal mast cell protease-1 (mMCP-1)[35] and BLG-specific IgE.[31] All strains lowered the IL-4 secretion and the BLG-specific $sIgG_1$-to-$sIgG_{2a}$ ratio[31] which indicates the overall T_h2-to-T_h1 response.[36] The rest of the responses were strain-dependent. *Lactobacillus rhamnosus* and *Bifidobacterium longum subspecies infantis* increased T_h1 IFN-γ and T_{reg} IL-10 secretion in stimulated splenocytes, whereas *Lactobacillus salivarius* declined IFN-γ secretion.[31] Post-challenge administration of those probiotic strains predominantly induced regulatory response.[28] All strains significantly increased TGF-β expression, while *Lactobacillus rhamnosus* and *Lactobacillus salivarius* interventions also increased FOXP3 and IL-10 expression. The post-sensitization intake resulted in overall cytokine suppression as well. The reduction in granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN-γ, IL-2, and IL-4 was common among the strains, while IL12p70, IL-10, IL-5 and IL-17A was strain-dependent.[28]

Kostadinova *et al.*[33,34] reported that synbiotic intake alone did not alleviate the acute allergic skin response but its combination with T cell-epitope-containing BLG peptides (PepMix) did.[33,34] Notably, the combined diet reestablished the lost T_h1/T_h2 balance as evidenced by the lymphocyte distribution in the small intestine lamina propria[33] as well as the increased

transcription factor (Tbet/GATA3) and cytokine (IFN-γ/IL-13) gene expression in the Peyer's Patches (PP).[34] Right after the intervention the immune response was predominantly regulatory. It was characterized by an increase in the mRNA expression of FOXP3 over the GATA3 and RORγT in the PP, as well as higher FOXP3+ over GATA3+ and T_{reg} over T_h cell frequencies in mesenteric lymph node.[34] Synbiotic addition had a site-dependent effect on IL-22 mRNA expression and also silenced the whey-stimulated splenocyte secretion of cytokines (IL-10, IL-5, IL-13, IL-17A, IFN-γ) which were induced by the PepMix intake.[33] Kleinjans *et al* showed that the effect of prebiotics on allergic symptoms varied with the composition and treatment duration.[32]

4. Discussion and conclusion

In general, no clear conclusion can be drawn about the GM diversity modification in CMA children, because of limited data on β-diversity[21,30] and discordant results regarding α-diversity in both human[16,19,20] and animal[30] studies.

Taxonomic findings showed that the *Bifidobacteriaceae* family, including *Bifidobacterium* spp., were consistently reported lower in CMA-children.[14,16,18,19] This result aligns with the consensus on the protective function of *Bifidobacterium* spp. in early life.[37,38] Another noteworthy observation concerning GM in CMA-children is the consistent increase of the Firmicutes phylum,[14–19,21] primarily associated with the Clostridia class. Conversely, decreased levels of bacteria of the Lactobacillales order were observed.[16,21] The trends of Firmicutes alterations align with the findings of an animal study which reported higher *Clostridium* cluster XIVa and lower *Lactobacillus* genus in CMA-mice.[27] However, CMA and healthy-colonized mice were both characterized with bacteria from the Clostridia class, with *Anaerostipes caccae*, a clostridial species, showing protective effects against CMA.[29] Additionally, infants who resolved CMA were reported to have enriched Clostridia class at 3-6 months.[22] Discordant results have also been reported regarding the protective or detrimental effect of the Clostridia class in food allergy.[39,40] Therefore, despite the conflicting findings of the Clostridia class in this review, we lean towards suggesting that GM with enriched Clostridia class, reduced Lactobacillales order and reduced *Bifidobacterium* genus is associated with CMA in early-life.

Various intervention approaches, including probiotics, prebiotics and synbiotics, were applied to restore the balance of GM and the metabolome in CMA-children. Elevated *Bifidobacterium* genus was consistently observed post-treatment with *Bifidobacterium* strains as probiotics[21,25,26] or after lactose-supplemented EHF treatment.[14] However, the impact on the Lactobacillales order in both CMA-children and CMA-mice was less clear. Increased levels of the *Lactobacillaceae* family were reported with *Bifidobacterium*-specific probiotics[26] and EHF in CMA-children,[18] while decreased *Enterococcus* and *Streptococcus* genera were noted in *Bifidobacterium*-treated CMA-children.[21] Additionally, decreased levels of *Lactobacillus* genus were reported in CMA-mice treated with *Bifidobacterium* and *Lactobacillus*-specific probiotics.[31,32] Similarly, the effect on the Clostridia class varied.

Higher levels of its members were reported in CMA-children and mice treated with probiotics.[16,21,26,28,31] Meanwhile, reduced Clostridia class members also noted in CMA-children treated with lactose-supplemented EHF or probiotics,[14,26] and in CMA-mice treated with probiotics.[28,31] Therefore, it is clear that the enhancement of *Bifidobacterium* after *Bifidobacterium*-specific treatment was commonly reported, however the treatment effect on other bacteria remain inconclusive. Despite the uncertainty of most GM profile modifications, there are studies which reported improved allergic symptoms or a high resolution rate in CMA-children treated with probiotics or prebiotics.[16,24,26]

In addition to GM modifications, CMA-children were reported to have decreased total SCFAs[14,16] and altered amino acids and nucleotides levels.[14,23] These findings are consistent with a recent review on the metabolic changes in children with IgE-mediated food allergies,[41] and these metabolome changes appear to be restored with interventions. Increased SCFAs and balanced amino acids were reported after treatment with LGG or lactose-supplemented EHF.[14,23] Enhanced levels of acetate,[33] butyrate,[33,34] and propionate[34] were also reported in synbiotic-treated CMA-mice.

This systematic review provides an overview of the modifications of the GM, metabolome, and immune response in IgE-mediated CMA-children and CMA animal models. Comparing microbiome data between studies is challenging due to methodological variations, diverse intervention approaches, and the reporting of different taxonomic levels. Consequently, only general conclusions can be drawn based on family or higher taxonomic levels. Meanwhile, insights into metabolomics are restricted by limited scope of studied metabolites. Thus, future work should examine broader range of metabolites known to be crucial in the crosstalk between the GM and host's immune system[41,42] and use untargeted metabolomics as hypothesis-generating strategy. Only a single human study reported microbiome and immune response data and their relationship.[26] Similarly, only a single animal study correlated transcriptomics and GM data,[29] including genes related to the immune response. Therefore, there is a need for both human and animal studies on the correlation of the GM to the immune response. Future animal studies can build on the general treatment outcome findings in the review, namely overall cytokine silencing,[28,33] restoration of the T_h2/T_h1 balance,[31,33,34] and induction of regulatory response.[28,31,34] Moreover, future work can focus on parameters already connected to allergic tolerance acquisition in human, such as induction of T_{reg} response, the production of TGF-β, IgG_4, IgA.[43] No proteomics studies met our inclusion criteria, but a study on the fecal microbiome and metaproteome relationships in CMA-children has been published after our inclusion date.[44] Overall, discussions on multi-omics connections are rare in the reviewed studies, and none of the studies reported shotgun meta-genomics, meta-transcriptomics, or meta-proteomics for microbiome function information. Therefore, there is a clear need for more comprehensive multi-omics studies to gain a better mechanistic understanding of CMA in early life. These efforts would eventually lead to the development of better and effective treatment and preventive strategies.

Author contributions

MVS: Formal Analysis, Investigation, Writing – Original Draft Preparation; **PZ**: Formal Analysis, Investigation, Writing – Original Draft Preparation; **ACH**: Supervision, Writing – Review & Editing; **RGvdM**: Investigation, Writing – Review & Editing; **CB**: Conceptualization, Funding Acquisition, Investigation, Supervision, Writing – Review & Editing; **DMH**: Formal Analysis, Investigation, Supervision, Writing – Review & Editing

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Conflict of interest statement

The authors declare that they have no known conflicts of interest.

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Supplementary Materials

Table S1. Abbreviations

Abbreviation	Full name/defination
16S rRNA	16S ribosomal ribonucleic acid
AAF	amino acid formula
AASR	acute allergic skin response (ear swelling)
AC	allergic control
AEDS	atopic eczema/dermatitis syndrome
BCSFAs	branched-chain short fatty acids
BLG	beta-lactoglobulin
body-T	body temperature
CFU	colony-forming unit
CM	cow's milk
CMA	cow's milk allergy
DC	dendritic cells
DGGE	denaturing gradient gel electrophoresis
DBPCFC	Double-blind, placebo-controlled food challenge
EHF	extensively hydrolyzed formula
ELISA	Enzyme-linked immunosorbent assay
ER/CC	Eubacterium rectale/Clostridium coccoides
ex-BLG	ex-vivo res-stimulation with BLG
ex-W	ex-vivo res-stimulation with whey
F	female
FC	flow cytometry
FF/Bb	short and long chain FOS and B. breve M-16V
FISH	fluorescent in situ hybridization
FOS	fructo-oligosaccharides
FOXP3	forkhead box P3
FT	fecal transplantation
G	group
GATA3	GATA Binding Protein 3
GC-FID	GC-flame ionization detector
GC-MS	gas-chromatography-mass spectrometry
GM	gut microbiome
GM-CSF	Granulocyte macrophage colony-stimulating factor
GOS	galacto-oligosaccharides
HC	healthy controls
HILIC	Hydrophilic interaction chromatography
HPLC-UV	high-performance liquid chromatography-ultraviolet detector
HWF	hydrolysed whey formula
IA	immunoassay (other than ELISA)
i.p.	intraperitoneal
i.g.	intragastric
i.d.	intradermally
IEC	Intestinal epithelial cell(s)
IFN-γ	Interferon-gamma

Abbreviation	Full name/defination
Ig(s)	immunoglobin(s)
IL	interleukin
LAB	lactic acid bacteria
lcFOS	long chain fructo-oligosaccharides
LGG	Lactobacillus rhamnosus GG
LP	lamina propria
M	male
MLN	mesenteric lymph node
mMCP-1	mucosal mast cell protease-1
MS	mass spectrometry
MS/MS	Tandem mass spectrometry
NMR	nuclear magnetic resonance
OTU	operational taxonomic unit
pAOS	pectin-derived acidic oligosaccharide
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PP	Peyer's Patches
qPCR	quantitative PCR
qRT-PCR	quantitative real-time PCR
RAAF	reference amino acid formula
Ror2	Receptor Tyrosine Kinase Like Orphan Receptor 2
RORγT	retinoid-Related Orphan Receptor gamma t
RP	reverse phase
SAS	systematic anaphylaxis scores
SCFAs	Short-chain fatty acids
scFOS	short chain FOS
scGOS	short chain galacto-oligosaccharides
sd	standard deviation
SI	small intestine
sIg	specific Immunoglobulin
SI-LP	small intestine lamina propria
sp.	single unnamed species (of a certain genus)
spp.	multiple species (of a certain genus)
SPT	skin prick test
TAAF	thickener amino acid formula
Tbet	T-box transcription factor
TC	tolerant control
Tgfbr3	Transforming growth factor beta receptor III
TGF-β	Transforming growth factor beta
T_h	T helper cell
T_{eff}	effector T cells
TMAO	trimethylamine-N-oxide
TNFα	tumor necrosis factor alpha
T_{reg}	T regulatory cell
TSLP	thymic stromal lymphopoietin

Abbreviation	Full name/defination
UPLC-MS/MS	ultra-performance liquid chromatography with tandem mass spectrometry
W	whey
Pre-S	pre-sensitization
Post-S	post-sensitization
WS	whole study
wk	week(s)

Table S2. Search queries

Database	Search query
MEDLINE	((((cow*.ti. OR cow*.ab. OR cow*.kw. OR cow*.kf.) AND (milk.ti. OR milk.ab. OR milk.kw. OR milk.kf.)) AND ((allerg*.ti. OR allerg*.ab. OR allerg*.kw. OR allerg*.kf.) OR (hypersensitiv*.ti. OR hypersensitiv*.ab. OR hypersensitiv*.kw. OR hypersensitiv*.kf.))) OR milk hypersensitivity.sh.) AND ((microb*.ti. OR microb*.ab. OR microb*.kw. OR microb*.kf.) OR (microflora.ti. OR microflora.ab. OR microflora.kw. OR microflora.kf.) OR (16S*.ti. OR 16S*.ab. OR 16S*.kw. OR 16S*.kf.) OR (bifido*.ti. OR bifido*.ab. OR bifido*.kw. OR bifido*.kf.) OR (bacter*.ti. OR bacter*.ab. OR bacter*.kw. OR bacter*.kf.) OR (lachno*.ti. OR lachno*.ab. OR lachno*.kw. OR lachno*.kf.) OR (rumino*.ti. OR rumino*.ab. OR rumino*.kw. OR rumino*.kf.) OR (veillo*.ti. OR veillo*.ab. OR veillo*.kw. OR veillo*.kf.) OR (entero*.ti. OR entero*.ab. OR entero*.kw. OR entero*.kf.) OR microbiota.sh. OR bifidobacterium.sh. OR bacteroidaceae.sh. OR bacteroides.sh. OR ruminococcus.sh. OR veillonellaceae.sh. OR veillonella.sh. OR enterobacteriaceae.sh.) AND ((child*.ti. OR child*.ab. OR child*.kw. OR child*.kf.) OR (infant*.ti. OR infant*.ab. OR infant*.kw. OR infant*.kf.) OR (baby.ti. OR baby.ab. OR baby.kw. OR baby.kf.) OR (babies.ti. OR babies.ab. OR babies.kw. OR babies.kf.) OR (toddler*.ti. OR toddler*.ab. OR toddler*.kw. OR toddler*.kf.) OR (newborn*.ti. OR newborn*.ab. OR newborn*.kw. OR newborn*.kf.) OR infant.sh. OR child.sh. OR child, preschool.sh. OR infant, newborn.sh.)
PubMed	(((((cow[Title/Abstract] OR cow's[Title/Abstract]) AND milk[Title/Abstract]) AND (allerg*[Title/Abstract] OR hypersensitiv*[Title/Abstract])) OR ((milk hypersensitivity[MeSH Terms]) OR (milk hypersensitivities[MeSH Terms]))) AND (((microb*[Title/Abstract]) OR (microflora[Title/Abstract]) OR (16S[Title/Abstract]) OR (bifido*[Title/Abstract]) OR (bacter*[Title/Abstract]) OR (lachno*[Title/Abstract]) OR (rumino*[Title/Abstract]) OR (veillo*[Title/Abstract]) OR (entero*[Title/Abstract])) OR ((microbiota[MeSH Terms]) OR (microbiotas[MeSH Terms]) OR (human microbiome[MeSH Terms]) OR (human microbiomes[MeSH Terms]) OR (microbiome[MeSH Terms]) OR (microbiome, human[MeSH Terms]) OR (microbiomes[MeSH Terms]) OR (16s ribosomal rna[MeSH Terms]) OR (ribosomal rna, 16s[MeSH Terms]) OR (rna, 16s ribosomal[MeSH Terms]) OR (bifidobacterium[MeSH Terms]) OR (bacteroidaceae[MeSH Terms]) OR (bacteroides[MeSH Terms])

	OR (ruminococcus[MeSH Terms]) OR (veillonellaceae[MeSH Terms]) OR (veillonella[MeSH Terms]) OR (enterobacteriaceae[MeSH Terms]))))) AND (((child*[Title/Abstract]) OR (infant*[Title/Abstract]) OR (baby[Title/Abstract]) OR (babies[Title/Abstract]) OR (toddler*[Title/Abstract]) OR (newborn*[Title/Abstract])) OR ((infant[MeSH Terms]) OR (child[MeSH Terms]) OR (child, preschool[MeSH Terms]) OR (infant, newborn[MeSH Terms]))))
Scopus	(TITLE-ABS-KEY (cow* W/6 milk)) AND ((TITLE-ABS-KEY (allergy)) OR (TITLE-ABS-KEY (hypersensitiv*))) AND ((TITLE-ABS-KEY (microb*)) OR (TITLE-ABS-KEY (microflora)) OR (TITLE-ABS-KEY (16s*)) OR (TITLE-ABS-KEY (bifido*)) OR (TITLE-ABS-KEY (bacter*)) OR (TITLE-ABS-KEY (lachno*)) OR (TITLE-ABS-KEY (rumino*)) OR (TITLE-ABS-KEY (veillo*)) OR (TITLE-ABS-KEY (entero*))) AND ((TITLE-ABS-KEY (child)) OR (TITLE-ABS-KEY (infant)) OR (TITLE-ABS-KEY (baby)) OR (TITLE-ABS-KEY (toddler)) OR (TITLE-ABS-KEY (newborn)))
Web of Science	(TI=(cow* AND milk) OR AB=(cow* AND milk) OR AK=(cow* AND milk) OR KP=(cow* AND milk)) AND ((TI=(allergy) OR AB=(allergy) OR AK=(allergy) OR KP=(allergy)) OR (TI=(hypersensitiv*) OR AB=(hypersensitiv*) OR AK=(hypersensitiv*) OR KP=(hypersensitiv*))) AND ((TI=(microb*) OR AB=(microb*) OR AK=(microb*) OR KP=(microb*)) OR (TI=(microflora) OR AB=(microflora) OR AK=(microflora) OR KP=(microflora)) OR (TI=(16s*) OR AB=(16s*) OR AK=(16s*) OR KP=(16s*)) OR (TI=(bifido*) OR AB=(bifido*) OR AK=(bifido*) OR KP=(bifido*)) OR (TI=(bacter*) OR AB=(bacter*) OR AK=(bacter*) OR KP=(bacter*)) OR (TI=(lachno*) OR AB=(lachno*) OR AK=(lachno*) OR KP=(lachno*)) OR (TI=(rumino*) OR AB=(rumino*) OR AK=(rumino*) OR KP=(rumino*)) OR (TI=(veillo*) OR AB=(veillo*) OR AK=(veillo*) OR KP=(veillo*)) OR (TI=(entero*) OR AB=(entero*) OR AK=(entero*) OR KP=(entero*))) AND ((TI=(child) OR AB=(child) OR AK=(child) OR KP=(child)) OR (TI=(infant) OR AB=(infant) OR AK=(infant) OR KP=(infant)) OR (TI=(baby) OR AB=(baby) OR AK=(baby) OR KP=(baby)) OR (TI=(toddler) OR AB=(toddler) OR AK=(toddler) OR KP=(toddler)) OR (TI=(newborn) OR AB=(newborn) OR AK=(newborn) OR KP=(newborn)))

Table S3. Information and reasons for the 28 papers excluded after careful consideration

Index	Author and year	Exclusion reason
1	Pohjavuori *et al.*, 2004[1]	Diagnosed IgE-mediated CMA based on a CM challenge and skin prick tests or antigen-specific IgE of any antigen tested (including also egg-white, cat, dog and birch).
2	Viljanen *et al*., 2005a[2]	
3	Barros *et al*., 2017[3]	Distinguished between IgE-mediated and non-IgE mediated CMA in the description of the allergic subjects but did not report any specific results for IgE-mediated CMA.
4	Viljanen *et al*., 2005b[4]	
5	Burks *et al*., 2015[5]	
6	Dong *et al*., 2018[6]	

Index	Author and year	Exclusion reason
7	Jarvinen *et al*., 2014[7]	Reported 29 infants with IgE-mediated CMA in their table with clinical characteristics. However, elevated levels of cow's milk specific IgE were reported in only 13 infants. The corresponding author was contacted by email, but was unable to supply additional data because the research was done in a previous institution
8	Mercer *et al*., 2009[8]	CMA was diagnosed based on total and CM specific IgE levels and CMA-related symptoms, but no oral food challenge was used to confirm CMA.
9	Taniuchi *et al*., 2005[9]	Included several subjects whose diagnosis was not confirmed by an oral food challenge, but by a cow's milk elimination diet
10	Kendler *et al*., 2006[10]	Did not confirm CMA by oral food challenge
11	Hol *et al*., 2008[11]	Used a food challenge, but diagnosed children based on their late response, which does not point to IgE-mediated CMA
12	Shek *et al*., 2005[12]	Included both children below 12 years old as well as adolescents and/or adults, but results for children were not reported separately
13	Yamamoto-Hanada *et al*., 2023[13]	
14	Hill *et al*., 1989[14]	Did not include any gut microbiome data or intervention targeting the gut microbiome
15	Hauer *et al*., 1997[15]	
16	Szabó and Eigenmann, 2000[16]	
17	Paparo *et al*., 2016[17]	Studied infants that received probiotics in the past, before entering the study, and therefore could not be compared to the probiotic intervention studies discussed in this review
18	Gotteland *et al*., 1992[18]	Studied CM protein absorption after *E. coli* infection
19	Morin *et al*., 2012[19]	Animals models were sensitized to CM, but did not receive a food challenge, thus focus on CM sensitization rather than CMA
20	Shandilya *et al*., 2016[20]	
21	Wróblewska *et al*., 2020[21]	
22	Maiga *et al*., 2017[22]	
23	Pescuma *et al*., 2019[23]	Two of the three experiments had no challenge, while in the third one there was no comparison between (allergy or treatment) groups
24	Graversen *et al*., 2021[24]	Focused on antibiotics instead of treatment for CMA.
25	Liu *et al*., 2023[25]	Studied the effect of pre-treatment with whey or beta-lactoglobulin (BLG) before sensitization
26	Mauras *et al*., 2019[26]	The CMA donor used for fecal transplantation had multiple food allergy
27	Schouten *et al.*,2009[27]	No GM-related data, do not mention how the treatment changed the GM
28	Adel-Patient *et al.*,2020[28]	

Table S4. CMA diagnosis and measured variables for all human studies. Abbreviations: see Table S1

Author and year	CMA diagnosis	Measured variables		
		Microbiome	**Metabolomics**	**Immune response**
Thompson-Chagoyan et al., 2010[29]	CM-specific IgE, SPT, DBPCFC	Aerobes, Anaerobes, Enterobacteria, Bifidobacteria, *Lactobacilli*, Clostridia	-	-
Salmi et al., 2010[30]	CM-specific IgE, SPT, DBPCFC	-	**Urine**: 37 organic acids, Creatinine	-
Thompson-Chagoyan et al., 2011[31]	CM-specific IgE, SPT, DBPCFC	10 targeted probes: *Bifidobacterium*, *Bacteroides,* Enterobacteria*, Streptococcus, Lactobacillus, Atopobium, Clostridium coccoides, Clostridium leptum, Clostridium perfringens sps., Clostridium difficile sps.*	**Feces**: Lactate, SCFA (acetate, propionate, butyrate, isocaproic acid), Branched-chain short fatty acids (BCSFA).	-
Francavilla et al., 2012[32]	CM-specific IgE, SPT, DBPCFC	13 targeted probes: Domain bacteria, negative control, *Bifidobacterium*, *Bacteroides/Prevotella*, Eubacterium rectale/Clostridium coccoides, *Lactobacillus/Enterococcus*, *Streptococcus/Lactococcus group*, *Escherichia coli*, Sulfate–reducing bacteria (SRB), *Atopobium* group, *Coriobacterium* group, *Clostridium histolyticum*, *Clostridium lituseburense*	**GC-MS (feces)**: 15 organic metabolites (esters, ketones, Alcohols, sulfur compounds, hydrocarbons, SCFA); **NMR (feces):** pyruvic acid, lactic acid, uridine, histidine, tyrosine, threonine, methionine, proline, TMAO, arginine/histidine, valine / isoleucine, phenylalanine, gamma–amino–butyric acid/lysine	-
Guo et al., 2016[33]	Analysis of serum samples, SPT, DBPCFC	Dominant bacteria, *Bifidobacterium, Lactobacillus, C. coccoides*, Microbiota diversity (Shannon-Weaver index, dice similarity coefficient)	-	-

Table S4. Continued

Author and year	CMA diagnosis	Measured variables		
		Microbiome	**Metabolomics**	**Immune response**
Canani et al., 2016[34]	Clinical history, CM-specific IgE, DBPCFC	Dominant bacteria, Microbiota Alpha diversity (Shannon index) and Evenness (Pielou's evenness index)	**Feces**: butyrate	-
Dong et al., 2018	CM-specific IgE, SPT, DBPCFC	Dominant bacteria, Microbiota Alpha diversity (Chao1, ACE, Simpson, Shannon, and coverage indices)	**Feces**: SCFAs (acetate, butyrate, propionate, isobutyrate), lactate	-
Mennini et al., 2021[36]	CM-specific IgE, SPT, DBPCFC	PCR : Dominant bacteria; qRT-PCR: *B. breve, B. longum subsp. longum, B. longum subsp. infantis* Microbiota Alpha diversity (Observed, Chao1 and Shannon indices) and beta diversity(unweighted UniFrac)	-	-
Mera-Berriatua et al., 2022[37]	Clinical history of IgE-mediated food allergy, SPT	Dominant bacteria Microbiota Alpha diversity (Shannon index) and beta diversity (Bray-Curtis distance)	-	-
Bunyavanich et al., 2016[38]	CM-specific IgE, SPT, CM challenge or AD with CM-specific IgE	Microbiome (feces): Dominant bacteria; Microbiota Alpha diversity (Faith's phylogenetic diversity) and beta diversity (unweighted UniFrac)	-	-

Table S4. Continued

Author and year	CMA diagnosis	Measured variables		
		Microbiome	**Metabolomics**	**Immune response**
Dupont et al., 2015[39]	CM-specific IgE, SPT, or both positive cutaneous tests and IgE, DBPCFC	Total bacteria, *Clostridium* cluster IV, *Bacteroides/ Prevotella* group, *Bifidobacterium, Lactobacillus/ Leuconostoc/Pediococcus* group, *Clostridium* cluster XIVa, *Clostridium* cluster XI, *Clostridium* cluster I/II, *Staphylococcus, Enterococcus, Escherichia coli*	**Plasma:** Amino acids (cysteine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tyrosine, valine) **Feces:** butyrate	-
Chatchatee et al., 2022[40]	CM-specific IgE, SPT, DBPCFC	bifidobacteria and ER/CC group	-	-
Jing et al. 2020[41]	SPT, IgE, DBPCFC	dominant bacteria microbiota Alpha diversity (number of OTUs, Chao1, Shannon, Simpson index) and beta diversity (weighted and unweighted UniFrac)	-	**Immunoglobulins** Total IgE, IgG_2 (serum) **Cytokines** TNFα, IL-1β, IL-6, IL-10 (serum)

Table S5. Model information for all animal studies. Abbreviations: see Table S1

Sensitization					Challenge		Intervention details		n size/ group † †	Author and year
Animal/ Strain (gender)	**Allergen: Dose(mg)**	**Adjuvant: Dose (µg)**	**Period† (wk)**	**Admini-stration**	**Intradermal Allergen dose (ug)**	**Intragastric Allergen dose(mg)**	**Intro-duction**	**Duration**		
C3H/HeOuJ mice (F)	W:20	CT:10	5	i.g.	W:20	W:50	Pre-S	6-9d‡	6-8	Kostadinova et al.2017a[42]
C3H/HeOuJ mice (F)	W:20	CT:10	5	i.g.	W:20	W:50	Pre-S	6-9d‡	6-8	Kostadinova et al 2017b.[43]
C3H/HeOuJ mice (F)	W:20	CT:10	5	i.g.	W:6	W:50	**Long:** WS **Short:** Pre-S	**Long:** 7.5wk **Short:** 5d	7-10	Kleinjans et al.2019[44]
BALB/cByJ mice (F)	W:15	CT:10	5	i.g.	-	BLG:60	WS	6wk	30	Neau et al.,2016[45]
BALB/cByJ mice (F)	W:15	CT:10	5	i.g.	-	BLG:60	Post-S	20d	10 -12	Esber et al.2020[46]
Germ-free C3H/HeN	BLG:20	CT:10	5	i.g.	-	BLG:2*100	-	-	6-42	Feehley et al.2019[47]
C3H/HeN mice (M)	W/W/W: 10/100/0.5	CT/CT/Alum: 10/10/2	5/2/2	i.g./i.g./i.p.	-	W:50	-	-	3-7	Cao et al. 2022[48]
C57BL/6J and BALB/cJ (M and F)	BLG:1	CT:10	5	i.g.	-	W:50	-	-	5-10	Smith et al. 2021[49]

†All administrations are performed weekly
†† Intervention group sizes (not control group)
‡ Synbiotic diet for 9 days, peptide mix intake for 6 days

Table S6. Measured variables for all animal studies. Abbreviations: see Table S1

Author and year	Measured variables		
	Microbiome	**Metabolomics**	**Immune response**
Neau et al.[45]	11 bacteria primers, all bacteria	-	**Igs:** Total and BLG-s IgE, IgG_1, IgG_{2a} (plasma) **Cytokines:** IFN-γ, IL-12p70, IL-4, IL-5, and IL-10 (spleen, MLN) **mRNA expression:** ifn-g, il-4, il-10, tgf-b, il-17a, t-bet, gata3, rorγt, foxp3 (ileum)
Esber et al.[46]	α (Shannon index) and β (Bray-Curtis distance, UniFrac distance) diversity	**Feces**: SCFA, **Plasma**: other metabolites	**Igs:** BLG- sIgE, $sIgG_1$, $sIgG_2$ (plasma) **Cytokines:** IL-17A, IL-2, GM-CSF, IL-4, IFN-γ, IL-10, IL-5, IL-12p70 (spleen) **mRNA expression:** gata3, tbet, foxp3, rorγt, ifnγ, tnfα, il4, Il10, and tgfβ (ileal)
Kleinjans et al.[44]	All bacteria	-	**Igs:** W- sIgE, $sIgG_1$, $sIgG_{2a}$ (serum)
Kostadinova et al.[42]	-	**Feces**: acetic acid, propionic acid, butyric acid	**Igs:** W- and BLG- sIgE, $sIgG_1$, $sIgG_{2a}$ (serum) **Lymphocytes:** T cells, DC (spleen, MLN, SI-LP) **Cytokines:** IL-5, IL-13, IL-10, IL-17A, IFN-γ (Spleen, MLN, SILP)
Kostadinova et al.[43]	-	<u>Part 1: Post-oral tolerance</u> **Metabolites** **Feces**: acetic acid, propionic acid, butyric acid, valeric acid	<u>Part 1: Post-oral tolerance</u> **mRNA expression:** Foxp3, Tbet, GATA3, RorγT, IL-10, galectin-9, TGF-β, IL-13, IFN-γ, IL-22 (PP, SI (proximal, middle), colon) **Immunohistochemistry**: Foxp3+ cells (colon) <u>Part 2: Post-challenge</u> **mRNA expression:** Foxp3, Tbet, GATA3, RorγT, IL-10, galectin-9, TGF-β, IL-13, IFN-γ, and IL-22 (PP, spleen) **Lymphocytes:** T_{reg} (LP)
Smith et al.[24]	α (Shannon, Simpson indices) and β(Bray-Curtis) diversity	-	**Igs:** BLG-sIgE,s sIgG1; sIgG2a (serum) **Cytokines, chemokines, and acute phase proteins:** e.g. IL-10, IL-13, IL-15, IL-1β, IL-31, IL-21, CCL1, CCL9, CCL12, CCL17, FGF2, CDF1, CSF2, TNFFSF1A, TNFRSF1B, ICAM-1 (plasma)

Table S6. Continued

Author and year	Measured variables		
	Microbiome	**Metabolomics**	**Immune response**
Cao et al.[23]	All bacteria, α and β diversity	-	**Igs:** W- sIgE, $sIgG_1$, $sIgG_{2a}$ (serum) **Cytokines:** IL-6, IL-10 (serum) **mRNA expression:** IL-4, IL-8, IL-33, IL-1β, TGF-β, GAPDH, mTOR mRNA
Feehley et al.[47]	α (Shannon index) and β (weighted UniFrac) diversity Pielous' s evenness	-	**Igs:** BLG-specific IgE, IgG_1 (serum) **Cytokines:** IL-13, IL-4 (spleen) ex-W **Transcriptome:** 32 genes (IEC)

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Chapter 3

Fecal metabolome alterations in infants at risk of developing allergies during the first year of life

Based on:

Fecal metabolome alterations in infants at risk of developing allergies during the first year of life

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Metabolomics (Under revision)

Abstract

Background: Disturbances in the gut microbiome (GM) in infancy may contribute to the risk of allergy. This period is characterized by rapid microbial colonization, influenced by factors like delivery mode and infant feeding practices. The present study investigated changes in key GM taxa and fecal metabolites in relation to allergy development, delivery mode, age, and infant feeding practices during the first year of life.

Methods: Seventy-two infants at risk of allergies, exclusively breastfed for at least 16 weeks, were followed in their first year. During this period allergy manifestations were recorded and fecal samples were collected at three time points: before 16weeks, at 6 months, and at 12 months of age. The samples were subjected to metabolic profiling covering host and microbial metabolites and fluorescent in situ hybridization to quantify Bifidobacterium spp. and the *Eubacterium rectale/Clostridium coccoides* group.

Results: Strong age-associated metabolic shifts were observed, particularly in aromatic amino acid metabolites, bile acids, B vitamins, and short and long-chain fatty acids. Feeding practices, specifically the introduction of complementary feeding and the cessation of breastfeeding were significantly associated with changes to the fecal metabolome. Delivery mode had a pronounced impact on the metabolome, with differences between vaginal and Cesarean deliveries persisting until 6 months of age. Infants who developed an allergy (n=20) during this period had lower *Bifidobacterium* spp. and higher polyunsaturated fatty acid levels before the age of 16 weeks.

Conclusion: This study offers valuable insights into the longitudinal development of the fecal metabolome and factors influencing it during infancy, a critical period for immune system development.

Keywords

early life, birth mode, stool metabolomics, solid food introduction, infant, children, allergy

Abbreviations

HMOs: human milk oligosaccharides; **FISH**: Fluorescence *in situ* hybridization; **ER/CC**: *Eubacterium rectale/Clostridium coccoides*; **GC-FID**: gas chromatography coupled - flame ionization detector; **LC-MS**: liquid chromatography – mass spectrometry; **LMM**: linear mixed model; **AAs**: amino acids; **ppBMI** - pre-pregnancy BMI**, PUFA**: polyunsaturated fatty acids;

1. Introduction

Our guts are home to trillions of bacteria that live in a symbiotic relationship with us as hosts.[1] The first year of life is crucial for the development and maturation of the gut microbiome (GM).[2] This period also coincides with the development of the immune system[3] and is a key window in which GM colonization shapes the host's immune system.[4] An accumulating body of research links the disturbances of the GM composition in early life to a multitude of immune-mediated diseases,[5] including allergies.[6,7] Allergic disease often follows a temporal progression from atopic dermatitis and food allergy in infancy to allergic asthma and rhinitis in childhood, also known as "atopic march".[8] The study of early life GM composition and function in relation to allergy development is therefore a topic of considerable interest.

Many factors are known to influence the GM composition in infancy, including use of antibiotics, mode of delivery (vaginal versus C-section), milk feeding practices (breastfeeding versus formula feeding), and the transition to solid foods (complementary feeding).[9] The use of antibiotics and C-section have been associated with dysbiosis in early life and risk of developing of atopic dermatitis and other diseases later in life.[10,11] Even though the effect of breastfeeding on allergy is still a topic of debate, breastfeeding is the recommended infant nutrition for allergy prevention.[12] Breastmilk is considered the optimal nutrition for infants due to its balanced composition of macronutrients and bioactive compounds satisfying the infant's nutritional and physiological requirements.[13] It is also an important source of bifidobacteria and lactobacilli as well as human milk oligosaccharides (HMOs).[14] Bifidobacteria, e.g. *B. breve*, *B. bifidum*, *B. longum*, capable of utilizing HMOs and derivatives for energy, thrive in the guts of healthy breastfed infants and are crucial for immune system development.[15]

While the impact of the above-mentioned factors on the GM composition is relatively well-studied, their influence on GM activity remains understudied. Similarly, research examining the link between allergies and the GM have mainly focused on compositional analysis.[7] Since the GM influences the host's physiology via the production of metabolites, researchers are increasingly examining the metabolome to get insights into host-microbiota interactions.[16]

In this study, healthy breastfed infants at increased risk of developing allergies were followed during their first year. Data on delivery mode, allergy development, feeding practices were collected, and key gut microbial taxa along with the fecal metabolome were analyzed at three time points. This allowed us to assess microbiome and metabolomic changes associated with allergy, delivery mode, age, milk feeding practices (breastfeeding and formula feeding), and complementary feeding.

2. Experimental Section

2.1 Study design, sample collection and storage

The samples for this work arise from a randomized, double-blind, controlled, parallel-group, multi-country study called TEMPO (clinicaltrials.gov identifier: NCT03067714). Detailed information on ethics committees, institutional review boards, and regulatory authorities that approved the study was previously published.[17] TEMPO enrolled healthy term infants (age: <16 weeks) at increased risk of developing allergy based on family history. Subjects who began formula feeding before 16 weeks entered one of the two intervention arms, while those exclusively breastfed for at least 16 weeks comprised the breastfed reference group. Exclusive breastfeeding was defined as receiving only breastmilk, with no other liquids or solids except water or formula in the first 72 hours of life, disregarding vitamins, minerals, or medicines. All participants were followed for a year, during which events of allergic manifestations were diagnosed by qualified physicians and classified as skin, food, or respiratory allergies. Allergy manifestations were considered IgE-mediated if either the skin prick test to any tested allergen or specific IgE blood test was positive at 12m. In this study, we selected a subset of 72 subjects solely from the breastfed reference group based on the availability of fecal samples collected before 16 weeks (baseline), at 6 months (6m), and at 12 months (12m) of age. Sample collection and storage procedure is available in Supplementary materials.

2.2 Microbiome data acquisition

Fluorescence *in situ* hybridization (FISH) quantification of *Bifidobacterium* genus and *Eubacterium rectale/Clostridium coccoides* group (ER/CC) was performed on a subset of subjects as described previously.[18]

2.3 Metabolomic data acquisition

Liquid chromatography–mass spectrometry (LC-MS) metabolomic data acquisition and preprocessing were performed as previously described.[19] Briefly, wet fecal samples went through lyophilization and liquid-liquid extraction prior to the analysis by reverse phase LC-MS (RPLC-MS) using two separate assays one covering polar to semi-polar metabolites and a second covering bile acids (BAs) and long-chain fatty acids (LCFAs). In case of coelution, the targets were reported using the name or abbreviation of one of the targets followed by a "#" (**Table S1**). Data quality inspection, including between-batch correction and removal of metabolites with high technical variance (quality control RSD > 30%) was conducted using mzQuality.[20] The analysis of short-chain fatty acids (SCFAs) and lactic acid was conducted as already described.[21]

2.4 Data analysis

Data handling and statistical analyses were performed in R (version 4.3.3). After dry weight normalization, metabolites with a median signal below five times the mean signal of the procedure blanks were excluded. To detect group bias in missing data, the Fisher's exact test was applied to metabolites with any missing measurements. The 57 metabolites with missingness >20% were subjected to unpaired Mann-Whitney U test to assess the difference between visits and between the study groups (allergic vs non-allergic, vaginal vs C-section delivery, complementary-fed vs non-complementary-fed, formula-fed vs non-formula-fed, breastfed vs non-breastfed) at the relevant visits. Missing values of the 162 metabolites with missingness <20% were imputed after log2 transformation. Then, linear mixed models (LMMs) were used to examine the metabolomic difference between the study groups over time. Clinical characteristics were checked for associations to allergy, delivery mode, and feeding practices using Mann-Whitney U-test for numeric variables and the Fisher's exact test for binary variables. Differences in the microbiome data across visits and between the study groups at each visit were assessed using the Mann-Whitney U test. Spearman's correlation analysis was conducted to assess the relationship between LCFAs and microbiome taxa. Multiple testing correction was performed using the Benjamini-Hochberg method where Q<0.1 was considered as statistically significant. Further data analysis details are available in Supplementary Materials.

3. Results

3.1 Patient characteristics

Table 1 summarizes the characteristics of the 72 infants at risk of developing allergy who were followed throughout their first year. The associations between the clinical characteristics and allergy manifestation, delivery mode, and feeding practices, were examined (**Table S2-3**). Potential confounders excluded from this analysis include: i) clinical characteristics describing symptoms of allergy and its treatment, as well as gestational age and maternal pre-pregnancy body mass index (ppBMI) associated with C-section; ii) patient characteristics such as country and mineral supplementation which were excluded due to low sample size.

3.2 Age has a significant impact on the fecal metabolome

To explore the impact of age, diet, delivery mode, and allergy on the fecal metabolome, a range of host and gut microbial metabolites, including (aromatic) amino acids (AAs) and derivatives, vitamins, nucleobases, nucleosides, BAs, LCFAs, and SCFAs were examined (**Table S1**).

Table 1. Clinical characteristics. Numeric variables are presented as median [range]; categorical variables are presented as numbers of participants.

Variable	Whole cohort	Allergic	Non-Allergic	C-section	Vaginal
Sex (female/male)	35 / 37	9 / 11	26 / 26	12 / 8	23 / 29
Allergy manifestation (Allergic/Not allergic)	20 / 52†	-	-	7 / 13	13 / 39
Type of allergy (IgE/non-IgE)	10/10	10/10	-	3 / 4	7 / 6
Type of allergy† (skin/food/respiratory)	18 / 2 / 2	18 / 2 / 2	-	7 / 1 / 0	11 / 2 / 1
Age onset allergy (days)	-	126.5 [69, 299]	-	-	-
Mode of delivery (Vaginal/C-section)	52 / 20 ‡	13 / 7	39 / 13	-	-
Country					
Belgium	1	1	0	0	1
Czech Republic	36	15	21	8	28
United Kingdom	1	0	1	0	1
Hungary	14	2	12	5	9
Slovakia	20	2	18	7	13
Gestational age (weeks)	39.3 [37.6-41.9]	39.2 [37.6-41.7]	39.3 [37.6-41.9]	38.8 [37.6-40.9]	39.6 [37.6-41.9]
Birth head circumference (cm)	34.5 [32-39]	34.0 [33-38]	35.0 [32-39]	35.0 [33-39]	34.0 [32-38]
Birth weight (kg)	3.4 [2.6-4.2]	3.4 [2.9-4.2]	3.4 [2.6-4]	3.4 [2.9-4]	3.4 [2.6-4.2]
Birth length (cm)	50 ± 2.4	50 ± 1.8	50 ± 2.6	50 ± 1.8	50 ± 2.6
Birth length (cm)	50 [47-58]	50 [47-54]	50 [47-58]	50 [47-53]	50 [47-58]
Mother's ppBMI	23.3 [18.4-40]	22.2 [18.4-35]	23.9 [18.6-40]	24.6 [19.9-40]	22.8 [18.4-35]
Age (days)					
baseline	41.5 [1-111]	23 [1-111]	52.5 [2-111]	53 [2-108]	37 [1-111]
6 months	180 [166-227]	179 [166-192]	180 [167-227]	180 [168-227]	180 [166-206]
12 months	364 [345-383]	365.5 [348-383]	363.5 [345-378]	365 [345-377]	363.5 [348-383]
Breastfeeding (yes/no)					
baseline	72 / 0	20 / 0	52 / 0	20 / 0	52 / 0
6 months	71 / 1	20 / 0	51 / 1	20 / 0	51 / 1
12 months	58 / 14	17 / 3	41 / 11	18 / 2	40 / 12
Formula Feeding (yes/no)					
baseline	0 / 72	0 / 20	0 / 52	0 / 20	0 / 52
6 months	7 / 65	2 / 18	5 / 47	1 / 19	6 / 46
12 months	23 / 49	6 / 14	17 / 35	4 / 16	19 / 33
Milk feeding§ (BF / FF / MMF)					
baseline	72 / 0 / 0	20 / 0 / 0	52 / 0 / 0	20 / 0 / 0	52 / 0 / 0
6 months	65 / 1 / 6	18 / 0 / 2	47 / 1 / 4	19 / 0 / 1	46 / 1 / 5
12 months	49 / 12 / 11	12 / 2 / 4	35 / 10 / 7	48 / 2 / 2	33 / 10 / 9
Complementary Feeding (yes/no)					
baseline	0 / 72	0 / 20	0 / 52	0 / 20	0 / 52
6 months	55 / 17	13 / 7	42 / 10	15 / 5	40 / 12
12 months	72 / 0	20 / 0	52 / 0	20 / 0	52 / 0

†The two subjects who had developed IgE-mediated food/respiratory allergy were also diagnosed with IgE-mediated skin allergy
‡Even though the numbers for allergy and delivery mode are the same (20 / 52), the infants in the four groups are different. More specifically 39 non-allergic and 13 allergic subjects were delivered vaginally; while 13 were non-allergic and delivered via a C-section; 7 were allergic and delivered via a C-section.
§BF – breastfed, infants receiving breastmilk and no formula milk; FF – formula-fed, infants receiving infant formula milk and not breastmilk, MMF – mixed milk-fed – infants receiving breastmilk and formula milk

Age had a strong effect on the metabolome, with LMM analysis identifying 99 metabolites that significantly changed within the first 6 months of life and 92 metabolites in the second half of the first year (**Figure 1A**). B vitamins and derivatives, AAs and derivatives, BAs, nucleobases, nucleosides and derivatives, SCFAs, and phenolic acids increased significantly throughout the whole first year, between baseline and 6m or between 6m and 12m. Among those the primary BAs, CA and CDCA, increased in the first six months, glyco-conjugated BAs in the latter six months, and secondary BAs during either or both halves of the year (**Figure 1A**).

Host-derived tryptophan metabolites also increased with age, whereas the microbial aromatic AA metabolites followed varying time trends. Aromatic lactic acids (PLA, ILA, 4-OH-PLA#) increased until 6m. Then, while PLA remained unchanged, ILA and 4-OH-PLA# decreased. The tryptophan-derived indoxyl sulfuric acid and phenylalanine-derived PAGIn and hippuric acid also declined after 6m. Meanwhile, the acetic aromatic acids 4-OH-PAA# and IAA increased after 6m, with IAA decreasing before 6m (**Figure 1A**).

LCFAs declined through the first year, except for ALA#, LA, and mead acid which declined significantly only until 6m. Even though an overall decline in acylcarnitines was observed after 6m, before 6m the long-chain acylcarnitines increased, whereas the short- and medium-chain acylcarnitines remained unchanged (**Figure 1A**).

The metabolites that could not be analyzed using LMMs were assessed using a Mann-Whitney U test (**Figure S1A**). Consistent with LMM findings, acylcarnitines decreased, whereas AAs and derivatives; B vitamins and derivatives; nucleobases, nucleosides and derivatives; SCFAs; phenolic acids; and host tryptophan metabolites increased over time. The acetic and propionic aromatic acids, PAA and 4-OH-PPA, also increased whereas IPA levels remained stable despite the decline in its missingness with age (missingness of 91.7%, 79.2%, 34.7% for baseline, 6m, 12m respectively). TUDCA and secondary BAs also increased, particularly in the second half of the first year (**Figure S1A**). Although LCA and DCA did not pass QC, visual inspection suggested a rise, especially in some subjects at 12m (**Figure S3**).

3.3 Dietary changes were associated with fecal metabolome alterations

Infant diets evolved during the first year (**Table 1**), where at baseline (<16 weeks), all infants were breastfed, at 6m 90% infants were breastfed, 8.3% mixed-milk-fed (breastfed and formula-fed), and 1.4% formula-fed, while at 12m, 68% were breastfed, 15% mixed-milk-fed, and 17% formula-fed. Meanwhile, complementary feeding had started for 76% of the participants by 6m and for all by 12m.

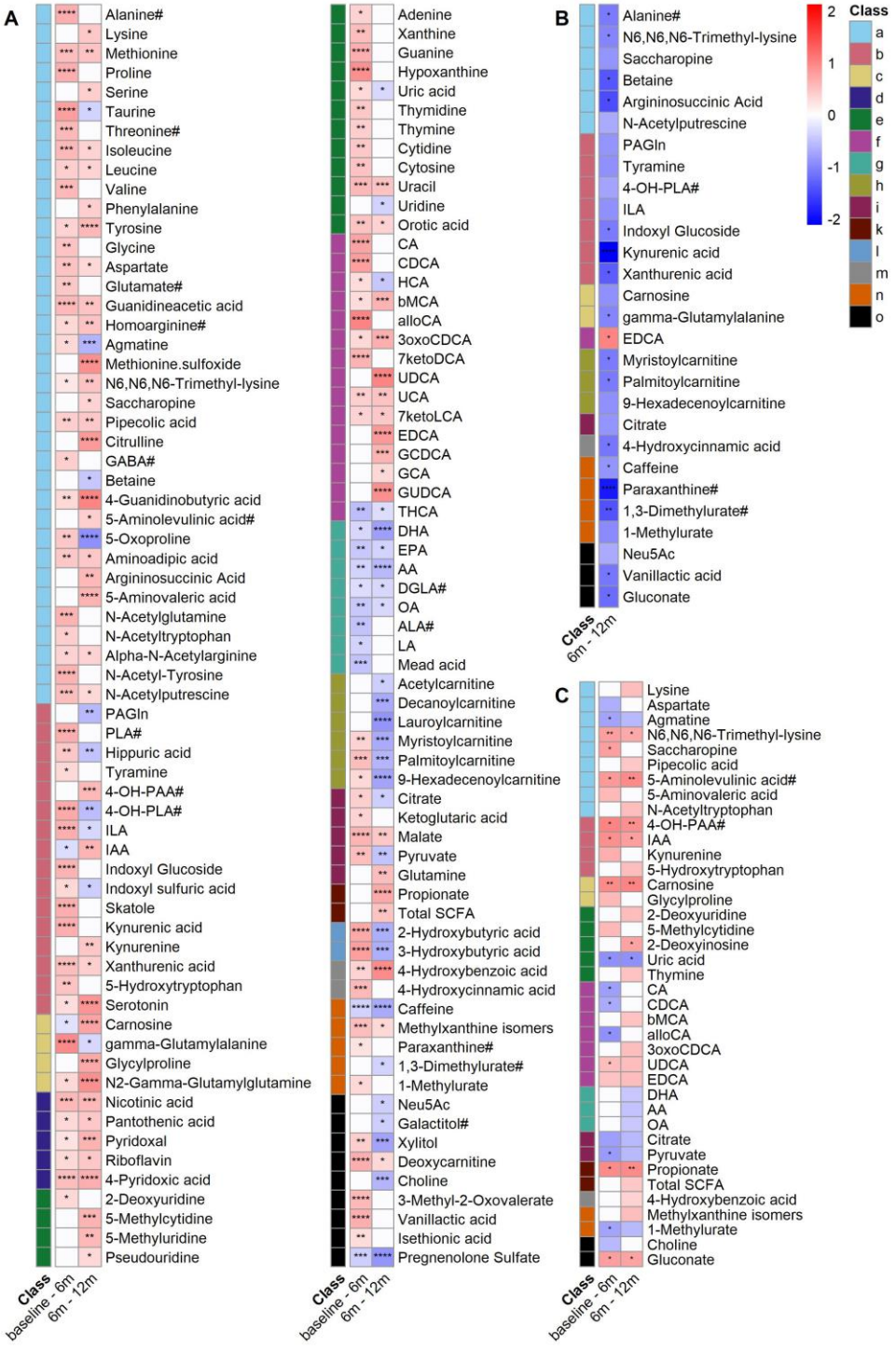
A
Alanine#
Lysine
Methionine
Proline
Serine
Taurine
Threonine#
Isoleucine
Leucine
Valine
Phenylalanine
Tyrosine
Glycine
Aspartate
Glutamate#
Guanidineacetic acid
Homoarginine#
Agmatine
Methionine.sulfoxide
N6,N6,N6-Trimethyl-lysine
Saccharopine
Pipecolic acid
Citrulline
GABA#
Betaine
4-Guanidinobutyric acid
5-Aminolevulinic acid#
5-Oxoproline
Aminoadipic acid
Argininosuccinic Acid
5-Aminovaleric acid
N-Acetylglutamine
N-Acetyltryptophan
Alpha-N-Acetylarginine
N-Acetyl-Tyrosine
N-Acetylputrescine
PAGln
PLA#
Hippuric acid
Tyramine
4-OH-PAA#
4-OH-PLA#
ILA
IAA
Indoxyl Glucoside
Indoxyl sulfuric acid
Skatole
Kynurenic acid
Kynurenine
Xanthurenic acid
5-Hydroxytryptophan
Serotonin
Carnosine
gamma-Glutamylalanine
Glycylproline
N2-Gamma-Glutamylglutamine
Nicotinic acid
Pantothenic acid
Pyridoxal
Riboflavin
4-Pyridoxic acid
2-Deoxyuridine
5-Methylcytidine
5-Methyluridine
Pseudouridine
Class
baseline - 6m
6m - 12m
Adenine
Xanthine
Guanine
Hypoxanthine
Uric acid
Thymidine
Thymine
Cytidine
Cytosine
Uracil
Uridine
Orotic acid
CA
CDCA
HCA
bMCA
alloCA
3oxoCDCA
7ketoDCA
UDCA
UCA
7ketoLCA
EDCA
GCDCA
GCA
GUDCA
THCA
DHA
EPA
AA
DGLA#
OA
ALA#
LA
Mead acid
Acetylcarnitine
Decanoylcarnitine
Lauroylcarnitine
Myristoylcarnitine
Palmitoylcarnitine
9-Hexadecenoylcarnitine
Citrate
Ketoglutaric acid
Malate
Pyruvate
Glutamine
Propionate
Total SCFA
2-Hydroxybutyric acid
3-Hydroxybutyric acid
4-Hydroxybenzoic acid
4-Hydroxycinnamic acid
Caffeine
Methylxanthine isomers
Paraxanthine#
1,3-Dimethylurate#
1-Methylurate
Neu5Ac
Galactitol#
Xylitol
Deoxycarnitine
Choline
3-Methyl-2-Oxovalerate
Vanillactic acid
Isethionic acid
Pregnenolone Sulfate
Class
baseline - 6m
6m - 12m
B
Alanine#
N6,N6,N6-Trimethyl-lysine
Saccharopine
Betaine
Argininosuccinic Acid
N-Acetylputrescine
PAGln
Tyramine
4-OH-PLA#
ILA
Indoxyl Glucoside
Kynurenic acid
Xanthurenic acid
Carnosine
gamma-Glutamylalanine
EDCA
Myristoylcarnitine
Palmitoylcarnitine
9-Hexadecenoylcarnitine
Citrate
4-Hydroxycinnamic acid
Caffeine
Paraxanthine#
1,3-Dimethylurate#
1-Methylurate
Neu5Ac
Vanillactic acid
Gluconate
Class
6m - 12m
2
1
0
-1
-2
Class
a
b
c
d
e
f
g
h
i
k
l
m
n
o
C
Lysine
Aspartate
Agmatine
N6,N6,N6-Trimethyl-lysine
Saccharopine
Pipecolic acid
5-Aminolevulinic acid#
5-Aminovaleric acid
N-Acetyltryptophan
4-OH-PAA#
IAA
Kynurenine
5-Hydroxytryptophan
Carnosine
Glycylproline
2-Deoxyuridine
5-Methylcytidine
2-Deoxyinosine
Uric acid
Thymine
CA
CDCA
bMCA
alloCA
3oxoCDCA
UDCA
EDCA
DHA
AA
OA
Citrate
Pyruvate
Propionate
Total SCFA
4-Hydroxybenzoic acid
Methylxanthine isomers
1-Methylurate
Choline
Gluconate
Class
baseline - 6m
6m - 12m

Figure 1. *Fecal metabolome alterations associated with age (**A**), cessation of breastfeeding (**B**), introduction of complementary feeding (**C**) between baseline and 6m and/or 6m and 12m, assessed using LMM. Colors represent the model coefficient: positive (red), negative (blue), P>0.05 (white). In (**A**) a positive coefficient represents an increase of the metabolite between the visits; in (**B**) a positive coefficient represents an increase of the metabolite associated with cessation of breastfeeding between 6m and 12m; while in (**C**) a positive coefficient represents an increase of the metabolite with introduction of complementary feeding between baseline and 6m or between 6m and 12m. Class annotation: **a** - AAs and derivatives; **b** aromatic AAs metabolites; **c** - dipeptides and tripeptides; **d** - B vitamins and derivatives; **e** - nucleobases, nucleosides and derivatives; **f** – BAs; g – LCFAs; **h** – carnitines; **j** – energy metabolites; **k** – SCFAs; **l** - hydroxy acids and derivatives; **m** - phenolic acids; **n** – xanthines; **o** – other. Asterisks indicate statistical significance: Q < 0.1 (*), Q < 0.01 (**), Q < 0.001 (***), Q < 0.0001 (****). The "#" in the metabolite names indicates that the metabolite coeluted with another target metabolite. All abbreviations and coeluting metabolites can be found in Table S1.*

Initiation of formula-feeding had a minor effect on the metabolome (LMM, **Table S4**). It was associated with lower levels of B vitamins i.e. pyridoxal, pantothenic acid, nicotinic acid as well as thymine, 2-deoxyuridine, 2-deoxyinosine but with higher guanosine# and allantoin until 6m. However, following multiple testing correction, only the association of thymine remained significant.

Complementary feeding was associated with significantly higher propionate, carnosine and aromatic acetic acids 4-OH-PAA# and IAA but lower uric acid and pyruvate levels among others (**Figure 1C**). Until 6m, complementary feeding was also negatively associated with the primary BAs CA, CDCA, alloCA but positively with the secondary BA UDCA and syringic acid. The latter was detected only after the introduction of complementary food except for one infant (**Figure S2**).

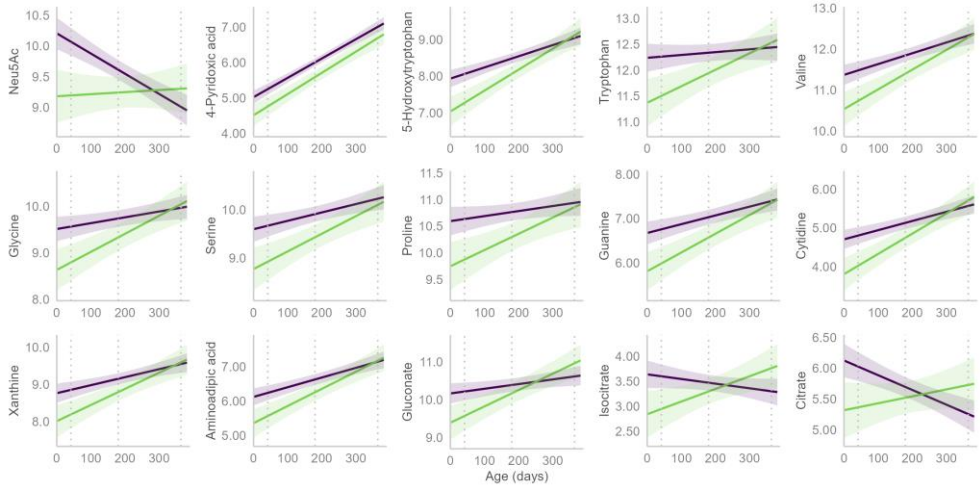

Figure 2. *Scaled abundance levels of the metabolites that significantly differed between infants delivered vaginally (purple, solid) and via C-section (green, solid) group as a function of age, based on LMM analysis. The shaded areas represent the 95% confidence intervals, while the dotted grey lines represent the median age at each visit (baseline, 6m, 12m). All abbreviations and coeluting metabolites can be found in Table S1.*

Meanwhile, the cessation of breastfeeding was associated with higher EDCA but lower long-chain acylcarnitines, caffeine and metabolites, Neu5Ac, gamma-glutamylalanine, and 4-hydroxycinnamic acid. The tryptophan and tyrosine metabolites kynurenic acid, indoxyl glucoside, xanthurenic acid (Q<0.1), ILA, tyramine, and 4-OH-PLA# (0.01<P<0.05, Q>0.1) were also negatively associated with cessation of breastfeeding (**Figure 1B**).

The effect of feeding practices for metabolites that could not be analyzed using LMM analysis, were assessed using the Mann-Whitney U test (**Figure S1B-C**). Butyrate, secondary BAs, and phenolic acids were higher in the complementary-fed versus non-complementary-fed infants at 6m (Q<0.1) and breastfed versus non-breastfed subjects at 12m (P<0.05). N2,N2-dimethylguanosine and 2-octenoylcarnitine were respectively higher and lower in the complementary-fed versus non-complementary-fed infants, whereas the tryptophan metabolite IPA was higher in the non-breastfed versus breastfed infants.

3.4 Delivery mode affected the fecal metabolome up to 6 months of age

TEMPO enrolled infants delivered vaginally and via a C-section, allowing an investigation into the effect of the delivery mode on the metabolome. Fifteen metabolites, including Neu5Ac, AAs and derivatives, pyrimidine and purine derivatives, and carboxylic acids, were significantly lower in the C-section compared to the vaginal group at baseline (**Figure 2**). For all, the group differences decreased with age until the groups completely overlapped at 12m. Notably, Neu5Ac levels remained stable over time in the C-section group, while they declined in the vaginal group. In contrast, proline and tryptophan were stable in the vaginal group but increased over time in the C-section group. Citrate and isocitrate also followed opposing trends, decreasing in the vaginal group while increasing in the C-section group.

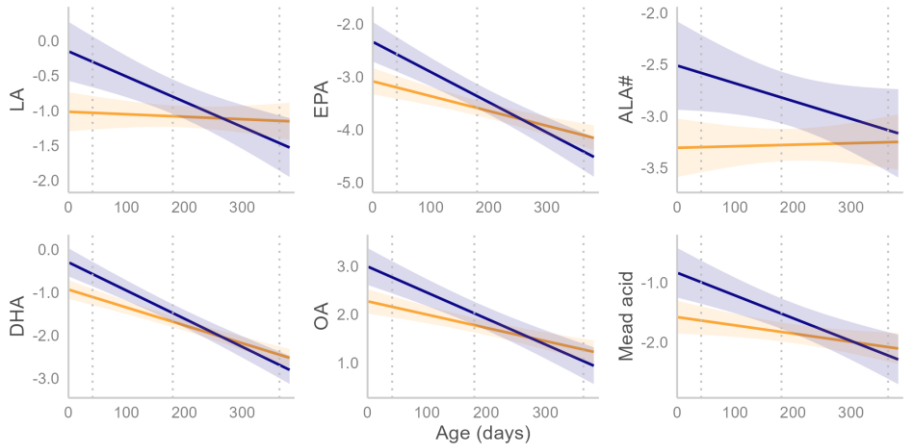

Figure 3. *Scaled relative abundance levels of LCFAs as a function of age in allergic (blue, solid) and non-allergic (orange, solid) groups, based on LMM analysis. The shaded areas represent the 95% confidence intervals, while the dotted grey lines represent the median age at each visit (baseline, 6m, 12m). The "#" in the metabolite names indicates that the metabolite coeluted with another target metabolite. All abbreviations and coeluting metabolites can be found in Table S1.*

3.5 Higher LCFA levels in infants who developed allergy

LMMs were used to examine the longitudinal metabolite alterations with age between the infants who developed allergies during the first year of life and those who did not. At baseline, no participants were allergic and allergies developed between 69 and 299 days (median age 126.5 days). A few LCFAs, namely LA, EPA, ALA#, DHA, OA, and mead acid, were found to be significantly higher at baseline in the allergic compared to the non-allergic group. However, the group separation disappeared over time and the groups overlapped at 6m and 12m (**Figure 3**).

3.6 Lower Bifidobacterium spp. in infants prior to allergy development

FISH was applied to quantify the *Bifidobacterium* spp. which are characteristic GM members in breastfed infants and ER/CC, which is primarily composed of *Lachnospiraceae* species and is more common in adults.[22] The analysis showed that the *Bifidobacterium* spp. levels were significantly lower at 12m compared to baseline and 6m, whereas the opposite was the case for ER/CC ($P < 0.05$, $Q < 0.1$, **Figure 4A-B**). ER/CC was also significantly lower in infants that were still breastfed versus non-breastfed infants ($P < 0.05$, $Q < 0.1$, **Figure 4D**) and those not receiving formula versus those that did at 12m ($P < 0.05$, $Q > 0.1$, **Figure S4**). Complementary feeding and delivery mode were not associated with significant differences in the examined taxa (**Figure S4**). The baseline *Bifidobacterium* spp. levels of the infants who developed allergy by 12m were lower than those that did not ($P < 0.05$, $Q > 0.01$, **Figure 4C**). A follow-up Spearman correlation analysis showed no evidence of a correlation between reduced *Bifidobacterium* spp. levels and elevated LCFAs levels (**Figure S5**).

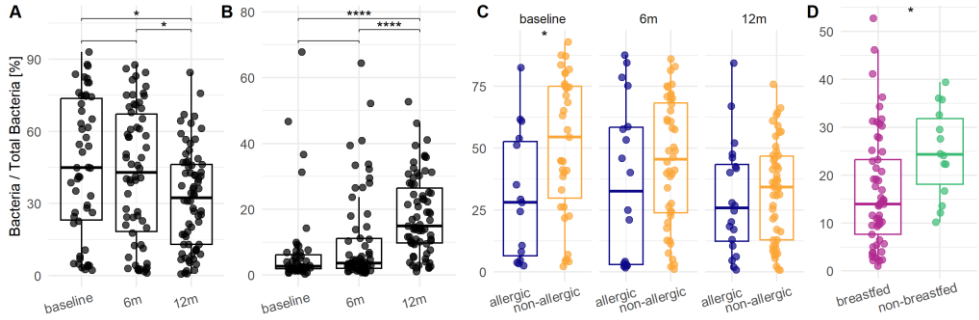

Figure 4. *The levels of A) Bifidobacterium spp. between visits; B) ER/CC between visits; C) Bifidobacterium spp. between the allergic (blue) and non-allergic (orange) infants at each visit; D) ER/CC between breastfed (pink) and non-breasted (green) at 12m as proportion of the total bacteria. Statistical analysis was performed using Mann-Whitney test: P < 0.05 (*), P < 0.01 (**), P < 0.001 (***), P < 0.0001 (****). Number of measurements per group and visit: Bifidobacterium spp.: n = [50, 62, 70]; ER/CC: n = [48, 60, 71] for baseline, 6m and 12m, respectively.*

4. Discussion

In this study, healthy breastfed infants at risk of allergy were followed throughout their first year. During this period, alteration in the fecal metabolome and key microbiome members

(*Bifidobacterium* spp., ER/CC) were examined in relation to age, feeding practices, mode of delivery, and allergy development. Strong age-associated alterations were observed including an overall increase in AAs and derivatives, BAs, nucleobases, nucleosides and derivatives, B vitamins and derivatives, SCFAs, and phenolic acids, along with a decrease in LCFAs and acylcarnitines. Feeding practices, specifically the intake of complementary food and cessation of breastfeeding were significantly associated with changes to the metabolome. Delivery mode had a pronounced impact on the metabolome with distinct differences observed mainly at baseline, some of which persisted until 6m. Meanwhile, infants who developed allergy had significantly lower *Bifidobacterium* spp. and higher LCFA levels at baseline.

These strong age-associated metabolome changes align with previous studies examining the fecal metabolome in early life.[23,24] These pronounced shifts are expected, given the rapid physical growth[2] and GM diversification associated with the transition from milk to solid food during this period.[25] In our cohort, the diversification is evident by the significant decline in bifidobacteria and the increase in the adult-like ER/CC at 12m as well as higher ER/CC in non-breastfed versus breastfed infants at 12m.

The shift to a diet richer in fiber and protein, and the resulting GM diversification, is also clearly reflected at the metabolomic level. The decline in pyruvate after 6m following an initial increase, and its negative association with complementary feeding, likely reflects its conversion to downstream metabolites as the GM diversifies.[26] The increase in fiber intake was also evident by the rise of butyrate and propionate after 6m and their positive association with complementary feeding and cessation of breastfeeding, respectively, in agreement with Tsukuda *et al.*[27] That aligns well with the observed increase in the well-known butyrate producers within ER/CC.[28] The observed temporal increase in phenolic acids, alongside associations with breastfeeding and complementary feeding, is consistent with their diverse origins, including plants,[29] breastmilk,[30] and microbial flavonoid and tyrosine transformation.[29] Meanwhile, the higher levels of carnosine[31] and N2,N2-dimethylguanosine[32] in complementary-fed infants may indicate meat consumption.

A shift from a bifidobacteria-rich to a more adult-like microbiome was also evident by the change in microbial aromatic AA metabolites. As expected, the aromatic lactic acids ILA, 4-OH-PLA#, and PLA#, known to be produced by infant-type bifidobacterial species,[33] increased until 6m. Subsequently PLA# levels remained unchanged, whereas those of ILA and 4-OH-PLA# declined and were lower in infants who received no breastmilk at 12m, supporting Sillner *et al.*'s findings.[34] The observations align with the bifidobacterial decline at 12m. The aromatic acetic and propionic acids IAA, 4-OH-PAA, PAA, and 4-OH-PPA increased after 6m and were positively associated with complementary feeding, likely reflecting increased microbial protein degradation.[26] In contrast, IPA did not rise with age, however, it was detected in more infants at 12m compared to 6m and was positively associated with cessation of breastfeeding, suggesting that IPA producers are more common GM members at 12m. The phenylalanine-derived PAGIn and tryptophan-derived

indoxyl sulfuric acid, declined with the introduction of complementary feeding contrary to their expected increase.[26] These metabolites are of particular interest due to their known detrimental effects on health in adults and remain understudied in early life.[26] Though B vitamins can be obtained from the diet, including breastmilk,[35] their temporal rise is also likely attributed to microbial production as multiple GM members are well-established B vitamin producers.[36]

As anticipated, the abundance and diversity of secondary BAs increased with age and the two drivers of GM diversification: introduction to complementary foods and the cessation of breastfeeding. Similar to Sillner *et al.*[34] we report on less-studied secondary BAs (7ketoLCA, 3oxoCDCA, 7,12oxoLCA, 7oxoDCA, 3oxoCA, UCA) in infancy, along with almost complete absence of LCA and DCA until 12m.[34] The latter aligns with the observed increase in ER/CC well-known for its high 7α-dehydroxylating activity required for their production.[37] Meanwhile, the rise in glyco-BAs after 6m likely reflects the reduction in particularly effective glyco-BAs deconjugators bifidobacteria.[38]

The decline in acylcarnitines after 6m and their positive association with breastfeeding, along with the negative association of LCFAs with age suggest increasing reliance on beta oxidation. Production of conjugated linoleic and linolenic acid isomers by bifidobacteria[39] possibly also contributes to the decline in LA and ALA# before 6m, a period characterized by bifidobacterial dominance.

Multiple studies have shown strong fecal metabolome differences between breastfed and formula-fed infants.[23,34,40,41] However, unlike these studies, our cohort consisted of infants breastfed for at least 16 weeks, with formula-feeding often initiated alongside breastfeeding, mainly after the introduction of complementary feeding. The minor significant associations observed with formula feeding in this cohort, agree with He *et al.*,[40] who reported convergence of the metabolome profiles between breast-fed and formula-fed infants following complementary feeding.

Despite its known importance in shaping the GM,[42] delivery mode was not associated with microbiome differences in this cohort. It did, however, affect the metabolome, especially at baseline and up to 6m. Earlier studies reported no metabolome changes despite shifts in the microbiome composition,[43] or significant alterations that differ from our findings and between each other.[24,44] These discrepancies may reflect ethnic or age-related cohort differences. We found the HMO building-block Neu5Ac to be significantly higher in the vaginal compared to the C-section group and maternal ppBMI to be associated with C-section. Since ppBMI has been linked to HMO composition,[45] the difference in Neu5Ac may be attributed to variations in breastmilk composition. Another possibility is that vaginally-delivered infants' guts are richer in taxa like *Bacteroides* capable of cleaving sialic acids HMO residues.[46]

Unexpectedly, no metabolome differences were observed between the allergic groups at 6m and 12m, despite the emergence of allergic symptoms in this period. Feeding changes

and the resulting GM shifts may have masked these differences. Infants who developed allergy during the study did, however, have significantly higher baseline levels of the LCFAs, mainly polyunsaturated fatty acids (PUFAs), including n-6 LA and n-3 EPA, ALA#, DHA. Although elevated plasma n-3 and n-6 PUFA levels have also been reported in children with food allergy,[47] lower n-3 PUFA levels are generally associated with increased allergy risk.[48,49] Along with the higher LCFA levels, we observed lower *Bifidobacterium* spp. in the allergic group. The absence of significant correlation between the two, however, suggests that the lower LCFA levels in the allergic infants are unlikely to be due to bifidobacteria. Instead, the difference may be due to variations in mother's breastmilk composition,[50] microbial transformation,[39] or differences in intestinal absorption.

Our study has several limitations, including the small sample size, the wide age range at baseline, and the infrequent sampling. The limited sample size, especially in the allergic group, prevented a separate analysis of the different allergy types. To enhance metabolomic interpretation and clarify the GM–allergy link, future research should consider whole microbiome dynamics rather than focusing solely on specific taxa and prioritize integrated microbiome–metabolome analyses. Meanwhile, examining the circulating metabolome and breastmilk compositional analysis are of interest to respectively understand the plausible link between LCFAs and allergy and aid the interpretation of the delivery mode findings.

5. Conclusion

This study offers valuable new insights into the longitudinal fecal metabolome development in infancy, a critical period with lasting implications for immune system development. Our findings reveal substantial metabolomic shifts with age likely due to changes to the host metabolism, diet, and the GM. Notably, we show that C-section is significantly associated with fecal metabolome alterations up to 6m, though the health implications of these changes require further investigation. This study showed that low *Bifidobacterium* spp. and LCFAs precede allergy, indicating a temporal association that suggests a direction for follow-up studies on their potential role in allergy development.

Author contributions

M.V.S: Conceptualization, Investigation, Methodology, Formal Analysis, Visualization, Data curation, Writing – Original Draft Preparation; **P.Z.**: Conceptualization, Investigation, Methodology, Writing – Review & Editing; **A.K.**: Conceptualization, Supervision, Writing – Review & Editing; **The TEMPO study team**: Resources; **H.W.**: Conceptualization, Writing – review and editing **C.B.**: Conceptualization, Funding acquisition, Writing – review and editing; **A.C.H.**: Conceptualization, Supervision, Writing – Review & Editing; T.H.: Conceptualization, Supervision, Funding acquisition, Writing – review & editing.

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Conflict of interest statement

Harm Wopereis is an employee of Danone Research & Innovation. The project is part of a partnership programme between NWO-TTW and Danone Research & Innovation. The other authors declare that they have no known conflicts of interest.

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Data availability

The metabolomics data of this study are submitted in MetaboLights at www.ebi.ac.uk/metabolights/ with reference number MTBLS8954, along with limited clinical metadata. Additional individual-level metadata, even pseudonymized, are sensitive and are protected by the GDPR and not publicly available. Reasonable data sharing requests based on data processing and material transfer agreements can be made to Danone Research & Innovation (www.danoneresearch.com/).

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Supplementary Materials

Sample collection and storage procedure

Fecal samples were collected at home and immediately stored in freezers, then transferred on ice to the participant hospitals and stored at −80◦C until transfer to Danone Research & Innovation (Utrecht, The Netherlands) for wet sample aliquoting and fluorescence *in situ* hybridization (FISH), SCFAs and lactic acid analysis. Sample aliquots for LC-MS metabolomics analysis were transferred on dry ice to Leiden University and stored at −80◦C until analysis.

Data analysis details

For the 162 metabolites with missingness <20%, missing values were imputed using quantile regression imputation of left-censored data method.[1] Two LMMs were built to investigate the effect of age and diet on the fecal metabolome with age (days), breastfeeding (yes/no), formula feeding (yes/no), and complementary feeding (yes/no) used as fixed effects and subject ID as a random effect. The first model investigated the baseline and 6m measurements (*Metabolite ~ age + complementary feeding + formula feeding + (1|ID)*), whereas the second the 6m and 12m measurements (*Metabolite ~ age + complementary feeding + formula feeding + breastfeeding + (1|ID)*). Reference levels for the variables were the median age at baseline, "no" for complementary and formula feeding, and "yes" for breastfeeding. Breastfeeding was included only in the second model since a single subject was non-breastfed at 6m (**Table 1**). As infants' diet changed during the first year (**Table 1**), the feeding practices (breastfeeding, formula feeding, complementary feeding) at each visit were considered. Because of the change in feeding practices and due to the choice of reference levels, the breastfeeding, complementary feeding and formula feeding coefficients are interpreted respectively as cessation of breastfeeding, introduction of complementary feeding and introduction of formula feeding. To study the effect of delivery mode on the metabolome a LMM with age (days), delivery mode (Vaginal/C-section), and their interaction as fixed effects was used (*Metabolite ~ age + delivery mode + age:delivery mode + (1|ID)*). Reference levels for the variables were the median age at baseline and "Vaginal" delivery. Similarly, the LMM for allergy had age, allergy status at 12 months (allergic, non-allergic) and their interaction as fixed effects (*Metabolite ~ age + allergy status + age:allergy status + (1|ID)*). Median age at baseline and the non-allergic group were used as a reference. A separate LMM was then constructed after stratifying the allergy group by allergy type (IgE-mediated, non-IgE–mediated, and non-allergic), with the non-allergic group as the reference.

Supplementary References

1. Wei, R. et al. Missing Value Imputation Approach for Mass Spectrometry-based Metabolomics Data. Sci Rep 8, 663 (2018).

Table S1. Analyte names and abbreviations

Metabolite(s)	Abbreviation
1,3-Dimethylurate/1,7-Dimethylurate	1,3-Dimethylurate#
12-keto Chenodeoxycholic Acid	12ketoCDCA
12-keto Lithocholic Acid	12ketoLCA
p-Hydroxymandelate/3,4-Dihydroxyphenylacetic acid	3,4-Dihydroxyphenylacetic acid#
3-Hydroxyhippurate/2-Hydroxyhippurate/4-hydroxyhippurate	3-Hydroxyhippurate#
3-Methylhistidine/1-Methylhistidine	3-Methylhistidine#
3-Oxocholic Acid	3oxoCA
3-oxochenodeoxycholic acid	3oxoCDCA
3-oxo Deoxycholic Acid	3oxoDCA
Mandelic acid/4-Hydroxyphenylacetic acid	4-OH-PAA#
Dihydrocaffeic acid / 3-hydroxy-3-(3-hydroxyphenyl)propanoic acid/Hydroxyphenyllactic acid (4-OH-PLA/DHCA/HPHPA)	4-OH-PLA#
4-Hydroxyphenylpropionic acid	4-OH-PPA
5-Aminolevulinic acid/4-Hydroxyproline	5-Aminolevulinic acid#
7,12-Diketolithocholic Acid	7.12oxoLCA
7-Ketodeoxycholic acid	7ketoDCA
7-ketolithocholic acid	7ketoLCA
Arachidonic acid	AA
Adenosine/Deoxyguanosine	Adenosine#
Alpha-Linolenic acid/Gamma-Linolenic acid	ALA#
Alanine/beta-Alanine/Sarcosine	Alanine#
Allocholic acid	alloCA
Allolithocholic Acid	alloLCA
beta-Muricholic Acid	bMCA
Butyrylcarnitine/Isobutyrylcarnitine	Butyrylcarnitine#
Cholic acid	CA
Chenodeoxycholic acid	CDCA
Creatine/3-Guanidinopropanoate	Creatine#
Deoxycholic acid	DCA
Dihomo-alpha-linolenic acid; eicosatrienoic acid/Dihomo-gamma-linolenic acid (DGLA)	DGLA#
Docosahexaenoic acid	DHA
Docosapentaenoic acid	DPA
3-Epideoxycholic Acid	EDCA
Eicosapentaenoic acid	EPA
Alpha-aminobutyric acid/Gamma-aminobutyric acid	GABA#
Galactitol/Mannitol/Sorbitol	Galactitol#
Glycocholic acid	GCA
Glycochenodeoxycholic acid	GCDCA
Glycodeoxycholic acid	GDCA
Glycohyocholic Acid	GHCA
Glycohydeoxycholic acid	GHDCA
Glycolithocholic acid	GLCA
O-Acetylserine/Glutamic acid	Glutamate#
Guanosine/8-Hydroxy-2-deoxyguanosine	Guanosine#
Glycoursodeoxycholic acid	GUDCA

Metabolite(s)	Abbreviation
Hyocholic acid	HCA
Hyodeoxycholic acid	HDCA
Hyodeoxycholic acid	HDCA
Targinine/Homoarginine	Homoarginine#
Indoleacetic acid	IAA
Indolelactic acid	ILA
Indolepropionic acid	IPA
Isolithocholic Acid	isoLCA
Isoursodeoxycholic Acid	isoUDCA
Linoleic acid	LA
Lithocholic acid	LCA
Murideoxycholic acid	MDCA
3-Methylxanthine/1-Methylxanthine/7-Methylxanthine	Methylxanthine isomers
N-Acetylneuraminic Acid	Neu5Ac
Oleic acid	OA
Phenylacetylglutamine	PAGln
Paraxanthine/Theophylline	Paraxanthine#
Phenyllactic acid/3-(3-Hydroxyphenyl)propanoic acid	PLA#
Taurocholic acid	TCA
Taurochenodesoxycholic acid	TCDCA
Taurodeoxycholic acid	TDCA
Taurohyocholic acid	THCA
Taurohyodeoxycholic Acid	THDCA
Threonine/Homoserine	Threonine#
Taurolithocholic acid	TLCA
Taurolithocholic acid 3-sulfate	TLCA-3S
Trimethylamine N-oxide	TMAO
Total branched-chain short chain fatty acids	Total BSCFA
Total short-chain fatty acids	Total SCFA
Tauroursodeoxycholic Acid	TUDCA
Ursocholic acid	UCA
Ursodeoxycholic acid	UDCA

Table S2. Assessment of association between categorical clinical variables and either allergy status and delivery mode using Fisher's exact test. Only significant associations are displayed (P > 0.05)

Clinical Variables	Groups	P
dermatitis and eczema by 12 months	Allergic - Non allergic	2.8E-12
dermatitis and eczema by 6 months	Allergic - Non allergic	1.7E-09
rashes, eruptions and exanthems (unclassified cause) by 6 months	formula fed - not formula fed at 6 m	1.6E-04
rashes, eruptions and exanthems (unclassified cause) by 12 months	formula fed - not formula fed at 6 m	1.6E-04
mother's alcohol consumption prepregnancy	complementary-fed - not-complementary-fed at 6m	4.2E-03
mineral supplements by 12 months	Vaginal - C-section	4.7E-03
country	formula fed - not formula fed at 6 m	4.8E-03
ear infections by 12 months	Allergic - Non allergic	5.4E-03

Clinical Variables	Groups	P
analgesics by 6 months	complementary-fed - not-complementary-fed at 6m	1.0E-02
ophthalmological and otological preparations by 6 months	Vaginal - C-section	1.1E-02
analgesics by 12 months	formula fed - not formula fed at 6 m	1.2E-02
upper respiratory tract signs and symptoms by 12 months	Vaginal - C-section	1.5E-02
pets at home	complementary-fed - not-complementary-fed at 6m	1.6E-02
country	Allergic - Non allergic	1.8E-02
otologicals by 12 months	Allergic - Non allergic	1.9E-02
emollients and protectives by 6 months	Allergic - Non allergic	1.9E-02
emollients and protectives by 12 months	Allergic - Non allergic	1.9E-02
pets at home	breastfed - non breastfed at 12 m	1.9E-02
general signs and symptoms (unclassified cause) by 12 months	breastfed - non breastfed at 12 m	2.1E-02
dyspeptic signs and symptoms by 6 months	formula fed - not formula fed at 6 m	2.3E-02
dyspeptic signs and symptoms by 12 months	formula fed - not formula fed at 6 m	2.3E-02
rashes, eruptions and exanthems (unclassified cause) by 6 months	formula fed - not formula fed at 12 m	3.3E-02
rashes, eruptions and exanthems (unclassified cause) by 12 months	formula fed - not formula fed at 12 m	3.3E-02
non site specific injuries (unclassified cause) by 12 months	breastfed - non breastfed at 12 m	3.6E-02
ophthalmological and otological preparations by 12 months	Vaginal - C-section	3.7E-02
antihistamines for systemic use by 12 months	formula fed - not formula fed at 12 m	3.8E-02
daycare	complementary-fed - not-complementary-fed at 6m	3.9E-02
analgesics by 6 months	formula fed - not formula fed at 6 m	3.9E-02
antiinflammatory and antirheumatic products by 12 months	formula fed - not formula fed at 6 m	4.0E-02
antihemorrhagics by 6 months	breastfed - non breastfed at 12 m	4.0E-02
antihemorrhagics 12 months	breastfed - non breastfed at 12 m	4.0E-02
pets at home	Vaginal - C-section	4.1E-02
mother's alcohol consumption during lactation	complementary-fed - not-complementary-fed at 6m	4.9E-02
ophthalmological and otological preparations by 12 months	formula fed - not formula fed at 12 m	5.0E-02
nasal preparations by 6 months	breastfed - non breastfed at 12 m	5.7E-02

Table S3. Assessment of association between numeric clinical variables and either allergy status and delivery mode using Mann–Whitney U test. Only significant associations are displayed (P < 0.05)

Clinical Variable	Groups	P
gestational age	Vaginal - C-section	2.79E-03
maternal pre-pregnancy BMI	Vaginal - C-section	4.49E-02
number of vaccines by 6 months	Allergic - Non allergic	5.42E-02
age at baseline	Allergic - Non allergic	5.67E-02
number of vaccines by 12 months	Allergic - Non allergic	5.88E-02
birth length	breastfed - non breastfed at 12 m	7.44E-03
birth head circumference	breastfed - non breastfed at 12 m	2.26E-02
number of vaccines by 6 months	complementary-fed - not-complementary-fed at 6m	3.10E-02
number of vaccines by 12 months	complementary-fed - not-complementary-fed at 6m	3.54E-02

Table S4. Results of the LMM analysis examining the effect of age, breastfeeding, complementary feeding, and formula feeding. The table displays only the fixed-effect coefficients for formula feeding; coefficients for the other predictors are not shown and only estimates significant prior multiple testing correction (P < 0.05). Information on LMM used and all abbreviations can be found in Supplementary materials.

Metabolite	Model	Estimate	$P_{unadjusted}$	$P_{adjusted}$ (Q)
Thymine	baseline – 6m	-1.29E+00	3.19E-04	5.16E-02
2-Deoxyinosine	baseline – 6m	-9.42E-01	1.15E-02	4.08E-01
2-Deoxyuridine	baseline – 6m	-9.58E-01	8.88E-03	4.08E-01
Pantothenic acid	baseline – 6m	-9.06E-01	1.26E-02	4.08E-01
Pyridoxal	baseline – 6m	-9.61E-01	7.74E-03	4.08E-01
Nicotinic acid	baseline – 6m	-8.48E-01	1.60E-02	4.33E-01
Allantoin	baseline – 6m	7.88E-01	3.12E-02	4.77E-01
Guanosine#	baseline – 6m	8.01E-01	2.31E-02	4.77E-01
Propionylcarnitine	baseline – 6m	9.13E-01	3.24E-02	4.77E-01
Saccharopine	baseline – 6m	-7.06E-01	3.15E-02	4.77E-01
Uracil	baseline – 6m	-8.23E-01	2.61E-02	4.77E-01
Glutamate#	baseline – 6m	-8.03E-01	4.34E-02	5.86E-01

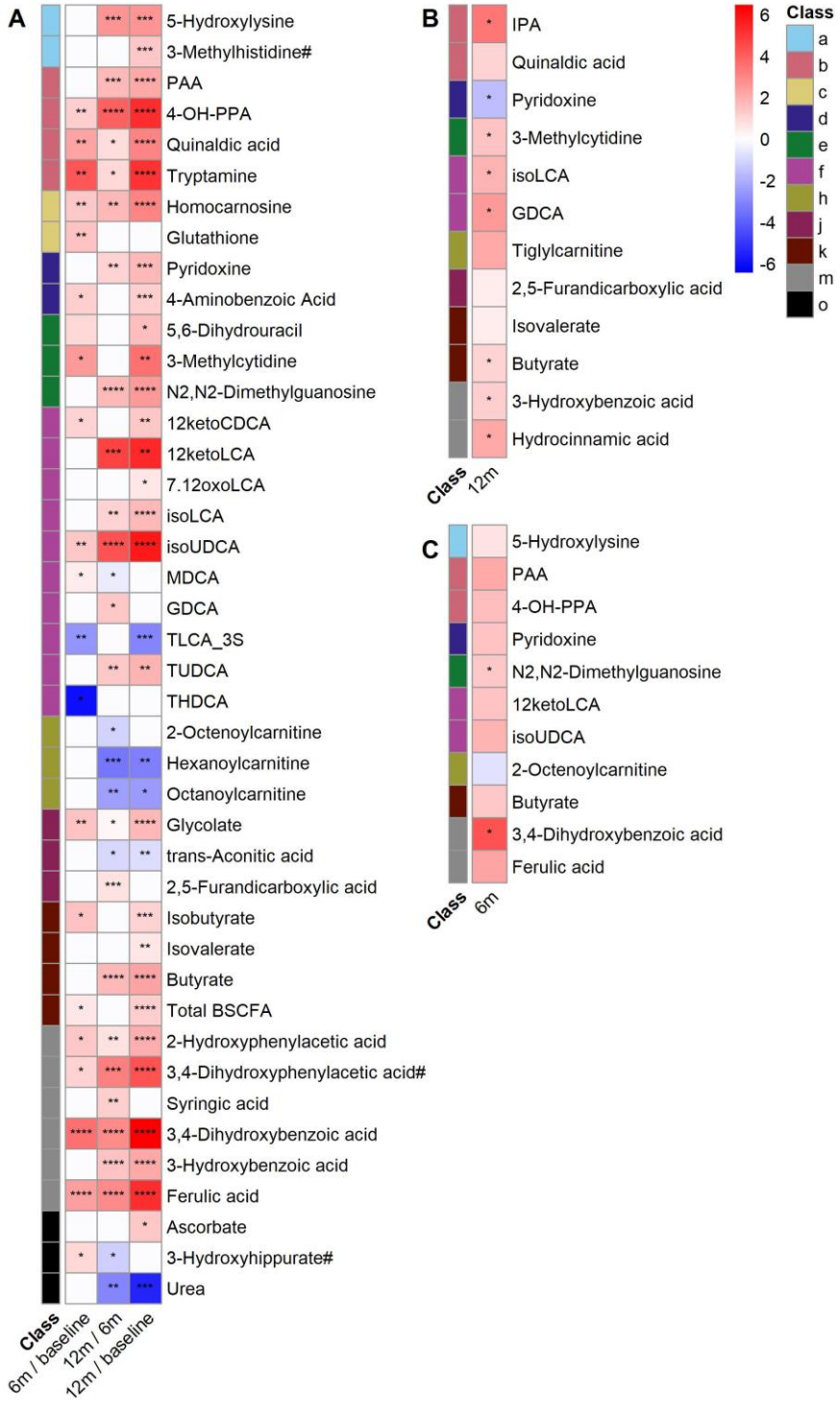
A
5-Hydroxylysine
3-Methylhistidine#
PAA
4-OH-PPA
Quinaldic acid
Tryptamine
Homocarnosine
Glutathione
Pyridoxine
4-Aminobenzoic Acid
5,6-Dihydrouracil
3-Methylcytidine
N2,N2-Dimethylguanosine
12ketoCDCA
12ketoLCA
7.12oxoLCA
isoLCA
isoUDCA
MDCA
GDCA
TLCA_3S
TUDCA
THDCA
2-Octenoylcarnitine
Hexanoylcarnitine
Octanoylcarnitine
Glycolate
trans-Aconitic acid
2,5-Furandicarboxylic acid
Isobutyrate
Isovalerate
Butyrate
Total BSCFA
2-Hydroxyphenylacetic acid
3,4-Dihydroxyphenylacetic acid#
Syringic acid
3,4-Dihydroxybenzoic acid
3-Hydroxybenzoic acid
Ferulic acid
Ascorbate
3-Hydroxyhippurate#
Urea
Class
6m / baseline
12m / 6m
12m / baseline
B
IPA
Quinaldic acid
Pyridoxine
3-Methylcytidine
isoLCA
GDCA
Tiglylcarnitine
2,5-Furandicarboxylic acid
Isovalerate
Butyrate
3-Hydroxybenzoic acid
Hydrocinnamic acid
Class
12m
6
4
2
0
-2
-4
-6
Class
a
b
c
d
e
f
h
j
k
m
o
C
5-Hydroxylysine
PAA
4-OH-PPA
Pyridoxine
N2,N2-Dimethylguanosine
12ketoLCA
isoUDCA
2-Octenoylcarnitine
Butyrate
3,4-Dihydroxybenzoic acid
Ferulic acid
Class
6m

Figure S1. *Comparison of the fold changes of metabolites A) between the visits, B) breastfeeding groups at 12m, C) complementary feeding at 6m. The comparison was performed using paired Mann-Whitney U test on the metabolites excluded for the LMM analysis due to high missingness. Colors represent the fold change: above 0 (red), below 0 (blue), p>0.05 (white). Asterisks indicate statistical significance: Q < 0.1 (*), Q < 0.01 (**), Q < 0.001 (***), Q > 0.001 (****). The "#" in the metabolite names indicates that the metabolite has coeluted with another target metabolite. Information on that and all abbreviations can be found in* ***Table S1***. *Class annotations: a - AAs and derivatives; b aromatic AAs metabolites; c - dipeptides and tripeptides; d - B vitamins and derivatives; e - nucleobases, nucleosides and derivatives; f – BAs; h – carnitines; j - organic acids; k – SCFAs; m - phenolic acids; o – other. The "#" in the metabolite names indicates that the metabolite coeluted with another target metabolite. All abbreviations and coeluting metabolites can be found in* ***Table S1***.

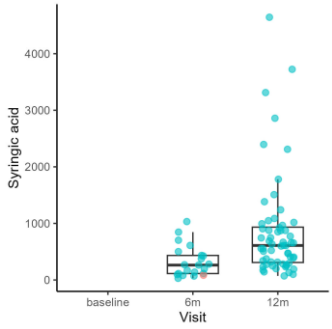

Figure S2. *Boxplots showing the distribution of syringic acid at the three visits (baseline, 6m, 12m). Individual data points are jittered and colored according to the complementary feeding status: blue – complementary-fed and orange: not complementary fed*

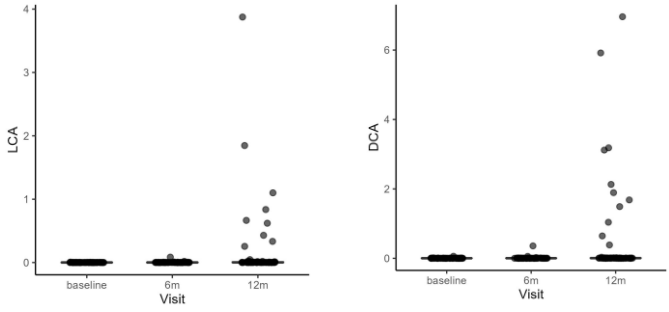

Figure S3. *Boxplots showing the distribution of LCA and DCA between the visits (baseline, 6m, 12m). Individual data points are jittered*

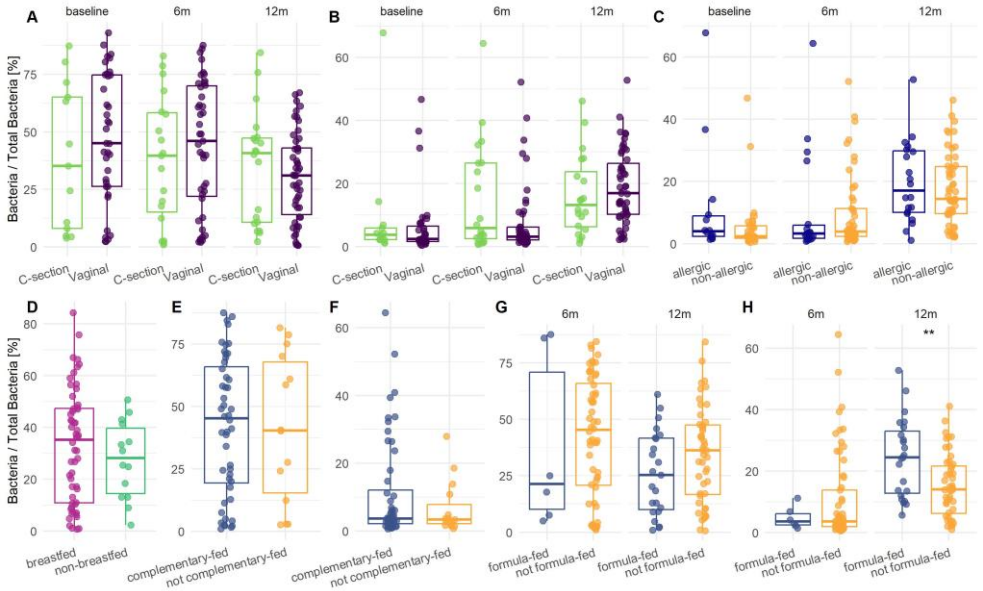

Figure S4. *FISH quantified levels of* ***A)*** *Bifidobacterium spp. between vaginal (purple) and C-section (green) deliveries;* ***B)*** *ER/CC between vaginal (purple) and C-section (green) deliveries;* ***C)*** *ER/CC between allergic (blue) and non-allergic (orange) infants);* ***D)*** *Bifidobacterium spp. between breastfed (pink) and non-breasted (green) infants at 12m;* ***E)*** *Bifidobacterium spp. between complementary-fed (blue) and not complementary-fed (orange) infants at 6m;* ***F)*** *ER/CC between complementary-fed (blue) and not complementary-fed (orange) infants at 6m;* ***G)*** *Bifidobacterium spp. between formula-fed (blue) and not formula-fed (orange) infants;* ***H)*** *ER/CC between formula-fed (blue) and not formula-fed (orange) infants. Statistical analysis was performed using Mann-Whitney test: P < 0.05 (*), P < 0.01 (**), P < 0.001 (***), P < 0.0001 (****). Number of measurements per group and visit: Bifidobacterium spp.: n = [50, 62, 70]; ER/CC: n = [48, 60, 71] for baseline, 6m and 12m, respectively.*

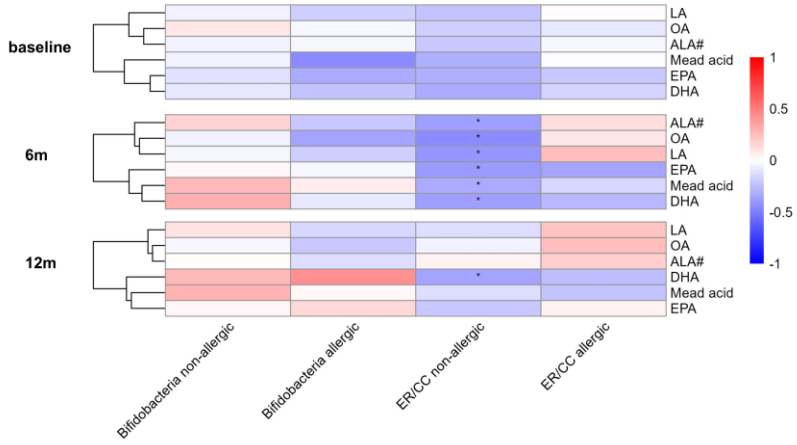

Figure S5. *Spearman correlation between the long-chain fatty acids found to be significantly higher at baseline in the allergic infants and Bifidobacterium spp. and ER/CC at baseline, 6m, 12m, Q < 0.1 (*). The "#" in the metabolite names indicates that the metabolite coeluted with another target metabolite. All abbreviations and coeluting metabolites can be found in* ***Table S1****.*

Chapter 4

Exploring the fecal metabolome in infants with cow's milk allergy: The distinct impacts of cow's milk protein tolerance acquisition and of synbiotic supplementation

Based on:

Exploring the fecal metabolome in infants with cow's milk allergy: The distinct impacts of cow's milk protein tolerance acquisition and of synbiotic supplementation

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Abstract

Scope: Cow's milk allergy (CMA) is one of the most prevalent food allergies in early childhood, often treated via elimination diets including standard amino acid-based formula or amino acid-based formula supplemented with synbiotic (AAF or AAF-S). This work aimed to assess the effect of cow's milk (CM) tolerance acquisition and synbiotic (inulin, oligofructose, *Bifidobacterium breve* M-16 V) supplementation on the fecal metabolome in infants with IgE-mediated CMA.

Methods and Results: The CMA-allergic infants received AAF or AAF-S for a year during which fecal samples were collected. The samples were subjected to metabolomics analyses covering gut microbial metabolites including SCFAs, tryptophan metabolites, and bile acids (BAs). Longitudinal data analysis suggested amino acids, BAs, and branched SCFAs alterations in infants who outgrew CMA during the intervention. Synbiotic supplementation significantly modified the fecal metabolome after 6 months of intervention, including altered purine, BA, and unsaturated fatty acid levels, and increased metabolites of infant-type *Bifidobacterium* species: indolelactic acid and 4-hydroxyphenyllactic acid.

Conclusion: This study offers no clear conclusion on the impact of CM-tolerance acquisition on the fecal metabolome. However, our results show that 6 months of synbiotic supplementation successfully altered fecal metabolome and suggest induced bifidobacteria activity, which subsequently declined after 12 months of intervention.

Keywords

early life, metabolomics, food allergy, bifidobacteria, fructooligosaccharides

Abbreviations

AAF: amino acid-based formula; **AAF-S**: amino acid-based formula with synbiotic; **BSCFA**: branched short-chain fatty acid; **BSH**: bile salt hydrolase; **CI**: confidence interval; **CM**: cow's milk; **CMA**: cow's milk allergy; **DBPCFC**: double-blind, placebo-controlled food challenge; **FOS**: fructooligosaccharide(s); **GM**: gut microbiome; **HMO**: human milk oligosaccharide; **IgE**: immunoglobin E; **LMM**: linear mixed model; **QC**: quality control; **RM-ASCA+**: repeated measures analysis of variance simultaneous component analysis+; **SCORAD**: SCORing Atopic Dermatitis; **T_{reg}**: regulatory T cell

1. Introduction

Cow's milk allergy (CMA), characterized by an immune-mediated response to cow's milk protein(s), is one of the major food allergies in early life.[1,2] Over the past decades, the estimated CMA prevalence in children of developed countries is approximately 0.5%–3%.[3,4] The allergic symptoms typically occur in the first year of life, whereas the resolution age varies and is related to the type of CMA.[5] Based on symptoms and pathophysiology, CMA is categorized into immunoglobin E (IgE)-mediated, non-IgE mediated, and mixed IgE CMA.[6] Subjects with IgE-mediated CMA, constituting approximately 60% of all CMA cases,[3] require longer time for tolerance acquisition to CM than non-IgE mediated CMA subjects.[7,8] In recent decades, the relevance of the gut microbiome (GM) in CMA has been highlighted, and studies show that compared to healthy counterparts, children with IgE-mediated CMA exhibit a reduction in bifidobacteria.[9]

Bifidobacteria, the prototypical health-promoting bacteria, are dominant inhabitants in a breast-fed infant's gut[10] and play a pivotal role in GM development in early life.[11,12] As coevolved bacteria, bifidobacteria possess unique glycosidases to digest complex host-derived glycans, particularly the human milk oligosaccharides (HMOs).[13,14] The oligosaccharide fermentation products not only satisfy the energy and carbon demands of bifidobacteria but also benefit other bacteria through cross-feeding activities, thereby contributing to maintaining the GM homeostasis in early life.[10,11]

Thus, bifidobacteria-related probiotics and HMO-mimicked prebiotics have gained popularity in the management of CMA in early life, alongside the conventional interventions with extensively hydrolyzed formula or amino acid-based formula (AAF).[15] Indigestible oligosaccharides, such as fructooligosaccharides (FOS) and galactooligosaccharides, are used as prebiotics due to their bifidogenic effect on the GM.[16] Bifidobacterium species, including *B. bifidum*,[17] *B. longum*,[18] and particularly *B. breve*,[18–21] are widely used probiotics for IgE-mediated CMA management in infants. These bifidobacteria have key immunomodulatory roles in the cross-talk between GM and host immune system: *B. bifidum*, for example, can induce the expression of FoxP3 in the regulatory T (Treg) cells through cell surface polysaccharides,[22] while B. longum in neonatal microbiota can alleviate the risk of allergy by promoting the Treg maturation;[23] *B. breve*, particularly the *B. breve* M-16V, can trigger the antiallergic process in early infancy by regulating the intestinal microbiota, intestinal epithelial barrier, and immune system.[24] Overall, bifidobacteria with HMO-utilization genes are found to induce intestinal IFN-β and silence Th2 and Th17 cytokines, thereby regulating the systemic immune balance in infants.[25] Additionally, by breaking down HMOs, bifidobacteria can indirectly enhance the production of butyrate[26] which is essential for the interplay between GM and systemic immunity,[27] possibly through epigenetic mechanisms.[28] Bifidobacteria-derived indolelactic acid (ILA) also actively engages in the immunoregulation during infancy.[25,29] However, despite these findings and the wide application of bifidobacteria-related interventions for IgE-mediated CMA,[17–21] none of the studies have reported comprehensive metabolome exploration.

In this study, we investigated longitudinal fecal metabolome changes of infants with IgE-mediated CMA undergoing dietary management with AAF, with and without synbiotic (*B. breve* M-16V; FOS: oligofructose, inulin). By applying linear mixed models (LMMs) and repeated measures analysis of variance simultaneous component analysis+ (RM-ASCA+), we compared the longitudinal fecal metabolome of infants with persistent CMA to those who developed CM-tolerance and identified key metabolic changes, associated with the synbiotic intervention.

2. Experimental section

2.1 Study design and dosage information

This study arises from a multicenter, randomized, double-blind, controlled clinical study PRESTO (registered as NTR3725 in Netherlands Trial Register). Detailed information on ethics committees, institutional review boards, and regulatory authorities that approved the study was previously published.[30]

PRESTO enrolled infants diagnosed with IgE-mediated CMA who then received either AAF (Nutricia, Liverpool, UK) or amino acid-based formula with synbiotic (AAF-S) to manage their CMA. The synbiotic blend consisted of chicory-derived neutral FOS: oligofructose and inulin in a 9:1 ratio (total concentration of 0.63g/100 mL formula, BENEO-Orafti SA, Oreye, Belgium) and *B. breve* M-16V (1.47×10^9 cfu/100 mL formula, Morinaga Milk Industry, Tokyo, Japan). Caretakers were instructed to provide subjects with a minimum daily dose of 450mL, 350mL, and 250mL for infants aged 0 to 8 months, 9 to 18 months, and older than 18 months, respectively.[19] After 12 months of intervention, the allergy status was re-evaluated through double-blind, placebo-controlled food challenge (DBPCFC) with CM. Detailed information on the diagnosis and reassessment was previously published.[19] Out of the 169 participants enrolled in PRESTO, 40 subjects (aged 3-13 months) were selected for this study based on sample availability. One subject was excluded due to unclear allergy status after 12 months.[30] Of the 16 AAF and 23 AAF-S participants, 10 and 14 infants, respectively, outgrew CMA within 12 months. Stool samples were available at 0 (baseline, TP0), 6 (TP1), and 12 months (TP2) after the start of the intervention, resulting in a total of 117 samples.

2.2 Sample collection and storage

The sample collection procedure has been described previously.[30] In short, fecal samples were collected at home and immediately stored in freezers, then transferred on ice to the participant hospitals and stored at -80°C until transfer to Danone Research & Innovation (Utrecht, the Netherlands) for wet sample aliquoting and SCFAs and lactic acid analysis. Sample aliquots for LC-MS metabolomics analysis were transferred on dry-ice to Leiden University and stored at -80°C until analysis.

2.3 Metabolomic analysis

2.3.1 SCFAs and lactic acid analysis

Quantitative SCFAs, including branched SCFAs (BSCFAs) analysis was performed using GC coupled to flame ionization detector and lactic acid was measured using lactic acid assay kit (Megazyme, Wicklow, Ireland) as previously described.[31]

2.3.2 LC-MS metabolomics analysis

The wet sample aliquots were lyophilized at 4 mbar and -110°C for 20h (Martin Christ Gefriertrocknungsanlagen GmbH, Germany), weighed (20±0.2mg), and stored at -80°C until extraction. Liquid-liquid extraction was performed as described by Hosseinkhani *et al.*[32] with adjusted sample amount and doubled solvent-to-feces ratio. Detailed information on the chemicals, the sample preparation, and the quality control (QC) is available in Supplementary Materials.

Polar to semi-polar metabolites, including acetylcarnitines, amines, benzenoids, organic acids, indoles, nucleosides, and nucleotides, were analyzed using reverse phase LC coupled with quadrupole (Q)-TOF-MS operated in full-scan positive and negative ionization modes, as described previously[33] and in the Supplementary Materials. Bile and fatty acids were measured using reverse phase LC separation using Q-TOF-MS operated in full scan negative ionization mode, as described in the Supplementary Materials.

Targeted peak integration was performed using SCIEX OS (version 2.1.6., SCIEX) with a maximum mass error of 10 ppm. The retention times were verified against authentic standards. In case of coelution, the targets were reported using the name or abbreviation of one of the targets followed by a "#". Details on the abbreviations used are listed in **Table S1** in Supplementary materials. For the polar to semi-polar metabolites, peak area was used for further data analysis, whereas for the bile and fatty acids, the area ratio of compounds to stable isotopically labeled standards (**Table S1** in Online Supplementary Materials) was used. Data quality inspection was performed using an in-house quality assurance software performing between batch correction and removal of metabolites with high technical variance (RSD of QC>30%).

2.3.3 Data analysis

Data handling and statistical analyses were performed in R (version 4.3.2). Metabolites with missingness above 20% and with median signal of the samples less than five times the mean signal of the procedure blanks were removed, leaving 166 metabolites. To identify group bias in missingness, Fisher's exact test was performed for metabolites with missingness above 20% at each time point after grouping the subjects by intervention or CM-tolerance status, and the results are summarized in **Table S2** in Online Supplementary Materials. Ratios of secondary to primary and unconjugated to conjugated bile acids (BAs) were added, resulting in a total of 177 variables. A list of the reported metabolites and their

abbreviations can be found in **Table S1**. The raw data were normalized by dry weight and subsequently log2-transformed. Missing values were imputed per metabolite using the quantile regression imputation of left-censored (QRILC) method.[34] Available clinical characteristics that potentially associated with CM-tolerance status at TP2 or intervention were analyzed with the two-sided Mann-Whitney U-test for numeric variables and the Fisher's exact test for binary variables as reported previously.[30,35]

To assess the change from TP0 to TP1 and TP2, LMMs were built using the lme4 package in R. Before building the model, the data was scaled by the standard deviation of all baseline samples. The metabolites were modelled as response variables with group and time as fixed effects and subject ID as a random effect. After grouping the subject by either their CM-tolerance status at TP2 (CM-allergic versus CM-tolerant) or intervention (AAF versus AAF-S), two models were built, namely tolerance-allergy and intervention. For the tolerance-allergy model (Metabolite ~ time + CM-tolerance_status + time:CM-tolerance_status + (1|ID)), TP0 and the CM-allergic group were used as references. Pairwise comparisons between groups at each time point and within a group between the time points were performed using the emmeans package in R. For the intervention model (Metabolite ~ time + time:intervention + (1|ID)), TP0 and the AAF group were used as references. The main effect of the intervention was removed from the model but its interaction with time was kept ensuring the groups are equal at baseline. The p values were calculated to assess a change from baseline with the Satterthwaite's degrees of freedom method using the lmerTest package within the ALASCA package.[36] In this study, the combined CM-tolerance status–intervention model was not performed because CM-tolerance acquisition as investigated in the parent study did not differ between the interventions at TP2 and aligned with natural rates of CMA outgrowth in infants.[19] For most metabolites, the addition of age as a covariate to models led to no improvement of the performance based on akaike information criterion (**Tables S4**-**S5** in Online Supplementary Materials). Therefore, age was not used as a covariate in the LMMs. Multiple testing correction was performed using the Benjamini-Hochberg method where Q<0.1 was considered as statistically significant.

Visualization of the longitudinal metabolomic alterations was achieved using RM-ASCA+ with ALASCA package,[36] as detailed in the Supplementary Materials. Performances of the analysis were validated using nonparametric bootstrapping, and the 95% confidence intervals (CI) were estimated based on 1000 resampling iterations.

2.4 16S rRNA gene sequencing and pre-processing

Extraction of DNA from stool samples and the subsequent gut microbiota profiling by 16S rRNA gene sequencing was performed as described previously.[30] Correlations between the changes in metabolites and the relative abundance of Bifidobacterium were examined using Spearman's rank correlation analysis. Relative abundance comparisons of Bifidobacterium between and within the AAF and AAF-S groups were evaluated with two-sided unpaired t-tests.

3. Results

3.1 Patient Characteristics

The statistical results of important clinical characteristics are summarized in **Tables S6-S7** in Online Supplementary Materials. When grouping the subjects by the CM-tolerance status at TP2, the father allergy occurrence and the SCORing Atopic Dermatitis (SCORAD) at baseline were significantly higher in the CM-allergic group than in the CM-tolerant group (**Table S6** in Online Supplementary Materials). None of the clinical characteristics were significantly different between AAF and AAF-S groups (**Table S7** in Online Supplementary Materials).

3.2 More pronounced fecal metabolome changes in the CM-tolerant group

Firstly, RM-ASCA+ was used to examine the longitudinal metabolome alterations within and between infants that remained allergic and those that acquired tolerance to CM by TP2 (CM-allergic vs. CM-tolerant). The PC1 score plot (**Figure 1A**) describes the direction of maximum variance in the modeled data, whereas the loadings plot (**Figure 1B**) highlights the top metabolites contributing to PC1. Metabolites with positive loadings follow the trend described by the score, whereas the opposite holds for metabolites with negative loadings. **Figure 1B** shows that almost half of the variation (47%) described by the fixed effects of the tolerance-allergy model was explained by PC1 (**Figure 1A**). The scores and loading for PC1 showed that over time ferulic acid, desaminotyrosine, pipecolic acid, 3-hydroxybenzoic acid increased, whereas dodecanoylcarnitine, pregnenolone sulfate, betaine, pyruvate decreased (**Figure 1**). Few BAs also showed overall change with time. The primary BAs cholic acid (CA), chenodeoxycholic acid (CDCA), and hyocholic acid (HCA) declined over time. In contrast, the secondary BAs deoxycholic acid (DCA) and the ratios of secondary to primary BAs, including DCA/CA, lithocholic acid (LCA)/CDCA, increased. Although with overlapped CIs between the two groups, those changes were more pronounced for the CM-tolerant group where the PC1 score declined more sharply than the CM-allergy group and for which the CI between the time points were separated, suggesting a significant time effect in this group.

Univariate marginal means comparison showed that around five times more metabolites were significantly altered over time in infants that acquired CM-tolerance versus those that remained CM-allergic (TP0-TP1: 9 metabolites in CM-tolerant vs. 2 metabolites in CM-allergic; TP0-TP2: 30 metabolites in CM-tolerant and 7 in CM-allergic; **Figure S1** and **Table S2**). Pregnenolone sulfate, pyroglutamic acid, pyruvate, oxoglutaric acid, and ferulic acid were significantly affected by time for both groups and followed comparable time-development trends (**Figure S1**). Similarly, arginine decreased, whereas 3-hydroxybenzoic acid, hydrocinnamic acid, LCA, DCA increased simultaneously in both groups, but significantly only in the CM-tolerant group (**Figure S1**). Pipecolic acid levels increased over time in both groups, but the rise was steeper and significant only in the CM-tolerant group.

Dodecanoylcarnitine followed the trend described by PC1 of the combined effect matrix (**Figure 1A**) with a decline in time at both TP1 and TP2 significant only in the CM-tolerant group. The rest of the significantly altered metabolites showed dissimilar longitudinal profiles between the groups (**Figure S1**). Butyric acid, PLA#, desaminotyrosine, and phenylacetic acid were significantly increased, whereas 5-hydroxytryptophan and the primary BAs CA and CDCA showed significant decreases in the CM-tolerant group only. In contrast, threonine#, and tryptophan significantly increased over time only in the CM-allergic group.

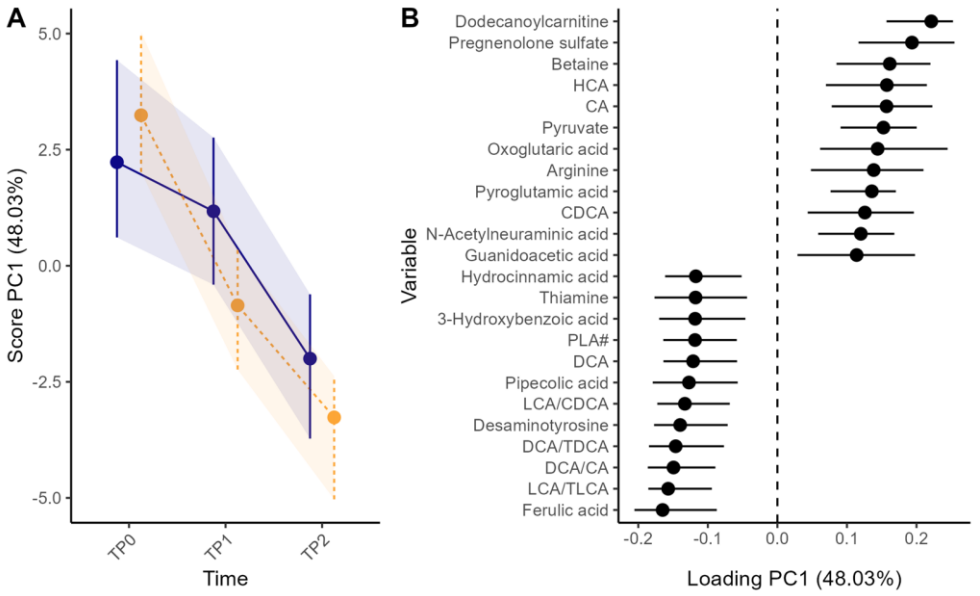

Figure 1. *RM-ASCA+ combined effect matrix showing the common metabolome development throughout the study for the CM-allergic (blue solid line, n=15) and CM-tolerant (orange dashed line, n=24) groups as scores (A) and loadings (B). Only the metabolites with 12 highest and 12 lowest loadings are shown in the plot. Error bars representing 95% CI were estimated based nonparametric bootstrapping.*

Next, the RM-ASCA+ interaction effect matrix was examined to focus on the alterations associated with CM-tolerance acquisition. The PC1 scores and loading of the interaction matrix, **Figure 2**, suggest that compared to the CM-allergic group, the CM-tolerant group showed overall alterations in amino acid metabolism with an increase in citrulline, lysine, N-acetyltyrosine, phenylacetic acid, gamma-aminobutyric acid (GABA#), glutamate, orotate, ornithine and a decrease in 5-hydroxytryptophan and serotonin. The BAs metabolism was also altered: decline in CDCA, CA, glycochenodeoxycholic acid (GCDCA), tauroursodeoxycholic acid (TUDCA), taurochenodeoxycholic acid (TCDCA) and increase in LCA/CDCA for the CM-tolerant group. The BSCFAs, isobutyrate and isovalerate, also contributed to PC1, showing higher levels in the CM-tolerant group. However, only citrulline and lysine were found significantly different at TP2 between the two groups univariately (**Table S6** in Online Supplementary Materials, **Figure S2**).

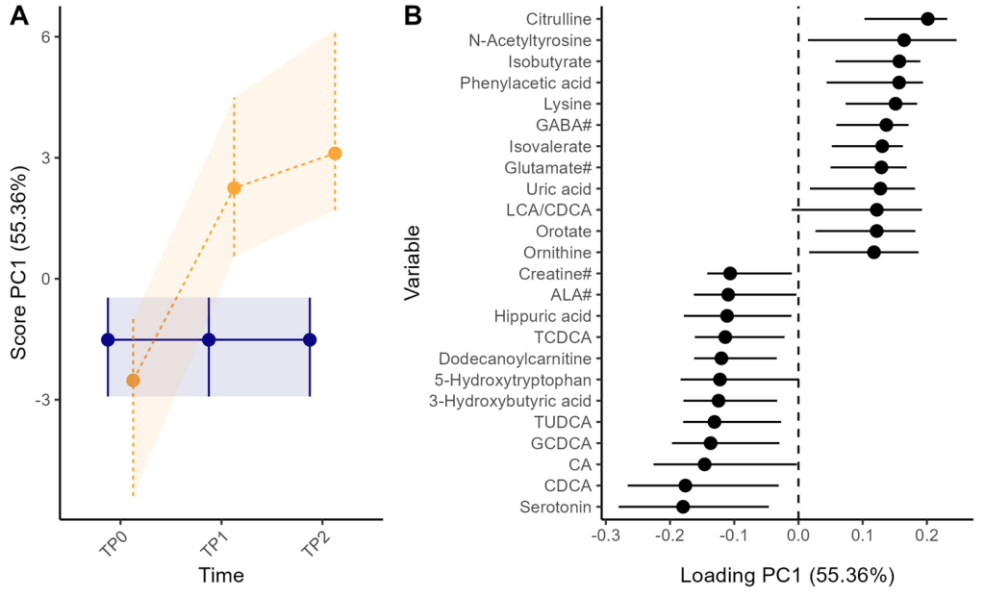

Figure 2. *RM-ASCA+ interaction effect matrix showing the metabolome differences between the CM-allergic (blue solid line, n=15) and CM-tolerant group (orange dashed line, n=24) over time as scores (A) and loadings (B). Only the metabolites with 12 highest and 12 lowest loadings are shown in the plot. Error bars representing 95% CI were estimated based nonparametric bootstrapping.*

3.3 Synbiotic supplementation altered fecal metabolome after six months of intervention

The longitudinal alterations of the fecal metabolome between the AAF and AAF-S group were studied to understand the effect of the synbiotic supplementation. As shown in **Figure 3**, clear group separation was observed in PC1 of the RM-ASCA+ interaction effect matrix, especially at TP1.

Among all the metabolites, 12 metabolites and three BA ratios were found to be statistically different between the AAF and AAF-S groups at TP1, and only inosine at TP2 (**Figure S3**, **Table S2**). The estimated marginal means plot of those analytes can be found in **Figure S3**. The synbiotic supplementation led to an increase of gut microbial metabolites indolelactic acid and 4-hydoxyphenyllactic acid (4-OH-PLA#) and a decline in the fatty acids linoleic acid (LA), alpha-linolenic acid (ALA#), and oleic acid (OA) at TP1 (**Figure 4**). Amino acid glutamine was also decreased in the AAF-S group at TP1. Three purine metabolites inosine, guanine, and adenine as well as the pyrimidine uridine were also affected by the intervention. Although adenine was higher upon the synbiotic addition, the opposite was true for inosine, guanine, and uridine. HCA and CDCA/GCDCA, CA/glycocholic acid (GCA), ursodeoxycholic acid (UDCA)/glycoursodeoxycholic acid (GUDCA) were all significantly higher in the AAF-S than in the AAF group at TP1, whereas GCDCA was significantly lower (**Figure 4**). A few other BAs were found to be among the main contributors to PC1 of the interaction matrix (**Figure 3**) or to have significant interaction coefficient at TP1 before multiple testing correction

(**Figure 4**), namely, the glyco-conjugated BAs GCA and GUDCA and the secondary BAs and their ratio to primary BAs: LCA, DCA, DCA/CA, and LCA/CDCA.

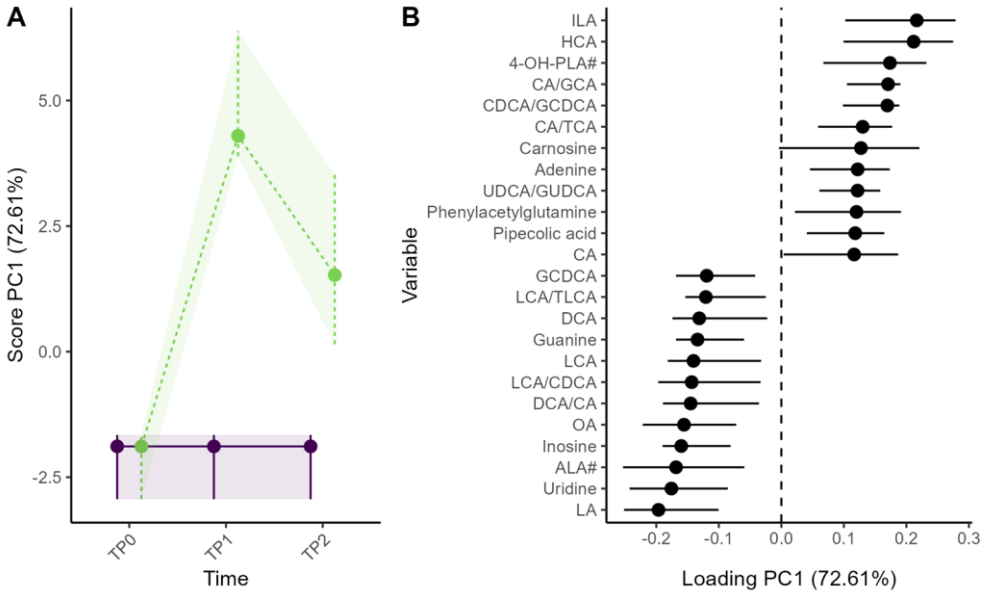

Figure 3. *RM-ASCA+ interaction effect matrix showing the metabolome differences between the AAF (purple solid line, n=16) and AAF-S (green dashed line, n=23) group over time as scores (A) and loadings (B). Only the metabolites with 12 highest and 12 lowest loadings are shown in the plot. Error bars representing 95% CI were estimated based nonparametric bootstrapping.*

3.4 Association between changes in Bifidobacterium and metabolites significantly altered by the synbiotic

The synbiotic supplementation significantly increased the relative abundance of Bifidobacterium in the AAF-S group from baseline to TP1 and TP2 compared to the AAF group (**Figure S4**).[35] To determine whether these increases were associated with the significantly changed metabolites, Spearman's rank correlation analysis was performed between the changes in metabolite levels and Bifidobacterium's relative abundance from baseline to TP1 (TP1-TP0) and TP2 (TP2-TP0), respectively (**Table S3**). In the AAF-S group, changes in ILA and 4-OH-PLA# from TP0 to later time points were positively correlated with those of Bifidobacterium ($r>0.6$, $p<0.005$), while changes in glutamine were negatively correlated ($r\leq-0.5$, $p<0.05$) (**Figure 5**). The changes in Bifidobacterium were positively correlated with those of adenine at TP1 and TP2 in both groups ($r>0.5$, $p<0.05$), and with CDCA/GCDCA and CA/GCA only at TP1 in the AAF-S group ($r>0.4$, $p<0.05$). Bifidobacterium also showed negative correlations with GCDCA and inosine in changes from TP0 to TP1 only in the AAF-S group ($r<-0.4$, $p<0.05$) (**Figure S5**).

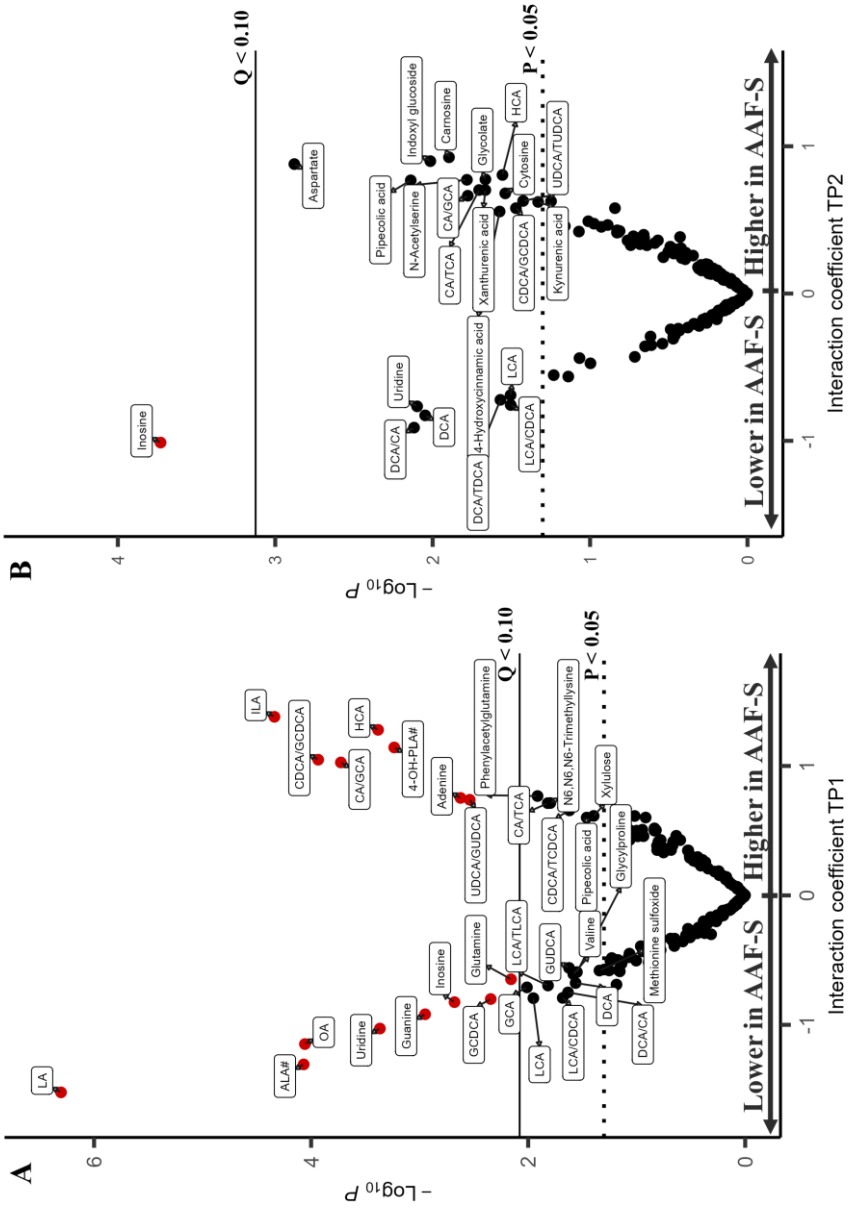

Figure 4. *Volcano plot showing the resulting p-value of the interaction coefficient for TP1 (left) and TP2 (right) in intervention LMM, dashed (p = 0.05), solid line (Q = 0.1) for TP1 (A) and TP2 (B). Red symbols indicate metabolites with Q<0.1 after Benjamini-Hochberg procedure.*

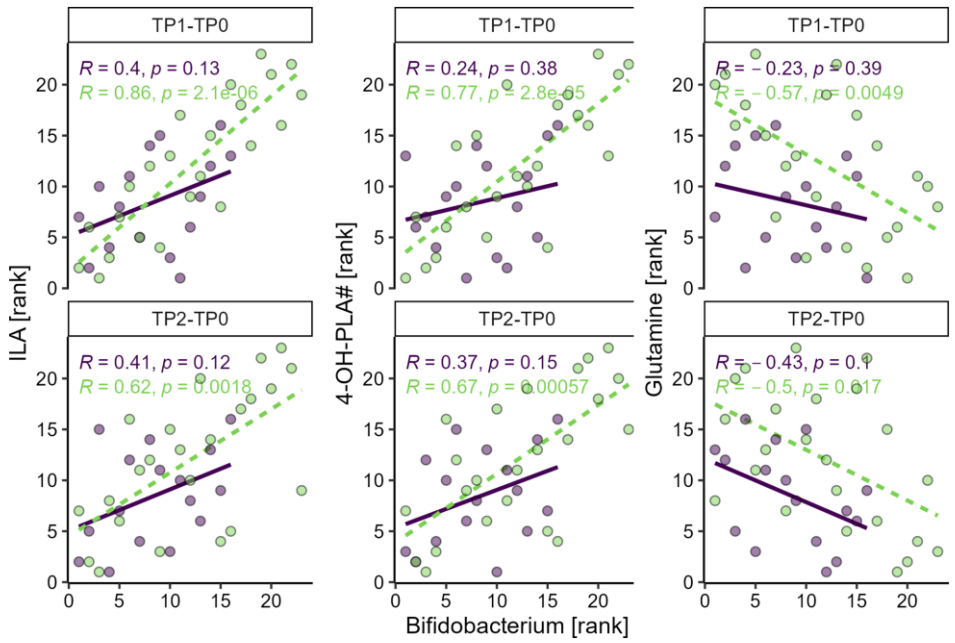

Figure 5. *Spearman's rank correlations between the changes in Bifidobacterium and ILA, 4-OH-PLA#, glutamine in AAF (purple solid line, n=16) and AAF-S (green dashed line, n=23) groups from baseline to TP1 (TP1-TP0) and TP2 (TP2-TP0). The rank of the changes in metabolite response and relative abundance of Bifidobacterium within each group were used for plotting. The figure shows p values; the Q values after Benjamini-Hochberg procedure are provided in Table S3.*

4. Discussion

In this study we followed the fecal metabolome alterations in infants with IgE-mediated CMA who received AAF with or without synbiotics for a year. Firstly, we examined the effect of CM-tolerance acquisition on the fecal metabolome over time. Time, reflecting growth and diet diversification, had a more pronounced impact on the metabolome than CM-tolerance acquisition (**Figure 1**, **Figure S1**). The diet enrichment was evidenced by the overall increase of the phenolic acids which are ubiquitously produced in plants,[37] including ferulic acid, 3-hydroxybenzoic acid, and hydrocinnamic acid. The decrease in the steroid hormone (pregnenolone sulfate), energy metabolites (pyruvate, oxoglutaric acid, and dodecanoylcarnitine), and the altered amino acids and derivatives (pyroglutamic acid, arginine, pipecolic acid) suggest metabolome modification associated with somatic growth.[38,39]

The multivariate RM-ASCA+ analysis showed an association of CM-tolerance acquisition status with alterations in amino acids, BAs, and (B)SCFAs (**Figure 2**). Compared to infants with persistent CMA, citrulline and lysine were significantly higher in the infants who developed CM-tolerance at TP2 (**Figure S2**). Lower plasma citrulline levels are known

marker of increased gut permeability,[40] which can raise the chance of allergen(s) passing the intestinal barrier and triggering the immune system.[41] The increase in fecal citrulline in the CM-tolerant group in this study might suggest improved gut barrier function and gut health. Although not significantly different between the two groups, the amino acids GABA#, glutamate#, threonine#, and ornithine were also higher in the CM-tolerant group compared to the CM-allergic group (**Figures S1-S2**). Lower fecal threonine levels have previously been reported in infants with IgE-mediated CMA compared to healthy controls.[42] Interestingly, although not significant, 5-hydroxytryptophan and serotonin were higher in the CM-allergic group at TP1 and TP2 (**Figure 2**), while their precursor tryptophan significantly declined only from TP0 to TP2 in this group (**Figure S1**). As serotonin is involved in intestinal epithelial proliferation[43] and plays an essential role in regulating intestinal inflammation,[44] the upregulated tryptophan-serotonin metabolism in the CM-allergic group may reflect an inflammatory state of the intestine in the CMA infants.

Children who outgrew CMA showed differences in their BAs profile. The primary BAs (CA, CDCA) significantly decreased, while the secondary BAs (DCA, LCA) and the secondary/primary BAs ratios (DCA/CA, LCA/CDCA) significantly increased from TP0 to TP2 only in the CM-tolerant group (**Figure S1**). A recent study found that, compared to healthy children, children with IgE-mediated CMA had lower ratios of fecal secondary/primary BAs from the CA pathway, with DCA and other oxidized keto BAs included in the calculation.[45] Secondary BAs from the CDCA pathway, including LCA, were reported lower in children with food allergy compared to healthy controls as well.[46] Although the secondary BAs and secondary/primary BAs ratios were not significantly different between the two groups in our study, the altered BAs profiles in the CMA-tolerant group likely indicate a more mature GM for secondary BAs production. This may contribute to improved intestinal functions in infants outgrowing CMA, as LCA is known to attenuate disruption in the intestinal barrier.[47]

(B)SCFAs were also altered during the CMA tolerance acquisition process. Butyrate significantly increased from TP0 to TP2 only in the CM-tolerant group (**Figure S1**). Isobutyrate and isovalerate tended to have group separation at TP1, with a continuous elevation in the CM-tolerant group over time, and a decrease at TP1 in the CM-allergic group (**Figure S2**). Consistent with our finding, those (B)SCFAs, specifically butyrate, are known for their antiinflammatory effects,[27,48] and are generally observed to be lower in feces of children with IgE-mediated food allergy.[42,48] Additionally, phenylalanine, phenyllactic acid (PLA#), and desaminotyrosine, which are GM metabolites from amino acids and dietary polyphenols,[49–51] were significantly increased from TP0 and TP2 only in the CM-tolerant group (**Figure S1**). The significant elevations of these metabolites may promote CM-tolerance acquisition, especially considering the recently recognized antiinflammatory property of desaminotyrosine.[52,53]

The synbiotic (*B. breve* M-16V, FOS: inulin, oligofructose) significantly altered the levels of aromatic lactic acids, purine metabolites as well as fatty acids and BAs, particularly after six months of intervention. The intervention enhanced ILA and 4-OH-PLA levels (**Figure S3**), and

their increases from baseline to TP1 and TP2 were positively correlated with those of bifidobacteria (**Figure 5**). This finding aligns with reports that ILA and 4-OH-PLA are metabolites of tryptophan[29,54,55] and tyrosine[29] produced by infant-type Bifidobacterium species, including *B. breve*. Earlier published microbiome and metaproteomics analysis of stool samples from the same clinical trial revealed that the synbiotic raised the level of bifidobacteria,[19,35] as well as bifidobacterial carbohydrate-active enzymes,[35] known to metabolize FOS.[56] Although the proportion of *Bifidobacterium* was significantly higher in the AAF-S group compared to the AAF group at both time points (**Figure S4**),[19,35] the increases in ILA and 4-OH-PLA# were significantly higher in the AAF-S group only at TP1. These results suggest that the synbiotic promoted the growth and/or the activity of aromatic lactic acids producers, for example, infant-type Bifidobacterium species, especially at TP1. This can be evidenced by stronger positive correlations between changes in the two aromatic lactic acids and bifidobacteria from baseline to TP1 than to TP2 in the AAF-S group (**Figure 5**). To validate our observations, *Bifidobacterium* species should be quantified. Alternatively, aromatic lactate dehydrogenase reported to convert tryptophan and tyrosine to respectively ILA and 4-OH-PLA in infant-type Bifidobacterium species should be analyzed.[29] The possibility that the ILA and 4-OH-PLA# were produced by some lactic acid bacteria should not be ignored either.[57,58] Overall, the increased ILA and 4-OH-PLA# levels in the AAF-S group suggest enhanced abundance or activity of infant-type bifidobacteria, supporting the successful synbiotic supplementation together with the microbiome and metaproteomics findings.[19,35] Although the parent study found that the CM-tolerance acquisition after 12 (TP2) and 24 months of synbiotic intervention aligned with natural outgrowth,[19] our findings, along with the reported antiinflammatory effect of ILA,[25,29,55,59] suggest that the synbiotic intervention may pose beneficial effects on infants' immune system. Further metabolomics studies on larger cohorts are required to verify this hypothesis.

In addition to the increase in ILA and 4-OH-PLA, the synbiotic lowered inosine, guanine, and uridine and raised adenine levels. The same purine-pyrimidine trend was observed in conventionally raised and core microbiota-colonized mice in comparison to germ-free mice,[60] indicating the importance of the GM in purine and pyrimidine metabolism.[60] A decline of inosine and uridine has also been reported in co-culture of *B. breve* with small intestinal-like epithelial cells.[61] *Lactobacillus brevis*, belonging to the *Lactobacillaceae* family, was found to be elevated in the AAF-S group for the same set of samples[35] and was also reported to have inosine degradation capabilities.[62] To link the purine-pyrimidine metabolism to the gut microbiome, and the role of *Bifidobacterium* spp. and *Lactobacillaceae* spp. herein, more research is required.

The AAF-S intervention lowered LA, ALA#, and OA levels, suggesting high consumption of these fatty acids by gut bacteria. This may be a result of hydration by bacteria of the Lactobacillus and Bifidobacterium genera[63] or production of conjugated fatty acids.[64–68]

Bifidobacterium strains, especially *B. breve*, are among the best producers of conjugated linoleic acids[66,67] and conjugated linolenic acids.[66,68]

The synbiotic enhanced the deconjugation of BAs, especially at TP1, where significantly decreased GCDCA and increased CDCA/GCDCA, CA/GCA, and UDCA/GUDCA were observed in the AAF-S compared to AAF group (**Figure 4**). Bifidobacterium, in general, are active bile salt hydrolase (BSH) producers,[69] which perform preferred deconjugation activity on glyco-conjugated BAs.[70] This aligns with our results showing that *Bifidobacterium* changes from baseline correlated negatively with those of GCDCA, and positively with those of CA/GCA and CDCA/GCDCA at TP1 in the AAF-S (**Figure S5**). These correlations in changes disappeared at TP2, possibly due to increased GM diversity. Compared to TP0, families from other phyla, including Bacteroidetes, Firmicutes, and Proteobacteria, were more abundant at later timepoints in both groups, especially at TP2.[35] These bacteria have also been identified as active BSH producers,[71] thus might eliminate the correlation between the activity of BAs deconjugation and Bifidobacterium. Unexpectedly, the increased deconjugation activity of BAs failed to promote the production DCA and LCA. In contrast, although not significant, their levels and ratios to precursors (DCA/CA, LCA/CDCA) were lower in the AAF-S than the AAF group (**Figure 4**). Considering that the conversion of primary BAs to secondary ones is highly conserved in bacteria with the bai operon,[72] and that the host liver can further hydroxylate secondary BAs to tertiary BAs after gut-liver circulation,[73] it is likely that more complex mechanisms underlie the host-gut metabolism of BAs during the intervention.

Our study has several limitations, including the wide age range of the participants at baseline of 3-13 (9.00 ± 2.90) months. Considering the rapid development of the GM in the first two years of life,[39] the wide age range may obscure the observation of fecal metabolome alterations related to CM-tolerance acquisition and the effect of intervention. Another limitation is the lack of information on the CM-tolerance status at TP1. Knowing the status at TP1 could have aided in the interpretation of CM-tolerance acquisition results. The research carried out for this paper is exploratory due to the small samples size (39 subjects). Increasing the sample size is necessary to verify these findings and would also allow to build LMM and RM-ASCA+ models following the intervention and CM-tolerance acquisition simultaneously. In addition, the parent study concluded that the synbiotic supplementation did not significantly affect CMA-resolution. Thus, in this study we cannot draw any conclusions regarding the clinical benefits of the synbiotic supplementation on CM-tolerance acquisition based on fecal metabolome alterations. Despite those limitations, our study revealed several fecal metabolome pathway alterations which may contribute to CMA outgrowth. Most importantly, we found that the AAF-S significantly altered the fecal metabolome after six months of the intervention, not after 12 months, suggesting that early intervention is required to maximize the effect of synbiotics. These findings aid in understanding the link between IgE-mediated CMA-tolerance acquisition, GM, and synbiotics intervention.

Author contributions

M.V.S: Conceptualization, Investigation, Methodology, Formal Analysis, Visualization, Data curation, Writing – Original Draft Preparation; **P.Z.**: Conceptualization, Investigation, Methodology, Writing – Review & Editing; **A.K.**: Conceptualization, Supervision, Writing – Review & Editing; **The TEMPO study team**: Resources; **H.W.**: Conceptualization, Writing – review and editing **C.B.**: Conceptualization, Funding acquisition, Writing – review and editing; **A.C.H.**: Conceptualization, Supervision, Writing – Review & Editing; T.H.: Conceptualization, Supervision, Funding acquisition, Writing – review & editing.

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Conflict of interest statement

Harm Wopereis is an employee of Danone Research & Innovation. The project is part of a partnership programme between NWO-TTW and Danone Research & Innovation. The other authors declare that they have no known conflicts of interest.

Data Availability Statement

The data that support the findings of this study are openly available in MetaboLights at **www.ebi.ac.uk/metabolights/**, reference number MTBLS12775.

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Supplementary Materials

Online Supplementary Materials are available in the original manuscript:
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Chemicals

Methyl tert-butyl ether (MTBE, ≥99.8%) and ammonium formate (≥99.0%) were purchased from Sigma Aldrich (St. Louis, United States). LC-MS-grade methanol (MeOH), isopropanol and formic acid (FA) were purchased from Biosolve B.V. (Valkenswaard, Netherlands). LC-MS grade acetonitrile was purchased from Actu-all chemicals (Randmeer, The Netherlands) and Biosolve B.V. (Valkenswaard, Netherlacnjugnds). Purified water was obtained from a Milli-Q PF Plus system (Merck Millipore, Burlington, United States). List of the isotopically labelled standards (SILs), including supplier details, can be found in **Table S1** in Online Supplementary Materials.

Sample preparation

Briefly, 72 µL of water and 216 µL MeOH, containing stable isotopically labelled standards (SILs) (**Table S1** in Online Supplementary Materials), were added to the 20 mg dry-weight fecal sample. After a 3-minute vortex mixing (Marshall Scientific, Cambridge, UK) 120 µL ice-cold MTBE was added, followed by another 3-minute vortex mixing. Following a brief centrifugation (30s, 100g, 4 °C), 200 µL of water and 168 µL of MTBE were added. The samples were vortex mixed for another 3 min, incubated at 4°C for 10 minutes until centrifugation (20 min, 16 000g, 4°C) inducing aqueous and organic layer separation. All solvents used during the LLE were ice-cold and vortex mixing was always at maximum speed. Following layer separation, each layer was transferred to an Eppendorf tube, followed by 5 and 2.5 minutes of centrifugation (16000g, 4°C) for aqueous and organic layers respectively. After extraction, 150 µL of the aqueous layer was aliquoted for polar to semi-polar metabolites analysis, while 48.8 µL of aqueous and 28.8 µL of organic layer was combined for the bile and fatty acids analysis. The aliquots were dried in a Speedvac (Labcono, USA) and stored at -80°C. Prior to LC-MS analysis, the extracts were reconstituted in 50 µL of 0.1% FA in water for polar to semi-polar metabolites analysis, and 200 µL of MeOH for the bile and fatty acids analysis. The reconstitution solvents contained different SILs (**Table S1** in Online Supplementary Materials).

Quality Control

Samples were randomized into two batches, with those from the same subject prepared and measured in the same batch. For the preparation of the quality control sample, 30 study samples were weighed and extracted. After the extraction, equal volumes of each layer were taken from each sample and pooled, resulting in pooled QC aqueous and organic layers. Those pooled layers were used to prepare QC samples for each platform. The LLE and aliquoting steps were performed as described in Sample preparation.

LC-MS analysis of polar to semi polar metabolites

Analysis of polar to semi-polar metabolites was performed on a UPLC-TOF/MS system consisting of a Shimadzu LC system coupled to a TripleTOF 6600 mass spectrometer (SCIEX, Foster City, CA, USA) with an electrospray ionization source (ESI) that operated at both positive and negative ion modes. The ESI source parameters were set as follows: spray voltage ±4.5 kV, capillary temperature 400 ◦C, sheath gas 40, auxiliary gas 40, curtain gas 45. Data were acquired under full scan mode over the m/z range of 60-800 Da. The LC separation was carried out at 40 °C using a Waters Acquity UPLC HSS T3 column (1.8 µm, 2.1 mm × 100 mm) with pre-column in-line stainless steel filter (0.3 µm, Agilent Technologies, Waldbronn, Germany). The mobile phase A was 0.1% FA in water, and the mobile phase B was 0.1% FA in ACN (Actu-all chemicals). With a flow rate of 0.4 mL min-1 and 1 µL of injection volume, the gradient starts at 100% A; 0–0.5 min 80% A; 0.5–2.5 min 2% A; 2.5–7.5 min 2% A; 7.5–12 min 2% A; 12 – 15 100% A. The data were acquired under full scan mode over the m/z range of 60-800 Da with Analyst TF software 1.7.1 (SCIEX) in negative and positive ionization modes. The preferred ionization mode for metabolites detectable in both polarities was chosen based on lower RSD% and higher signal-to-noise ratio of the QC samples.

LC-MS analysis of bile acid and fatty acids

Analysis of bile and fatty acids was performed on an UPLC-TOF/MS system consisting of ExionLC™ AC UHPLC system and SCIEX ZenoTOF 7600 system (Darmstadt, Germany) equipped with an IonDrive™ Turbo V Source, operated in negative ESI mode. The ion source conditions were as follows: spray voltage of 4.5 kV, capillary temperature of 550°C, ion source gas 1 50 psi, ion source gas 2 50 psi, curtain gas 35 psi, CAD gas 7 psi. The MS data was acquired under full scan mode over the m/z range of 200-900 Da. Accumulation time was set to 0.25 s, delustering potential to -70V and collision energy to -10eV. Chromatographic separation was performed on a Waters Acquity UPLC HSS T3 column (1.8 µm, 2.1 mm × 100 mm) with pre-column in-line stainless steel filter (0.3 µm, Agilent Technologies, Waldbronn, Germany). The flow rate was set at 0.4 ml min-1, the column was kept at 45 °C, injection volume at 2 µL. Mobile phase A consisted of 10 mM ammonium formate in water/ACN (Biosolve B.V) (95:5, v:v), while mobile phase B was 10 mM ammonium formate in MeOH/water (99:1, v:v). The gradient was as follows: starting at 0% B; 0–0.2 min 70% B; 0.2–7.5 min 100% B; 7.5–11.5 min 100% B; 11.5–11.6 min 0% B; 11.6 – 15 0% B. Isopropanol was used as an external rinsing solution (2 s sip time + rinse port). The flow was directed to waste in the first minute of the run. The autosampler temperature was set at 10 °C. Data acquisition was carried out on SCIEX OS 2.1.6.

Visualization RM-ASCA+

Visualization of the longitudinal metabolomic alterations was achieved using RM-ASCA+, which is an extension of LMMs for multivariate data. In the first step, LMMs are used to decompose the response matrix into effect matrices. The effect matrices are then analyzed

using principal component analysis (PCA), and the results are summarized into PCA scores and loadings. The LMMs used for RM-ASCA+ were the LMMs used for the univariate analysis. The visualized effect matrices included the time effect matrix ('time') which shows time development of the reference group over time. The interaction matrix ('time:group') and the group-interaction matrix ('group + time:group') both show the deviations of the study group compared to the reference group over time with the latter also displaying the baseline differences. Lastly, the combined matrix ('time + time:group' or 'time + group + time:group') shows the time development of both the study and the reference group.

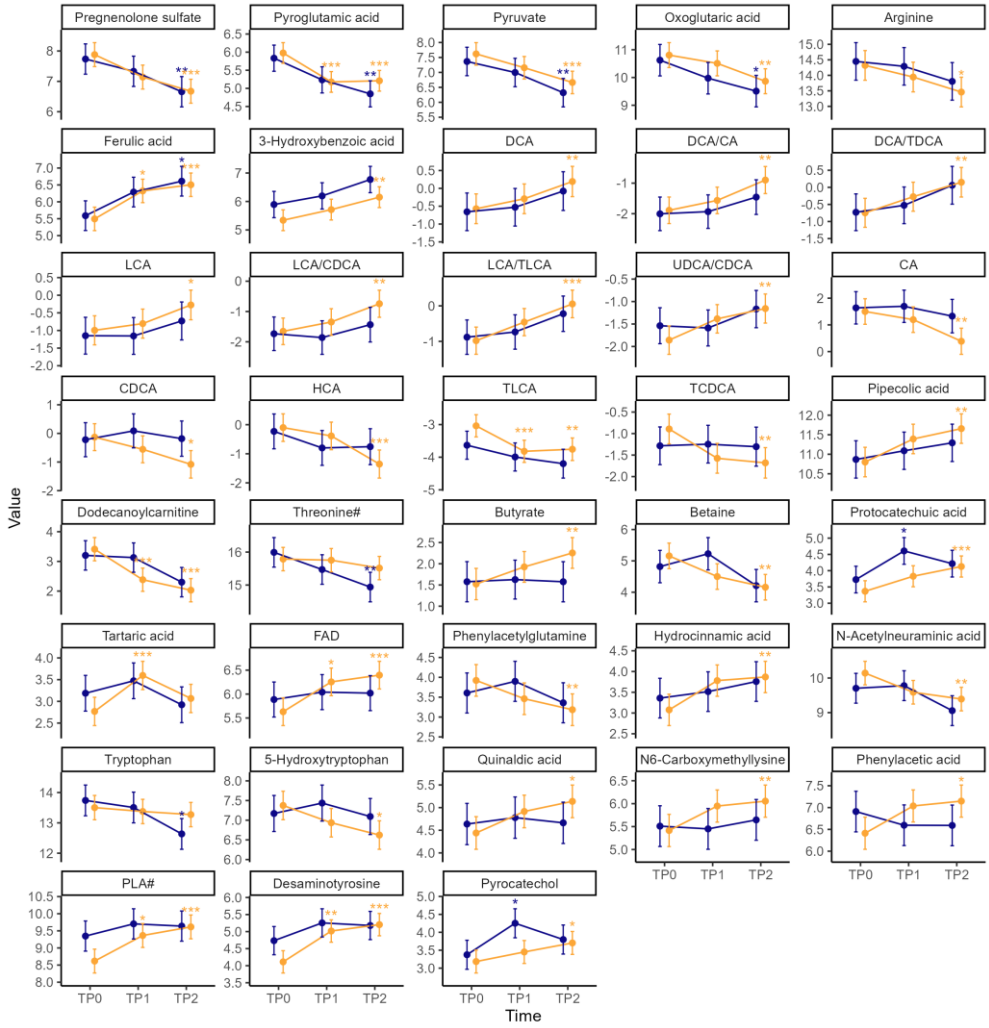

Figure S1. *Marginal means estimated from the LMMs for participants who acquired tolerance (CM-tolerant, orange) and those that remained allergic (CM-allergic, blue). Only the metabolites for which pairwise comparison in time was found significant are plotted. The q-values are based on the marginal mean comparison to TP0 for each group, q < 0.01 (***), q < 0.05 (**), q <0.1 (*).*

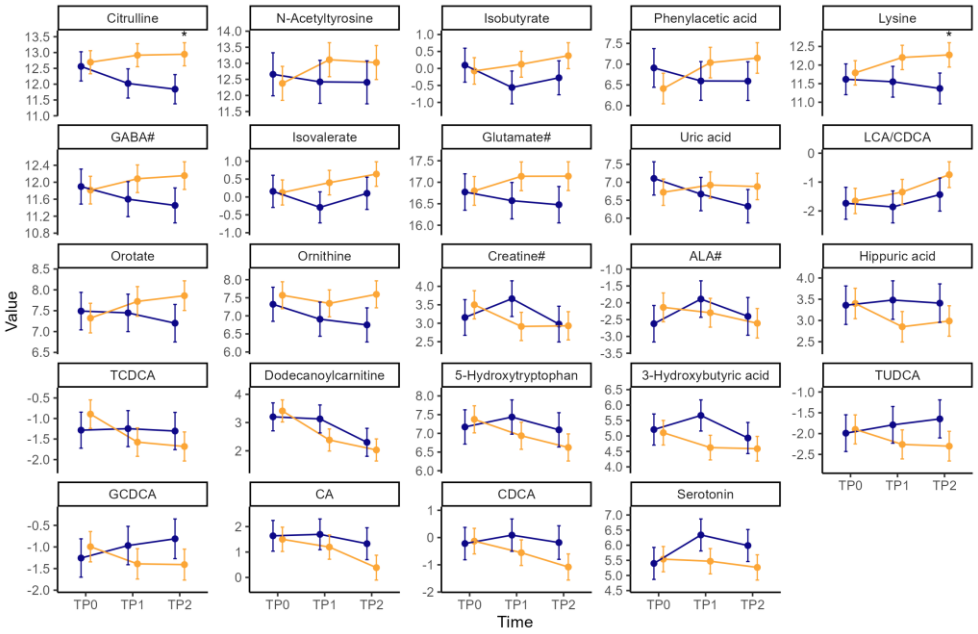

Figure S2. *Marginal means estimated from the LMMs for participants who acquired tolerance (CM-tolerant, orange) and those that remained allergic (CM-allergic, blue). The metabolites with top loadings in PC1 of the RM-ASCA+ interaction matrix are plotted. The q-values are based on the marginal mean comparison between the groups at each time point, q <0.1 (*).*

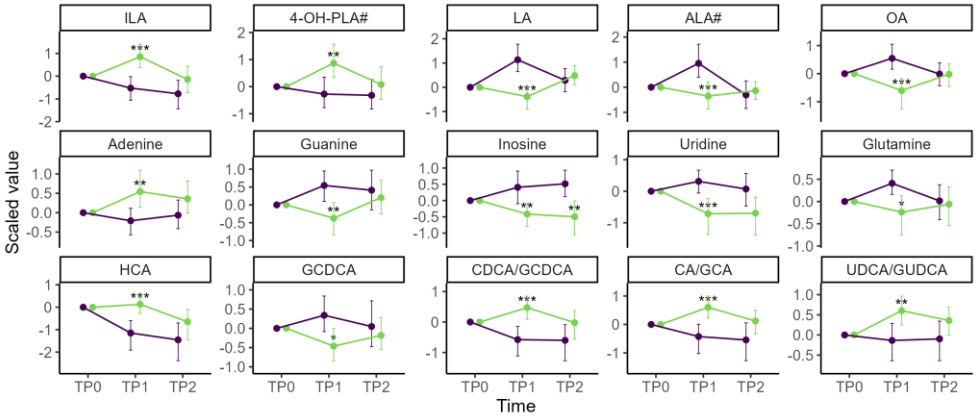

Figure S3. *Marginal means estimated from the LMMs for AAF (purple) and AAF-S (green) group. Only the metabolites for which an interaction coefficient was found significant are plotted. The response has been scaled. The q-values are based on/denote the significant between-group change in the within-group change from baseline. q < 0.01 (***), q < 0.05 (**), q <0.1 (*)*

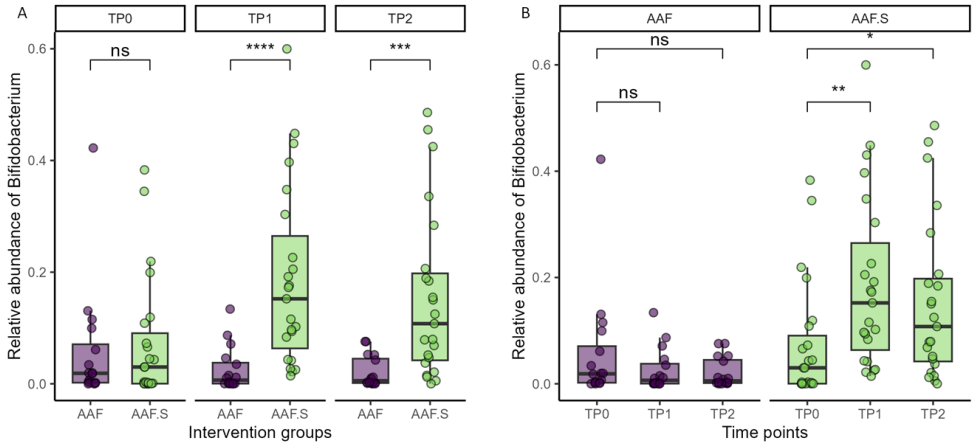

Figure S4. *Relative abundance of Bifidobacterium comparisons between AAF and AAF-S groups at each time point (A), and between time points in each group (B). Statistical significance was evaluated with two-side unpaired t-tests; p > 0.05 (ns), p ≤ 0.05 (*), p ≤ 0.01 (**), p ≤ 0.001 (***), p ≤ 0.0001 (****).*

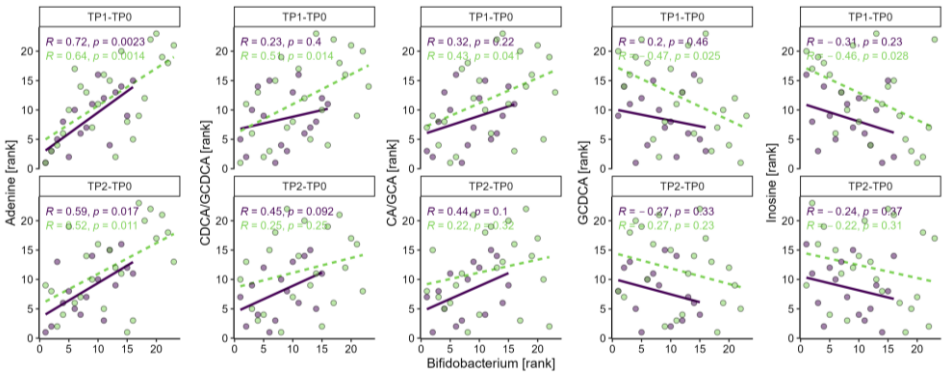

Figure S5. *Spearman's rank correlations between the changes in Bifidobacterium and adenine, CDCA/GCDCA, CA/GCA. GCDCA, inosine in AAF (purple solid line) and AAF-S (green dashed line) groups from baseline to TP1 (TP1-TP0) and TP2 (TP2-TP0). The rank of the changes in metabolite response and relative abundance of Bifidobacterium within each group were used for plotting.*

Table S1. Abbreviations of target analytes. A name or abbreviation followed by "#" indicates coelution with other targets.

Compound name	Abbreviations
Dihydrocaffeic acid/3-hydroxy-3-(3-hydroxyphenyl)propanoic acid/Hydroxyphenyllactic acid	4-OH-PLA#
3-Methylxanthine/1-Methylxanthine/ 7-Methylxanthine	Methylxanthine isomers
Phenyllactic acid/3-(3-Hydroxyphenyl)propanoic acid	PLA#
p-Hydroxyphenylacetic acid/Mandelic acid	p-Hydroxyphenylacetic acid#
alpha-Aminobutyric acid/gamma-Aminobutyric acid/3-Aminoisobutanoic acid/Dimethylglycine	GABA#
Indolelactic acid	ILA
myo-Inositol/ Galactose/ Fructose	Fructose#
O-Acetylserine/Glutamic acid	Glutamate#
1-Methyladenosine/N6-Methyladenosine/2'-O-Methyladenosine	1-Methyladenosine#
5-Aminolevulinic acid/4-Hydroxyproline	5-Aminolevulinic acid#
Adenosine/Deoxyguanosine	Adenosine#
Alanine/beta-Alanine/Sarcosine	Alanine#
Creatine/Beta-Guanidinopropionic acid	Creatine#
Cytidine	Cytidine
Targinine/Homoarginine	Homoarginine#
N1-Methyl-4-pyridone-3-carboxamide/Nudifloramide	Nudifloramide#
Symmetric dimethylarginine/Asymmetric dimethylarginine	SDMA#
Threonine/Homoserine	Threonine#
Cholic acid	CA
Chenodeoxycholic acid	CDCA
Deoxycholic acid	DCA
Oleic acid	OA
Linoleic acid	LA
alpha-Linolenic acid/gamma-Linolenic acid	ALA#
Dihomo-gamma-linolenic acid/Dihomo-alpha-linolenic acid	DGLA
Arachidonic acid	AA
Eicosapentaenoic acid	EPA
4,8,12,15,19-Docosapentaenoic acid	DPA
Docosahexaenoic acid	DHA
Glycocholic acid	GCA
Glycochenodeoxycholic acid	GCDCA
Glycoursodeoxycholic acid	GUDCA
Hyocholic acid	HCA
Lithocholic acid	LCA
Taurocholic acid	TCA
Taurochenodesoxycholic acid	TCDCA
Taurodeoxycholic acid	TDCA
Tauroursodeoxycholic acid	TUDCA
Taurolithocholic acid	TLCA
Ursodeoxycholic acid	UDCA

Table S2. Significantly altered metabolites in CM-allergic and CM-tolerant groups from marginal means comparison

CM-Allergic				
Metabolite	TP0	TP1	P value	Q value
Protocatechuic acid	3.727 (3.317, 4.137)	4.607 (4.197, 5.017)	0.0006	0.0674
Pyrocatechol	3.374 (2.969, 3.778)	4.252 (3.847, 4.656)	0.0008	0.0674
CM-Allergic				
Metabolite	TP0	TP2	P value	Q value
Pyroglutamic acid	5.833 (5.472, 6.194)	4.849 (4.489, 5.21)	0.0002	0.0328
Threonine#	15.992 (15.544, 16.44)	14.939 (14.491, 15.387)	0.0004	0.0340
Pyruvic acid	7.365 (6.89, 7.841)	6.322 (5.847, 6.798)	0.0006	0.0347
Pregnenolone sulfate	7.735 (7.236, 8.234)	6.658 (6.159, 7.157)	0.0011	0.0460
Tryptophan	13.74 (13.233, 14.247)	12.636 (12.129, 13.142)	0.0030	0.0806
Oxoglutaric acid	10.624 (10.057, 11.191)	9.508 (8.941, 10.076)	0.0033	0.0806
Ferulic acid	5.59 (5.151, 6.029)	6.61 (6.171, 7.049)	0.0034	0.0806
CM-Tolerant				
Metabolite	TP0	TP1	P	Q
Tartaric acid	2.769 (2.443, 3.094)	3.597 (3.271, 3.922)	0.0001	0.0082
Pyroglutamic acid	5.977 (5.691, 6.262)	5.178 (4.893, 5.463)	0.0001	0.0082
Dodecanoylcarnitine	3.411 (3.02, 3.802)	2.383 (1.992, 2.774)	0.0002	0.0082
TLCA	-3.038 (-3.378, -2.698)	-3.819 (-4.159, -3.479)	0.0002	0.0082
Desaminotyrosine	4.112 (3.785, 4.44)	5.017 (4.689, 5.344)	0.0003	0.0095
Ferulic acid	5.495 (5.148, 5.842)	6.323 (5.976, 6.67)	0.0026	0.0707
PLA#	8.617 (8.268, 8.965)	9.364 (9.015, 9.712)	0.0042	0.0920
FAD	5.631 (5.342, 5.92)	6.255 (5.966, 6.544)	0.0044	0.0920
Pregnenolone sulfate	7.88 (7.486, 8.275)	7.141 (6.746, 7.535)	0.0051	0.0945
CM-Tolerant				
Metabolite	TP0	TP2	P	Q
Dodecanoylcarnitine	3.411 (3.02, 3.802)	2.03 (1.639, 2.421)	6.10E-07	0.0001
Pregnenolone sulfate	7.88 (7.486, 8.275)	6.675 (6.281, 7.07)	3.88E-06	0.0003
Desaminotyrosine	4.112 (3.785, 4.44)	5.204 (4.877, 5.532)	1.22E-05	0.0007
LCA/TLCA	-0.98 (-1.363, -0.598)	0.054 (-0.335, 0.443)	0.0001	0.0025
Pyruvate	7.619 (7.243, 7.995)	6.664 (6.288, 7.04)	0.0001	0.0025
PLA#	8.617 (8.268, 8.965)	9.615 (9.267, 9.963)	0.0001	0.0030
Protocatechuic acid	3.367 (3.043, 3.691)	4.128 (3.804, 4.452)	0.0002	0.0040
HCA	-0.1 (-0.574, 0.373)	-1.354 (-1.836, -0.871)	0.0002	0.0040
Ferulic acid	5.495 (5.148, 5.842)	6.505 (6.158, 6.852)	0.0002	0.0040
Pyroglutamic acid	5.977 (5.691, 6.262)	5.21 (4.924, 5.495)	0.0002	0.0043
FAD	5.631 (5.342, 5.92)	6.396 (6.107, 6.685)	0.0004	0.0064
TLCA	-3.038 (-3.378, -2.698)	-3.756 (-4.101, -3.41)	0.0008	0.0113
Pipecolic acid	10.801 (10.423, 11.178)	11.656 (11.279, 12.034)	0.0010	0.0141
DCA/CA	-1.888 (-2.322, -1.454)	-0.899 (-1.341, -0.456)	0.0015	0.0188
Oxoglutaric acid	10.807 (10.359, 11.256)	9.865 (9.417, 10.314)	0.0016	0.0188
DCA/TDCA	-0.745 (-1.17, -0.321)	0.149 (-0.283, 0.582)	0.0019	0.0209
3-Hydroxybenzoic acid	5.341 (4.976, 5.706)	6.151 (5.786, 6.516)	0.0023	0.0234
Betaine	5.159 (4.751, 5.567)	4.16 (3.752, 4.568)	0.0027	0.0262
TCDCA	-0.893 (-1.24, -0.547)	-1.681 (-2.035, -1.327)	0.0030	0.0278
N-Acetylneuraminic acid	10.142 (9.802, 10.483)	9.392 (9.051, 9.732)	0.0032	0.0279
CA	1.501 (1.022, 1.979)	0.386 (-0.104, 0.875)	0.0040	0.0327
Hydrocinnamic acid	3.076 (2.698, 3.454)	3.869 (3.491, 4.246)	0.0041	0.0327
UDCA/CDCA	-1.858 (-2.175, -1.542)	-1.156 (-1.479, -0.832)	0.0054	0.0392

CM-Tolerant				
Metabolite	TP0	TP2	P	Q
LCA/CDCA	-1.654 (-2.092, -1.216)	-0.744 (-1.191, -0.297)	0.0054	0.0392
Butyrate	1.522 (1.153, 1.891)	2.255 (1.893, 2.617)	0.0055	0.0392
Phenylacetylglutamine	3.922 (3.524, 4.321)	3.185 (2.786, 3.583)	0.0073	0.0481
DCA	-0.571 (-0.988, -0.155)	0.192 (-0.232, 0.616)	0.0076	0.0481
N6-Carboxymethyllysine	5.415 (5.064, 5.767)	6.055 (5.704, 6.406)	0.0076	0.0481
5-Hydroxytryptophan	7.375 (7.013, 7.737)	6.623 (6.261, 6.985)	0.0131	0.0802
CDCA	-0.131 (-0.601, 0.339)	-1.081 (-1.561, -0.601)	0.0139	0.0809
Quinaldic acid	4.44 (4.08, 4.8)	5.138 (4.777, 5.498)	0.015	0.08089
Arginine	14.321 (13.842, 14.799)	13.461 (12.983, 13.94)	0.015	0.08089
Pyrocatechol	3.184 (2.864, 3.504)	3.703 (3.383, 4.023)	0.015	0.08089
Phenylacetic acid	6.412 (6.044, 6.78)	7.148 (6.78, 7.516)	0.016	0.08089
LCA	-0.995 (-1.408, -0.583)	-0.276 (-0.696, 0.145)	0.016	0.08089
TP2				
Metabolite	Allergic	Tolerant	P value	Q value
Citrulline	11.841 (11.378, 12.303)	12.946 (12.58, 13.311)	0.0003	0.0537
Lysine	11.371 (10.957, 11.785)	12.273 (11.946, 12.601)	0.0010	0.0823

Table S3. Spearman's rank correlation between the changes of bifidobacterium and metabolites/ratios which are significantly altered in the AAF-S group. Only correlations significant following multiple testing correlation are displayed

Compound	Rho	P value	Time points	Intervention	Q value
ILA	0.859	2.13E-06	TP1-TP0	AAF-S	<2.00E-04
4-OH-PLA#	0.769	2.81E-05	TP1-TP0	AAF-S	2.00E-04
Adenine	0.637	0.00138	TP1-TP0	AAF-S	0.0069
Glutamine	-0.573	0.0049397	TP1-TP0	AAF-S	0.0185
Adenine	0.721	0.0023058	TP1-TP0	AAF	0.0346
CDCA/GCDCA	0.508	0.0144412	TP1-TP0	AAF-S	0.0433
Inosine	-0.462	0.0275279	TP1-TP0	AAF-S	0.059
GCDCA	-0.468	0.0254131	TP1-TP0	AAF-S	0.059
CA/GCA	0.431	0.0413402	TP1-TP0	AAF-S	0.0775

Chapter 5

Cytokine-induced barrier dysfunction and lipid signaling in a gut-on-chip model

Based on:

Cytokine-induced barrier dysfunction and lipid signaling in a gut-on-chip model

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Abstract

The intestinal epithelial barrier is crucial for gut homeostasis, with dysfunction linked to inflammatory disorders like inflammatory bowel disease (IBD). While cytokines are established mediators of barrier disruption, the role of lipid signaling remains poorly understood. Using a microfluidic platform, we cultured epithelial tubules and exposed their luminal (apical) side to TNF-α, IL-1β, and IFN-γ (0-200 ng/mL) under serum-containing or serum-free conditions. Barrier function was assessed primarily via transepithelial electrical resistance (TEER), with DRAQ7 staining and actin cytoskeletal analysis providing complementary indicators of membrane integrity and structural disruption. In parallel, a targeted liquid chromatography–tandem mass spectrometry approach was used to profile lipid mediators across apical and basolateral compartments. Cytokine exposure significantly impaired barrier integrity, as indicated by reduced TEER, alongside associated cell damage and structural changes reflected by DRAQ7 staining and actin remodeling. The remodeling effect was lower in serum-free medium. Lipid profiling revealed inflammatory signatures characterized by increase in prostaglandins in the luminal compartment, particularly under serum-free conditions. PGF1α increased under both media conditions whereas the rest of the changes were condition-specific with rise in PGE1, PGE2, and PGD2, among others, particularly under serum-free conditions. Simultaneously, changes in other eicosanoids, but not in prostaglandins, were detected in the basolateral compartment under serum-free conditions. This proof-of-principle study demonstrates how medium composition significantly influences inflammatory responses and lipid signaling patterns in a physiologically relevant gut-on-chip model. Our integrated approach reveals the complex spatial organization of lipid mediators during cytokine-induced barrier dysfunction and provides a valuable framework for investigating the interplay between inflammation, barrier integrity, and lipid metabolism in intestinal pathophysiology.

Keywords

intestinal integrity; intestine-on-chip; gut permeability; cytokines; signaling lipids; serum; inflammation

Abbreviations

CDCA – Chenodeoxycholic acid; **COX** – Cyclooxygenase; **EMEM** – Eagle's Minimum Essential Medium; **FBS** – Fetal Bovine Serum; **HBSS** – Hanks' Balanced Salt Solution; **IBD** – Inflammatory Bowel Disease; **IFN-γ** – Interferon-gamma; **IL-1β** – Interleukin-1 beta; **LC-MS/MS** – Liquid Chromatography–Tandem Mass Spectrometry; **LCA** – Lithocholic acid; **MRM** – Multiple Reaction Monitoring; **NF-κB** – Nuclear Factor kappa-light-chain-enhancer of activated B cells; **PBS** – Phosphate-Buffered Saline; **PG** – Prostaglandin; **SF-EMEM** – Serum-Free Eagle's Minimum Essential Medium; **TEER** – Transepithelial Electrical Resistance; **TDCA** – Taurodeoxycholic acid; **TNF-α** – Tumor Necrosis Factor-alpha

1. Introduction

The intestinal epithelial barrier is crucial for maintaining health. It facilitates nutrient absorption, supports immune defense, and prevents pathogen invasion.[1,2] It also regulates the selective permeability of the gut, allowing essential nutrients to pass into the bloodstream while blocking harmful substances. Disruption of this barrier is associated with various inflammatory and metabolic disorders, including inflammatory bowel disease (IBD) and food allergies.[3,4] Understanding the mechanisms that preserve gut barrier integrity is essential for developing therapeutic strategies for these conditions.

Cytokines play a central role in the disruption of intestinal barrier integrity. Elevated levels of cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interferon-gamma (IFN-γ) contribute to barrier dysfunction by disrupting tight junction integrity and increasing epithelial permeability. For instance, TNF-α and IFN-γ have been shown to synergistically impair barrier function by altering tight junction protein expression and distribution.[5,6] Additionally, IL-1β can induce barrier dysfunction by promoting inflammatory responses that compromise epithelial cell integrity.[7] These cytokines also induce actin cytoskeletal remodeling,[8] which is directly linked to compromised epithelial barrier function and increased permeability.[9]

While the role of cytokines in intestinal barrier dysfunction is well-documented, the role of other inflammatory mediators remains understudied. Eicosanoids, potent bioactive signaling lipids derived from arachidonic acid and eicosapentaenoic acid, may also play a role in maintaining intestinal barrier integrity.[10] Among them, prostaglandins (PG), more specifically increased levels of prostaglandin E2 (PGE2) have been associated with increased gut permeability in in vitro models[11,12] whereas elevated mucosal, fecal, and blood levels of eicosanoids have been reported in patients with ulcerative colitis.[13,14] Despite this, the role of eicosanoids and other signaling lipids in barrier regulation and inflammatory responses remains poorly understood.

To better understand lipid signaling pathways and their role in barrier regulation, appropriate experimental models are essential. However, traditional in vitro models such as static cell culture often fail to replicate the complex architecture and dynamic environment of the intestinal epithelium, limiting their relevance for studying gut physiology and pathology.[15] Advanced models, such as organ-on-chip systems, overcome these limitations by incorporating microfluidic technology to mimic the mechanical forces, spatial organization, and cell-cell interactions of the intestine.[16,17] The OrganoPlate, a membrane-free microfluidic platform, offers a robust solution by allowing the culture of up to 64 intestinal tubules with apical and basolateral access.[18] It provides simultaneous assessment of barrier function and cellular responses, making it ideal for complex analyses combining barrier permeability[19–21] and inflammation.[22,23]

While these advanced models provide powerful tools for studying intestinal biology, optimizing their experimental conditions is crucial for reliable results. Particularly, the composition of cell culture media is known to significantly influence cellular responses to external stimuli.[24] Serum starvation is a common practice in drug screening, primarily used to synchronize the cell cycle.[25] However, it has limitations, as prolonged starvation can have undesirable effects on cell viability and function,[26] and cells cannot be maintained in a starved state indefinitely. Conversely, serum provides essential nutrients, binds and transports small molecules including lipids,[27] and reduces non-specific binding to culture plastic surfaces,[28] but it introduces batch variability and is a possible source of contamination.[29] The impact of serum on signaling lipid secretion in intestinal barrier models remains underexplored. This gap highlights the need for systematic studies to evaluate how medium composition affects cytokine-induced barrier dysfunction and lipid signaling.

Here, we aim to address these gaps using the OrganoPlate to investigate the effects of cytokine exposure on intestinal barrier integrity and signaling lipid secretion under varying medium conditions. Specifically, we compare standard serum-containing and serum-free media to evaluate their influence on cytokine-induced changes in barrier function, assessed through transepithelial electrical resistance (TEER), DRAQ7 staining (as a marker of cell death), actin remodeling, and lipid signaling. By focusing on the interplay between cytokine responses and medium composition, this study provides a novel framework for understanding the mechanisms underlying gut inflammation.

This proof-of-principle study represents a significant advancement in intestinal research, offering a comprehensive analysis of cytokine-induced barrier dysfunction alongside lipid signaling in a microfluidic platform. By leveraging the OrganoPlate technology, we demonstrate how physiologically relevant models can uncover previously unexamined dynamics of gut inflammation, paving the way for innovative experimental designs and therapeutic strategies.

2. Experimental Section

A detailed list of all chemicals and reagents is provided in the Supplementary materials.

2.1 OrganoReady Colon Caco-2

The OrganoReady Colon Caco-2 3-lane 40 (MI-OR-CC-01, MIMETAS B.V.) cultures are pre-seeded Caco-2 tubules in the OrganoPlate platform - a multiwell plate format equipped with microfluidic chips designed to support up to 64 membrane-free microfluidic chips. Following Trietsch et al.[18], these cultures were prepared according to the manufacturer's instructions, with Caco-2 medium replaced upon receipt. Continuous perfusion flow was applied using the OrganoFlow rocker (MIMETAS B.V., MI-OFPR-L), set at a 7-degree angle with 8-min intervals optimized specifically for the 3-lane 40 configuration. On day 2 post-receipt (day 6 post-seeding), the medium was refreshed, and experimental exposures began

on day 4 post-receipt (day 8 post-seeding). See **Figure 1** for a visual representation of the OrganoPlate and OrganoFlow setup.

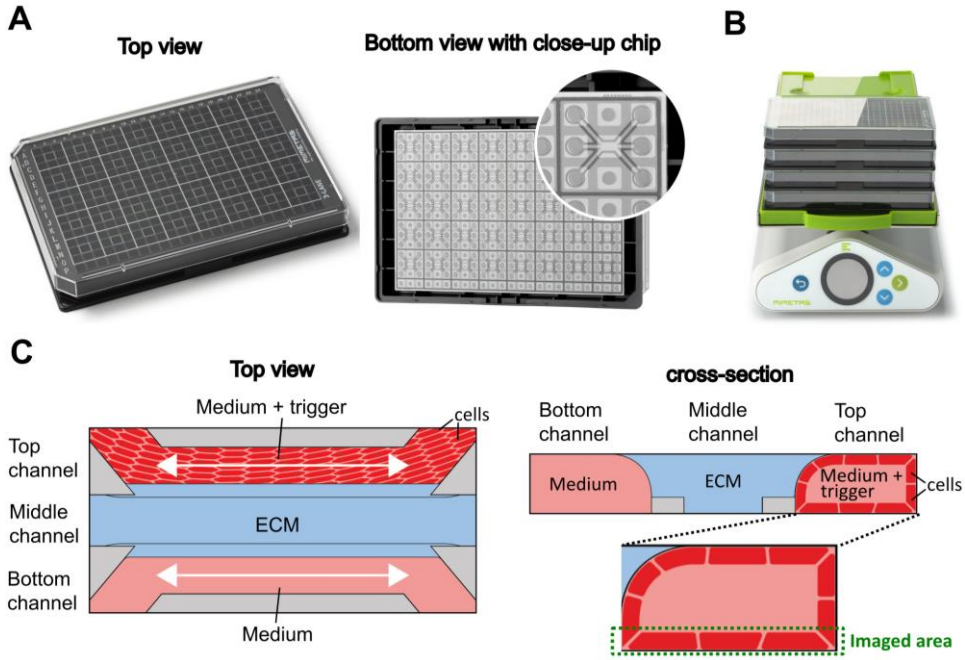

***Figure 1**. Overview of the OrganoPlate platform and experimental configuration. (A) Illustration of the OrganoPlate showing top and bottom view with a close-up on a microfluidic chip. (B) OrganoFlow rocker generating pump-free medium perfusion. (C) Detailed schematic of the three-channel structure (top and cross-sectional views), highlighting the top channel where the epithelial cell tubule is cultured. This top channel represents the luminal (apical) side of the tubule, where cytokines are administered. The middle channel contains ECM, and the bottom channel contains culture medium and represents the basolateral side of the tubule. The dashed green outline indicates the imaged area at the bottom of the tubule used for maximum projection analysis.*

2.2 Cytokines Exposures and Sampling

On day 8 post-seeding, Caco-2 tubules in the OrganoPlate were exposed to a cytokine mixture. The culture medium in the bottom and middle channels was replaced with complete Eagle's Minimum Essential Medium (EMEM) or serum-free EMEM (SF-EMEM). For the top channel, the medium was replaced with either EMEM or serum-free EMEM containing TNF-α, IL-1β, and IFN-γ at concentrations of 50, 100, or 200 ng/mL each. The plates were placed back in the incubator and maintained under continuous perfusion.

After 72 h of exposure and endpoint readouts, culture medium was collected from the top, middle, and bottom channels. All experiments were performed three times independently, (n = 3) and each independent experiment contained four chips per condition (n = 4). For the cellular assays, each replicate was considered during the statistical analysis. For the signaling lipid profiling, per condition and channel, media from four replicate chips (~80 µl each) were pooled to prepare a sample, resulting in three independent replicates per

condition (n = 3). The (pooled) samples were centrifuged for 10 min at 1500 rpm to remove dead cells. Media samples and media spiked with 200 ng/mL cytokine mixture were collected for background assessment. All samples were stored at -80°C immediately after collection. A close-up of the chip configuration and different channels (top, middle, bottom) is shown in **Figure 1C**.

2.3 Trans-Epithelial-Electrical-Resistance (TEER)

To evaluate the integrity of the gut barrier, TEER was measured with the OrganoTEER, an automated multichannel impedance meter.[20] An electrode board, designed to fit the 3-lane OrganoPlate was sterilized with 70% ethanol at least 1 h before measurement. The OrganoPlate was taken out of the incubator and equilibrated at room temperature for 30 min before the measurement to eliminate any flow or temperature effect. Baseline measurements were performed on day 8, after equilibration and right before exposure, then TEER was further measured after 72 h of exposure to the cytokine mixture. For Caco-2 the OrganoTEER was set on "high TEER" and we used a TEER threshold of 350 Ω/cm^2 at baseline, meaning that all tubules below 350 Ω/cm^2 at baseline were excluded from the analysis. The OrganoTEER setup is shown in **Figure 2A, B**.

2.4 Fluorescent Imaging

To visualize the effect of cytokine exposure on cell structure and membrane integrity, the tubules were directly fixed and stained on the OrganoPlate.

2.4.1 DRAQ7 Nuclear Staining

Before fixation, culture medium in the tubule inlets and outlets was replaced by 20 µL of DRAQ7 dye (Biostatus, DR71000) diluted 1:100 in serum-free EMEM, and cells were incubated for 30 min under continuous perfusion inside the incubator.

2.4.2 Fixation

Intestinal tubules were fixed with 3.7% formaldehyde (Sigma, 252549) in Hanks' Balanced Salt Solution (HBSS) with calcium and magnesium (Thermo Scientific, 14025092) for 15 min, washed twice with phosphate-buffered saline (PBS; Gibco, 70013065) for 5 min, and then stored with 50 µL PBS per well at 4°C until further staining.

2.4.3 Actin and Hoechst Nuclear Staining

Intestinal tubule cells were permeabilized with 0.03% Triton X-100 (Sigma, T8787) in PBS for 10 min, followed by two washes with 4% FBS in PBS. Cells were stained with NucBlue Fixed Cell ReadyProbes Reagent (Invitrogen, R37606) and ActinGreen (Invitrogen, R37110) as per the manufacturer's instructions. Two drops per ml of both NucBlue and ActinGreen were added to PBS to prepare the staining solution. Twenty microliters of the staining solution was introduced at both the inlet and outlet of the tube, allowing for incubation at room

temperature for 30 min under continuous perfusion. Cells were then washed twice with PBS for 5 min each.

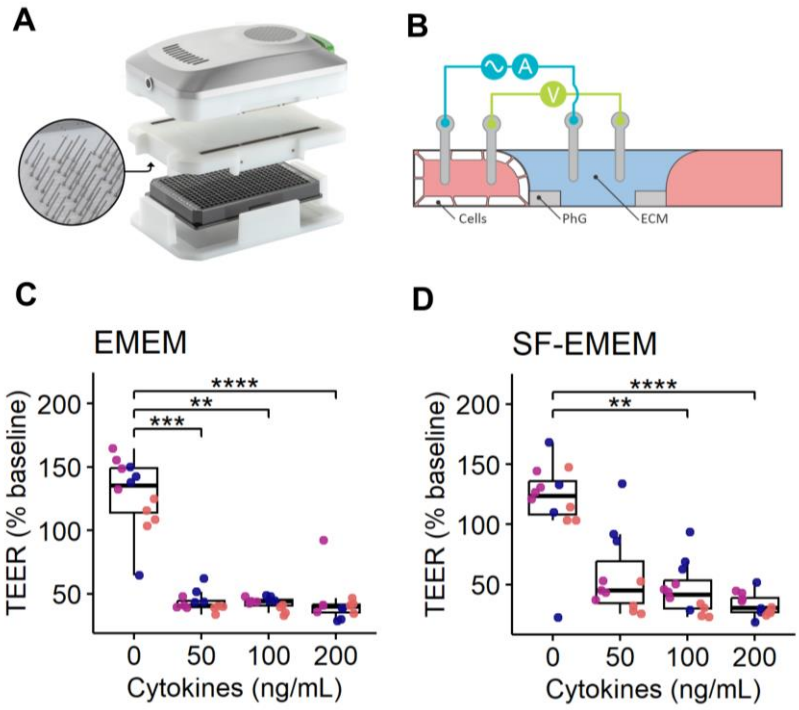

***Figure 2**. Impact of Cytokine exposure on TEER in complete (EMEM) and serum-free (SF-EMEM) media. (A) OrganoTEER setup to measure TEER in an OrganoPlate. (B) Schematic diagram showing a transversal view of the OrganoPlate chip and the electrode pair positioning across the medium channel and the Caco-2 tube channel containing the medium dilution with cytokine (TNF-α, IL-1β, and IFN-γ). The concentration is expressed as ng/nl of each cytokine. PhG: PhaseGuide, ECM: ExtraCelluar Matrix. (C-D) Dose-dependent decrease in TEER after exposure to cytokines in EMEM (C), and SF-EMEM (D). Data represents the percentage change in TEER compared to baseline measurements. Each dot represents a measurement from a single chip in the OrganoPlate. Colors indicate independent experiments. Results are derived from three independent experiments (N = 3), with four technical replicates per cytokine concentration (n = 4). Significance was determined using Kruskal-Wallis test followed by Dunn's post hoc test, with p-values adjusted using the Bonferroni correction. Adjusted p values are expressed as follows: ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05.*

2.5 Intestinal Tubules Visualization and Imaging

Imaging was conducted on the bottom of the tubules using the ImageXpress Micro XLS (Molecular Devices, USA) and Micro XLS-C High Content Imaging Systems (Molecular Devices, USA) and processed using Fiji34 to enhance contrast and improve visualization. Fixed and stained OrganoPlates were stored at 4°C until imaging and equilibrated at room temperature at least 30 min before imaging. Maximum intensity projection images were saved as TIFF files after confocal imaging of stained cells.

2.6 Quantification of DRAQ7 and Actin Signals

We used the open-source cell image analysis software CellProfiler (version 4.2.5) to process the images. We designed a pipeline to process the TIFF files capturing DRAQ7 and NucBlue staining, allowing the segmentation of DRAQ7-positive cells and total nuclei to quantify the percentage of DRAQ7-positive (DRAQ7 +) cells. Another pipeline was designed to quantify actin remodeling. We identified actin objects from the actin signal and analyzed three parameters: actin object count, object mean area, and total actin area, which together quantify the number, individual size, and total extent of actin reorganization. All parameters were divided by nuclei count (NucBlue staining) to normalize for cell number. Detailed methodology of the staining and CellProfiler pipeline was previously described.[30]

2.7 Signaling Lipids Profiling

2.7.1 Sample Collection and Preparation

An aliquot of 150 µL of pooled media was subjected to liquid-liquid extraction as described by Yang *et al.* (2024) with small modifications.[31] Briefly, to 150 µL of media 5 µL antioxidant solution (0.2mg/ml butylated hydroxytoluene (BHT) and 0.2/ml mg ethylenediaminetetraacetic acid (EDTA), 10 µL of internal standard solution, 150 µL of citrate buffer (0.2 M citric acid, 0.4 M disodium hydrogen phosphate buffer, pH 4.5) and 1 ml of the butanol:methyl tert-butyl ether (BuOH:MTBE) (1:1 v:v) were added. The samples were left on ice for 20 min, followed by homogenization for 4 min in a Bullet Blender. Following centrifugation at 15 800 rcf (10 min, 4°C), 900 µL of the upper organic phase were collected and dried under vacuum in Speedvac (Labcono, USA). The samples were reconstituted in 40 µL of methanol/acetonitrile, vortexed for 5 min, centrifuged at 15 800 rcf (10 min, 4°C) and transferred to injection vials for liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis.

2.7.2 LC-MS/MS Conditions

LC-MS/MS analysis, covering a range of isoprostanes, prostaglandins, endocannabinoids, and bile acids, was carried out as previously described.[31] Briefly, the analysis was carried out on a three-pump Shimadzu LC-30 AD system coupled to SCIEX Triple-Quad 7500 system (USA). The chromatographic separation was performed on a Waters Acquity BEH C18 column (50 mm × 2.1 mm, 1.7 µm) at 40^{o}C following a 16-min multistep gradient at a flow rate of 0.7 ml/min. Mobile phase A consisted of 0.1% (v/v) acetic acid in water, mobile phase B of 0.1% acetic acid in acetonitrile/methanol (90/10, v/v), and mobile phase C of 0.1% acetic acid in isopropanol. The data were acquired under multiple reaction monitoring (MRM) at unit resolution with polarity switch scanning mode. A detailed list of MRM transitions and retention times of each analyte and the corresponding isotopically labelled standard can be found in the original work.[31] Data acquisition of the main and follow-up experiments was carried out on SCIEX OS 2.0.0 and SCIEX OS 3.3.0.

2.7.3 Quality Control

Blank media samples (EMEM and SF-EMEM) were used to assess the background signal from each media type. In a follow-up experiment, the background signal coming from the cytokine mixture was assessed by analyzing blank media samples (EMEM and SF-EMEM) and media (EMEM and SF-EMEM) spiked with 200 ng/ml cytokine mixture.

2.8 Data Analysis

The statistical analyses for cellular assays (TEER measurement, actin remodeling, and DRAQ7 signal) were conducted in two steps. First, we performed a global analysis of main effects and interactions on the full dataset using Two-Way ANOVA when data met normality assumptions (verified by Shapiro-Wilk test n>50, or Anderson-Darling test n>50), or Scheirer-Ray-Hare test for non-normal distributions. This analysis evaluated the effects of cytokine cocktail concentration (0, 50, 100, 200 ng/mL) and medium type (EMEM vs. SF-EMEM) on cellular assays, as well as the interaction between both variables.

For variables showing significant main effects, we conducted independent analyses. When comparing cytokine concentrations, we used One-Way ANOVA followed by Tukey's HSD post hoc test for normal data, or Kruskal-Wallis followed by Dunn's post hoc test for non-normal data. When medium type comparisons were significant, we used an independent t-test for normal data or Wilcoxon Rank-Sum test for non-normal data. All p values were adjusted for multiple comparisons using Bonferroni correction, with adjusted values (q-values) ≤ 0.05 considered statistically significant. Global analysis results and detailed analyses of the medium are provided in supplementary materials.

Preprocessing of the LC-MS/MS data was performed using SCIEX OS 3.0. Data analysis and visualization were conducted using R version 4.3.2. per media, lipids with fewer than three measurements below three times the blank media signal (blank threshold) were excluded. Data were visually inspected, and only lipids detected above the blank threshold in at least two conditions (defined by media, channel, and concentration) were retained. The cytokine mixture was evaluated for potential background interference, and only lipids unaffected by it were retained.

3. Results

3.1 Cytokine Mixture Significantly Reduced TEER in EMEM and SF-EMEM Medium Conditions

TEER measurements reflect epithelial barrier integrity, with decreases indicating increased permeability, characteristic of inflammation-driven barrier dysfunction. We exposed Caco-2 tubules to a cytokine mixture (TNFα, IL-1β, and IFNγ) at concentrations of 0, 50, 100, and 200 ng/mL each for 72 h in two different media (EMEM and SF-EMEM). Using OrganoTEER technology (**Figure 2A,B**), we assessed barrier integrity through real-time, non-invasive

TEER measurements before and after exposure. Values were normalized to baseline (i.e., expressed as a percentage of the chip's pre-exposure value) and analyzed to evaluate cytokine effects in EMEM and SF-EMEM.

The Scheirer-Ray-Hare test revealed a significant effect of cytokine concentration on TEER ($p = 7.0 \times 10^{-7}$) but an insignificant effect of medium type ($p = 0.73$) and the cytokine–medium interaction ($p = 0.95$) (**Table S1** in Online Supplementary Materials). In EMEM, all cytokine concentrations (50, 100, and 200 ng/mL) significantly reduced TEER compared to control, with no differences between treatment groups (**Figure 2C**). In SF-EMEM, only higher concentrations (100 and 200 ng/mL) significantly reduced TEER versus control (**Figure 2D**).

3.2 Cytokine Mixture Increased DRAQ7 Signal in All Conditions Independently of Media Composition

The effect of cytokine exposure on Caco-2 tubule integrity was assessed using DRAQ7 staining (**Figure 3A**). DRAQ7 is a membrane-impermeant DNA dye that labels nuclei of cells with compromised plasma membranes. It is therefore commonly used as a marker of cell death. To quantify this effect, we performed DRAQ7 staining and developed a CellProfiler pipeline to analyze the percentage of DRAQ7-positive nuclei.

The Scheirer-Ray-Hare test showed a significant effect of cytokine concentration ($p = 1.05 \times 10^{-11}$) and medium ($p = 0.033$) on DRAQ7 positive-nuclei with no significant interaction between the two variables ($p = 0.62$) (**Table S2** in Online Supplementary Materials). DRAQ7 signal increased significantly when cytokine concentrations were raised from 0 (control) to each dose (50, 100, or 200 ng/mL) in both media types. However, no significant differences were observed between the doses themselves (e.g., between 50 and 100, or 100 and 200 ng/ml). (**Figure 3B,C**). Post hoc comparisons did not reveal significant differences between media at each cytokine concentration (**Table S2** in Online Supplementary Materials).

3.3 Cytokine Mixture induced Actin Remodeling in All conditions After Exposure

The effect of cytokine exposure on actin remodeling was assessed using Actin staining (**Figure 4A**). Actin remodeling reflects cytoskeletal reorganization, which can occur in response to environmental stressors, such as cytokine exposure, and may serve as an early indicator of barrier disruption and epithelial dysfunction under inflammatory conditions.

3.3.1 Actin Count

Two-Way ANOVA revealed significant effects of cytokine concentration ($p = 4.17 \times 10^{-20}$), medium type ($p = 2.98 \times 10^{-9}$), and their interaction ($p = 0.0019$) on actin object count, indicating dependent effects of cytokine mixture and medium (**Table S3** in Online Supplementary Materials). In EMEM, all cytokine concentrations increased actin count versus control, with 200 ng/mL showing higher counts than 50 or 100 ng/mL (**Figure 4B**). In

SF-EMEM, increases were significant only at 100 and 200 ng/mL versus control, with 200 ng/mL also higher than 50 ng/mL (**Figure 4E**). Medium-specific comparisons revealed significantly lower actin counts in SF-EMEM compared to EMEM at 50 and 200 ng/mL, but not at 0 or 100 ng/mL (**Figure S1**).

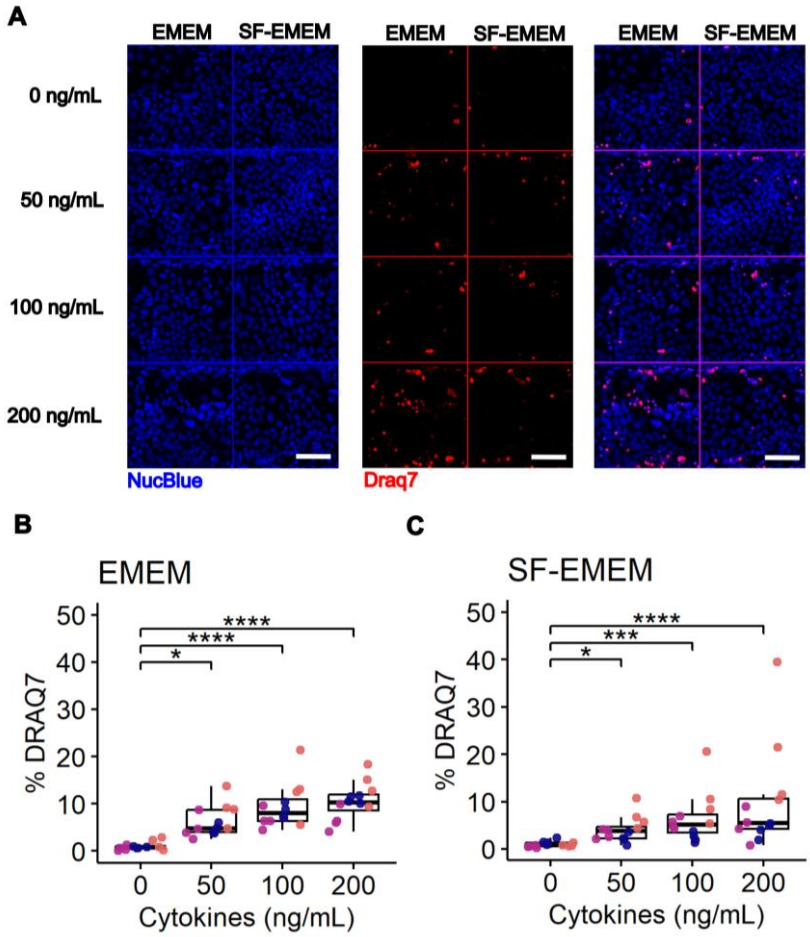

***Figure 3**. Impact of cytokine exposure on Caco-2 tubule membrane integrity to DRAQ7 in EMEM and SF-EMEM media. (A) Representative fluorescence images of NucBlue (blue, nuclei) and DRAQ7 (red, dead cells under various cytokine conditions). Scale bar = 100 µm. (B-C) Quantification of DRAQ7-positive cells (% DRAQ7) in Caco-2 tubules exposed to increasing concentrations of cytokines (TNF-α, IL-1β, and IFN-γ) in EMEM (B) and SF-EMEM (C). The concentration is expressed as ng/nl of each cytokine. Data represent the percentage of DRAQ7-positive cells normalized to total nuclei count. Each dot represents a measurement from a single chip in the OrganoPlate. Colors indicate independent experiments. Results are derived from three independent experiments (N = 3), with four technical replicates per cytokine concentration (n = 4). Statistical significance was determined using the Kruskal-Wallis test followed by Dunn's post hoc test, with p values adjusted using the Bonferroni correction. Adjusted p values are expressed as follows: ****p<0.0001, ***p<0.001, *p<0.05.*

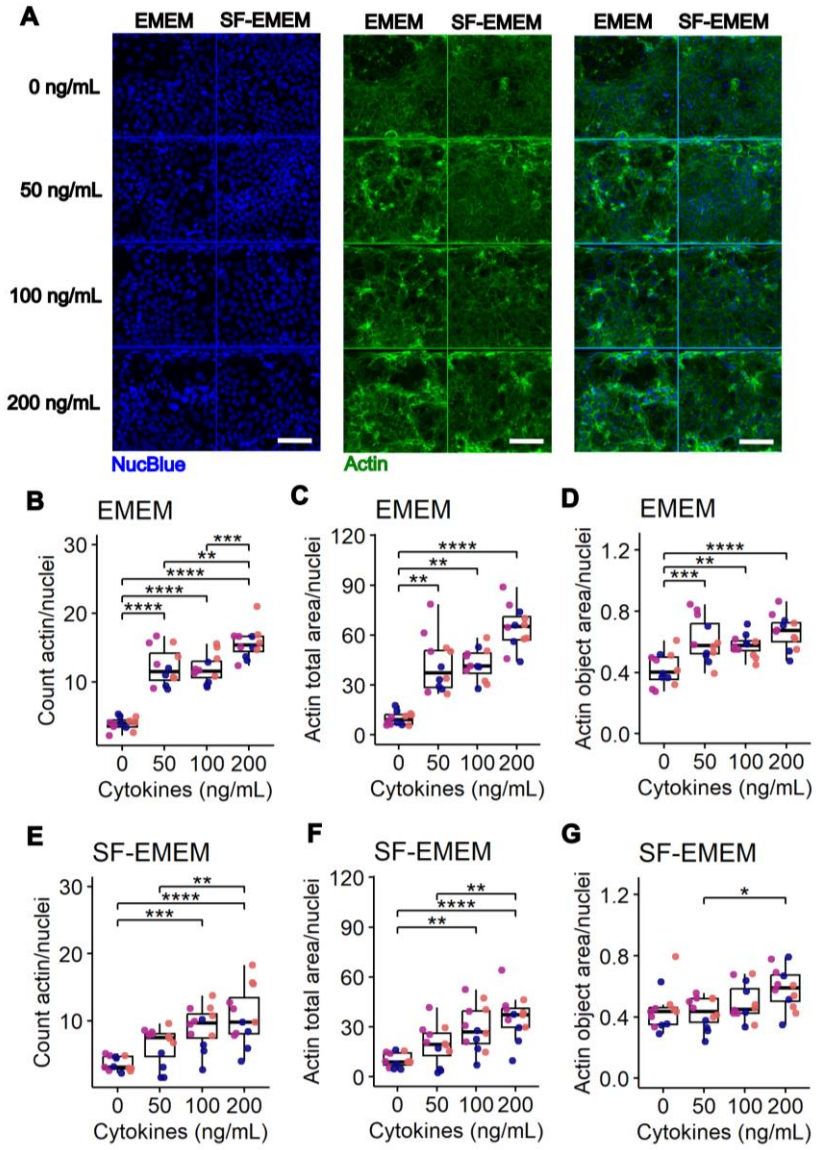

Figure 4. *Impact of cytokine exposure on Actin remodeling in EMEM and SF-EMEM media. (A) Representative fluorescence images of NucBlue (blue, nuclei) and Actin (green) under various cytokine conditions. Scale bar = 100 µm. (B-G) Quantification of Actin remodeling parameters, including the count of Actin objects per nucleus (B, E), total Actin area per nucleus (C, F), and mean Actin object area per nucleus (D, G), in Caco-2 tubules exposed to increasing concentrations of cytokines (TNF-α, IL-1β, and IFN-γ) in EMEM (B-D) and SF-EMEM (E-G). The concentration is expressed as ng/nl of each cytokine. Each dot represents a measurement from a single chip in the OrganoPlate. Colors indicate independent experiments. Results are derived from three independent experiments (N = 3), with four technical replicates per cytokine concentration (n = 4). Statistical significance was determined using either the Kruskal-Wallis test followed by Dunn's post hoc test or ANOVA followed by Tukey's post hoc test, depending on whether assumptions for parametric tests were met, with p values adjusted using the Bonferroni correction. Adjusted p values are expressed as follows: ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05.*

3.3.2 Total Actin Area

The Scheirer-Ray-Hare test showed significant effects of cytokine concentration ($p = 5.19 \times 10^{-11}$) and medium type ($p = 3.49 \times 10^{-4}$) on actin total area, while the cytokine–medium interaction was not significant ($p = 0.14$), indicating independent effects of cytokine mixture and medium (**Table S4** in Online Supplementary Materials). In EMEM, all cytokine concentrations increased the total actin area compared to control, with no differences between treated groups (**Figure 4C**). In SF-EMEM, increases were significant at 100 and 200 ng/mL versus control and at 200 ng/mL versus 50 ng/mL (**Figure 4F**). Medium comparisons within each cytokine condition showed significantly lower actin area in SF-EMEM compared to EMEM at 50, 100, and 200 ng/mL, but not at 0 ng/mL (**Figure S2**).

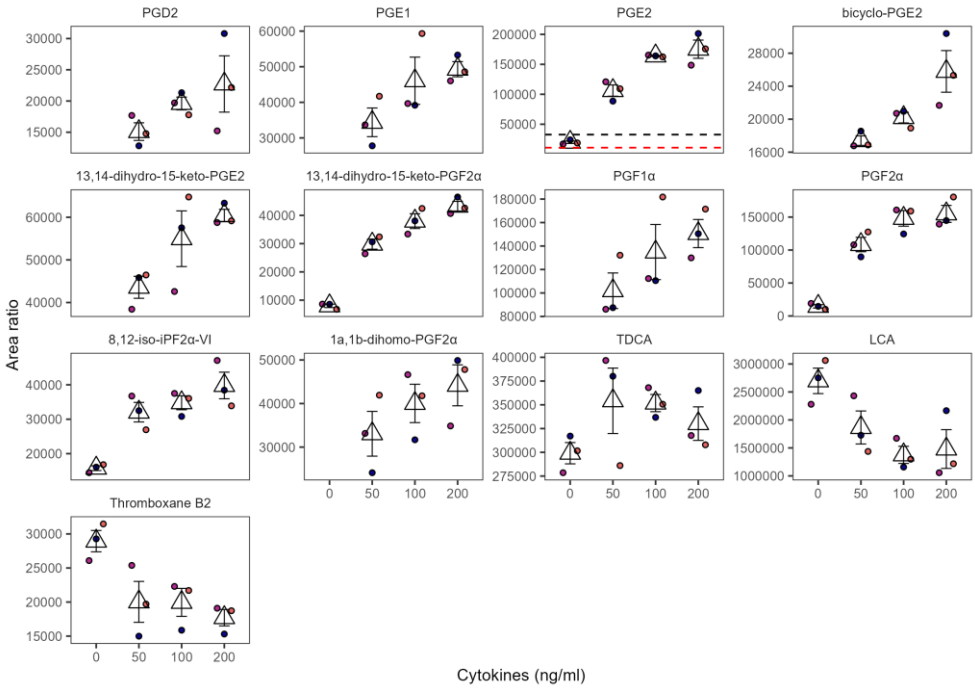

***Figure 5**. Impact of cytokine exposure on signaling lipid in SF-EMEM media, top channel. Results are derived from three independent experiments (N = 3). Missing measurement points correspond to signals below the detection limit. Dot colors indicate independent experiments. The triangular marker represents the mean of the three replicates, while the error bars are based on the standard deviation. The red and black dotted lines represent, respectively, one and three times the median level in blank SF-EMEM. The results for PGF2α, 8,12-iso-iPF2α-VI and 13,14-dihydro-15-keto-PGE2 should be treated with caution since EMEM media spiked with cytokine mixture contributed to the background signal of those lipids. The concentration is expressed as ng/ml of each cytokine.*

3.3.3 Mean Actin Object Area

Two-Way ANOVA showed significant effects of cytokine concentration ($p = 6.11 \times 10^{-6}$), medium type ($p = 0.00175$), and their interaction ($p = 0.0266$), indicating dependent effects of cytokine mixture and medium on actin object area (**Table S5** in Online Supplementary Materials). In EMEM, only 200 ng/mL significantly increased the mean object area versus control (**Figure 4D**). In SF-EMEM, cytokine exposure showed no significant effects (**Figure 4G**). Medium comparisons revealed a significantly lower the mean object area in SF-EMEM compared to EMEM only at 50 ng/mL (**Figure S3**).

3.4 Lipid Signaling Profiling

3.4.1 Cytokine Exposure Resulted in Higher Prostaglandin Levels in the Top Channel Under SF-EMEM Conditions

The effect that cytokine exposure has on the signaling lipid profile in Caco-2 cells was assessed by examining the changes to a range of isoprostanes, prostaglandins, endocannabinoids, and bile acids. Under the SF-EMEM and EMEM conditions, respectively, 40 of 105 and 9 of 125 detected signaling lipids met the quality control criteria and were studied further (**Table S6** in Online Supplementary Materials).

Under the SF-EMEM conditions, 12 signaling lipids were affected by the cytokine exposure in the top channel (**Figure 5**). Among those, nine prostaglandins (PGs) and derivatives, and the isoprostane 8,12-iso-iPF2α-VI were secreted in response to the cytokine exposure in a concentration-dependent manner. The cytokine exposure also affected the bile acids taurodeoxycholic acid (TDCA) and lithocholic acid (LCA), and thromboxane B2. The levels of the latter two declined upon exposure to the cytokine cocktail, which contrasted with the other observed changes.

Under EMEM conditions, PGF1α, 8-iso-PGF2α, and chenodeoxycholic acid (CDCA) were affected by the cytokine exposure in the top channel (**Figure 6**). Analogous to the SF-EMEM results, PGF1α was higher in the cytokine-exposed conditions compared to the unexposed conditions. Relative to the baseline, 8-iso-PGF2α was also higher when the tubules were exposed to 50 and 100 ng/ml of cytokine mixture. Other PGs and derivatives for example PGE2, and bicyclo-PGE2 were also higher in the cytokine-exposed conditions compared to the control; however, did they not meet our quality criteria due to the high EMEM or cytokine mixture background (**Figure S4**, **Table S6** in Online Supplementary Materials). The primary bile acid CDCA, in contrast, declined with the addition of cytokine mixture to the media (**Figure 6**).

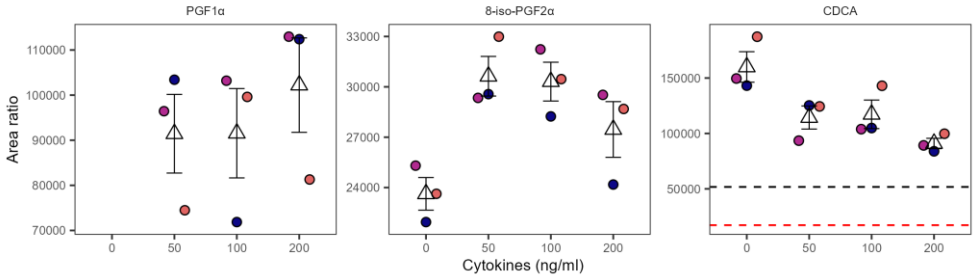

***Figure 6**. Impact of cytokine exposure on selected signaling lipid in EMEM media, top channel. Results are derived from three independent experiments (N = 3). Missing measurement points correspond to signals below the detection limit. Dot colors indicate independent experiments. The triangular marker represents the mean of the three replicates, while the error bars are based on the standard deviation. The red and black dotted lines represent respectively, one and three times the median level in blank EMEM. The concentration is expressed as ng/ml of each cytokine.*

3.4.2 Concentration-Dependent Decline in Eicosanoids After Exposure to Cytokines in the Bottom Channel Under SF-EMEM Conditions

None of the signaling lipids that changed with the cytokine exposure in the top channel exhibited a similar effect in the bottom channel. Instead, in the bottom channel under the SF-EMEM conditions, the eicosanoids 8,9-DiHETrE, 11,12-DiHETrE, 14,15-DiHETrE, and 11-HETE were affected in a cytokine concentration-dependent manner (**Figure 7**). For the 8,9-DiHETrE and 11,12-DiHETrE, the decline was evident at 50 ng/ml and 100 ng/ml of cytokines, whereas for 14,15-DiHETrE only at 50 ng/ml level (**Figure 7**). Meanwhile, in the EMEM model, no effect of the cytokine exposure was observed.

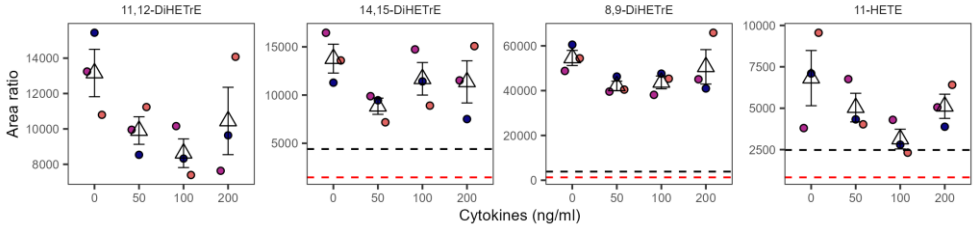

***Figure 7**. Impact of cytokine exposure on signaling lipid profiles in SF-EMEM media, bottom channel. Results are derived from three independent experiments (N = 3). Dot colors indicate independent experiments. The triangular marker represents the mean of the three replicates, while the error bars are based on the standard deviation. The red and black dotted lines represent, respectively, one and three times the median level in blank SF-EMEM. The concentration is expressed as ng/nl of each cytokine.*

3.4.3 Signaling Lipid Levels Differ Between the Channels

Under SF-EMEM conditions, 18 signaling lipids remained unaffected by the cytokine exposure, but their levels in the top channel differed from those in the middle and bottom channels (**Figure S5**). Few bile acids and 5-iPF2α-VI were higher in the top channel compared to the middle and bottom channels, whereas the opposite was observed for the three

eicosanoids, three docosanoids, and six long-chain fatty acids (**Figure S5**). Differences in the levels of the signaling lipids between the channels irrespective of the cytokine concentration were also observed under EMEM conditions for 8,9-DiHETrE, Δ17,6-keto PGF1α, and 5-iPF2α-VI (**Figure S6**). Under those conditions, similar changes were also observed for other signaling lipids that did not meet our quality control criteria (**Figure S7**). **Table 1** summarizes the key experimental findings across all measured parameters.

Table 1. Summary of results

Parameter	Effect of Cytokine Exposure	Mechanistic Explanation	SF-EMEM vs EMEM
TEER (Barrier Integrity)	↓ TEER (increased permeability)	Cytokines disrupt tight junctions, reducing resistance	No major difference in TEER reduction
Viability (DRAQ7)	↑ DRAQ7 (decreased cell death)	Cytokine exposure compromises plasma membrane integrity, allowing DRAQ7 to enter and stain the nucleus.	Similar DRAQ7 increase in both media
Actin Remodeling	↑ Cytoskeletal changes	Cytokines trigger cytoskeletal remodeling via NF-κB, MAPK	Stronger actin remodeling in SF-EMEM
PGs and derivatives	↑ Pro-inflammatory lipid mediators in top channel	COX-2 upregulation leads to increased prostaglandin synthesis	Prostaglandins more prominent in SF-EMEM
Eicosanoids (8,9-DiHETrE, 11,12-DiHETrE)	↓ Eicosanoids in bottom channel for some cytokine concentrations	-	Effects seen in SF-EMEM, not as clear in EMEM

4. Discussion

This study uses a gut-on-chip model to examine the effect of inflammatory cytokines on barrier function and lipid signaling in intestinal epithelium. Our findings demonstrate that cytokine exposure not only induces significant barrier dysfunction but also triggers distinct lipid signaling responses that are influenced by both medium composition and spatial location.

Cytokine exposure consistently compromised barrier integrity, as evidenced by decreased TEER and increased DRAQ7 signal. These effects align with established mechanisms whereby TNF-α, IL-1β, and IFN-γ disrupt tight junctions through NF-κB and MAPK signaling pathways.[7,32] The observed barrier dysfunction was accompanied by significant actin remodeling, reflecting early cytoskeletal reorganization that typically precedes junction breakdown.[8]

While TEER and DRAQ7 signal changes were comparable between media conditions, actin remodeling showed medium-dependent differences, with SF-EMEM generally displaying

lower values compared to EMEM for most parameters at specific cytokine concentrations. Serum factors may influence cytoskeletal reorganization, likely through integrin-mediated or growth factor-dependent pathways,[33] without directly affecting barrier integrity. The use of serum-free conditions can standardize responses through cell cycle synchronization.[25] Although autophagy-induced mechanisms can enhance barrier function under serum-free conditions,[34,35] our results indicate that these effects did not measurably impact TEER or DRAQ7 signal in this model. Future studies should investigate the specific mechanisms by which serum components influence cytoskeletal responses without affecting barrier integrity measures.

A novel aspect of our study was the comprehensive profiling of lipid mediators in a gut microphysiological system, revealing distinct spatial patterns and medium-dependent responses. In this work, a concentration-dependent increase in nine prostaglandins and derivatives was observed in the top channel (apical side) under SF-EMEM conditions. These findings align with other in vitro studies reporting PGE2 association with impaired barrier integrity[11,12] and its production by Caco-2 cells following exposure to proinflammatory cytokines IL-1b, TNFα, and IFN-γ.[36] Moreover, elevated levels of PGE2 have been observed in mucosal biopsies from patients with ulcerative colitis.[14,37] Under the studied conditions, the PGs are most likely produced by cyclooxygenase-2 (COX-2), which in the gastrointestinal tract are upregulated in case of inflammation, instead of COX-1 which produces PGs under physiological conditions.[38–40] While PGs increased, thromboxane B2 decreased, suggesting a shift in the COX pathway favoring prostaglandin rather than thromboxane formation. The levels of bile acids TDCA and LCA were also affected by cytokine exposure, but in opposing directions. These changes may reflect altered activation of Farnesoid X Receptor (FXR), which is increasingly recognized for its involvement in gut immunity and barrier function.[41]

In the top channel, unlike under SF-EMEM conditions, where overall PG upregulation was observed, under EMEM conditions, only PGF1α and 8-iso-PGF2α showed cytokine-induced increases. Similarly, the bile acid response to the cytokine exposure also varied between the media conditions. Other signaling lipids also appeared affected under the EMEM conditions; however, the high background signal compromised the data quality. These findings suggest that media conditions influence lipid signaling and that the discrepancy in response between the media conditions likely stems from a combination of biological differences and methodological constraints.

Under neither media conditions were the results in the top channel replicated in the bottom and medium channels. While no alterations were observed under EMEM conditions in the bottom channel, a concentration-dependent decline in eicosanoids was detected under SF-EMEM conditions. Even though the decline is not consistent across the cytokine concentrations, the observation supports the shift of the arachidonic acid metabolism toward prostaglandin synthesis, observed in the top channel under the same conditions.

Spatial organization in the OrganoPlate was also evident by the difference in the levels of signaling lipids, unaffected by the cytokine exposure, between the top and bottom channels. Such differences were observed under both media conditions, with clearer trends observed under SF-EMEM conditions, partly due to the high EMEM background signals. These findings demonstrate that lipid profiles can differ markedly between apical and basolateral compartments, emphasizing the importance of sampling location when interpreting epithelial lipid signaling. Future studies should explore whether these compartment-specific patterns reflect physiologically relevant lipid trafficking processes, such as directed secretion toward the lumen or circulation, particularly under inflammatory conditions.

A key strength of our study is its multiparametric approach, integrating TEER, viability (DRAQ7), actin remodeling, and lipid signaling to provide a comprehensive view of cytokine-induced barrier dysfunction. Real-time TEER monitoring offers non-invasive tracking, while lipid profiling adds a novel lipidomic dimension, linking cytokine exposure to inflammation and shifts relevant to inflammatory diseases like IBD. Actin remodeling complements these endpoints by capturing early cytoskeletal changes that contribute to tight junction disassembly and epithelial deformation, which are key features of barrier loss in IBD.[8]

Several oxylipin species, including eicosanoids, have increasingly been recognized in mediating inflammatory responses in IBD.[14,42,43] By following these lipid mediators in our gut-on-chip model, we demonstrate that this system captures clinically relevant inflammatory pathways, making it suitable for studying IBD-related mechanisms and screening therapeutic interventions targeting lipid signaling cascades.

Unlike previous studies in the field that focused on a narrow range of eicosanoids,[11,12,36] this work examined a broader spectrum of eicosanoids and other signaling lipids. The study also systematically compares serum-containing (EMEM) and serum-free (SF-EMEM) media, highlighting how medium composition affects inflammation-related lipid signaling. These findings emphasize the importance of standardized culture conditions when studying gut inflammation. Together, these strengths establish this study as a proof-of-concept for integrating gut barrier function and lipid signaling in inflammation research. Several limitations of this study should be considered. First, our model examines acute inflammation over 72 h and does not fully capture the chronic inflammatory conditions characteristic of many intestinal diseases.[44] Second, while the Caco-2 monoculture provides reproducible results, it lacks immune cells, microbiota, and a mucus layer, all of which play critical roles in intestinal barrier function and inflammatory responses.[30,45,46] Technical aspects of lipid analysis required pooling multiple chips for adequate sample volume, which reduced the number of replicates. Subsequent studies should therefore aim to develop analytical assays with enhanced sensitivity. The distinct spatial distribution of lipids between compartments and their varying responses to cytokine treatment suggest complex transport dynamics that warrant further investigation.

Important to note is that, although cytokine exposure consistently triggered changes in DRAQ7 staining, actin remodeling, and prostaglandin secretion, this study does not establish causal relationships between these outcomes. These endpoints should therefore be interpreted as parallel responses to inflammatory stress. Future studies will be needed to determine whether specific lipid mediators actively drive epithelial structural or functional changes.

Our study provides a comprehensive analysis of cytokine-induced intestinal barrier dysfunction and lipid signaling using a gut-on-chip model. By integrating TEER, DRAQ7 staining, actin remodeling, and lipid profiling, we reveal distinct inflammatory responses influenced by medium composition and spatial organization. Our findings demonstrate that cytokine exposure consistently impairs barrier integrity while inducing prostaglandin-mediated lipid signaling, with notable differences between serum-free and complete media conditions.

While this proof-of-principle study establishes a valuable framework for investigating gut inflammation, future research should extend to chronic inflammation models and refine lipidomic analysis. Follow-up experiments directly exposing gut epithelial tubules to selected prostaglandins could help establish causal links between specific lipid mediators and functional barrier responses, such as TEER reduction, viability, and actin remodeling. Future work could also explore the lipid signaling response to individual cytokines to clarify their specific contributions. Incorporating other cell types such as immune- or mucus-producing cells would further elucidate how these lipid signals mediate immune-epithelial crosstalk during inflammation. This could inform the development of targeted combination therapies addressing multiple inflammatory nodes simultaneously - particularly valuable for complex conditions like IBD.

Author contributions

M.M.: Conceptualization; Formal analysis; Investigation; Methodology; Visualization; Writing - Original Draft; Writing - Review & Editing; **M.V.S.**: Conceptualization; Formal analysis; Investigation; Methodology; Visualization; Writing - Original Draft; Writing - Review & Editing; **K.Q.**: Conceptualization; Funding acquisition; Resources; Supervision; Writing - Review & Editing; **A.C.H.**: Conceptualization; Resources; Supervision; Writing - Review & Editing; **T.H.**: Conceptualization; Funding acquisition; Project administration; Resources; Supervision; Writing - Review & Editing

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Conflict of interest statement

All authors have read the journal's policy on disclosure of potential conflicts of interest. K.Q. and M.M. are employees of Mimetas BV, which markets advanced in vitro system for drug development. T.H is shareholder of the same company. The authors declare they have no additional conflict of interests.

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Data availability

Preprocessed data used in this study are included in the Online Supplemental Materials. Raw data files can be obtained from the corresponding author upon request.

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Supplementary Materials

Online Supplementary Materials are available in the original manuscript:
DOI: 10.1096/fj.202501685R

List of reagents for cellular assays

Reagent	Supplier	Reference number	Note
ActinGreen™ 488 ReadyProbes™ Reagent	Sigma-Aldrich	R37110	2 drops/ml
NucBlue™ Live ReadyProbes™ Reagent	Sigma-Aldrich	R37605	2 drops/ml
DRAQ7™ Dye	Biostatus	DR71000	Working: 3 µM
Triton 100X	Sigma-Aldrich	T8787	
Formaldehyde	Sigma-Aldrich	252549	
HBSS	ThermoFisher	14025-092	
PBS	Gibco	70013-016	Diluted 10X in milliQ
EMEM	ATCC	30-2003	Complete EMEM: 440mL EMEM 50mL FBS 5mL P/S 5mL NEAA SF-EMEM 490mL EMEM 5mL P/S 5mL NEAA
FBS	Gibco	16140-071	
P/S	Gibco	15140-122	
MEN NEAA	Gibco	11140-050	
OrganoReady Colon Caco-2 3-lane 40	MIMETAS	MI-OR-CC-01	
TNF-α	Immunotools	11343015	50ug in 500uL water to get 100ug/mL, then 1:500 in culture medium
IL-1β	Immunotools	11340015	50ug in 500uL water to get 100ug/mL, then 1:500 in culture medium
IFN-γ	Preprotech	300-02	20ug in 200uL water to get 100ug/mL, then 1:500 in culture medium

Chemicals and reagents for lipid profiling

LC-MS grade acetonitrile, methanol, isopropanol, glacial acetic acid were acquired from Biosolve (Valkenswaard, Netherlands); 1-Butanol (99%) from Thermo Scientific (Belgium); Butylated hydroxytoluene (≥99%), ethylenediaminetetraacetic acid (≥99%) from Sigma-Aldrich; citric acid monohydrate was purchased from Roth (Karlsruhe, Germany); disodium hydrogen phosphate (≥99.5, Supelco, Merck, Darmstadt, Germany). Purified MilliQ water was obtained from Arium® pro VF system (Sartorius Stedim biotech, Göttingen, Germany). Isotopicaly labelled standards were purchased from Cayman Chemicals (Ann Arbor, MI, USA) and Avanti Polar Lipids (Alabaster, AL, USA) as previously described.[1]

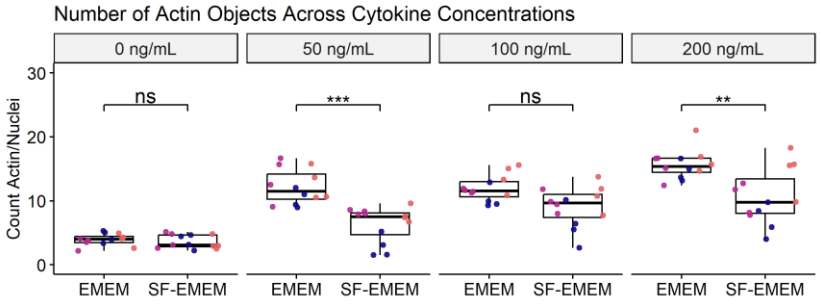

Figure S1. *Number of actin objects across cytokine concentrations in EMEM and SF-EMEM media. Boxplots show the count of actin objects per nucleus in Caco-2 tubules exposed to increasing cytokine concentrations (0, 50, 100, and 200 ng/mL) in EMEM and SF-EMEM. Each dot represents a measurement from a single chip in the OrganoPlate. Colors indicate independent experiments. Results are derived from three independent experiments (N = 3), with four technical replicates per cytokine concentration (n = 4). Statistical significance between media types at each cytokine concentration was determined using an unpaired t-test, with p-values adjusted for multiple comparisons using the Bonferroni correction. Significance levels are indicated as follows: *** p<0.001, * p<0.05, ns: not significant.*

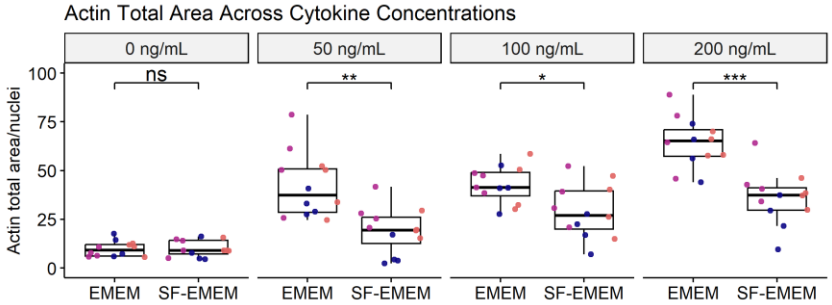

Figure S2. *Total area of actin objects across cytokine concentrations in EMEM and SF-EMEM media. Boxplots show the total area of actin objects per nucleus in Caco-2 tubules exposed to increasing cytokine concentrations (0, 50, 100, and 200 ng/mL) in EMEM and SF-EMEM. Each dot represents a measurement from a single chip in the OrganoPlate. Colors indicate independent experiments. Results are derived from three independent experiments (N = 3), with four technical replicates per cytokine concentration (n = 4). Statistical significance between media types at each cytokine concentration was determined using an unpaired t-test, with p-values adjusted for multiple comparisons using the Bonferroni correction. Significance levels are indicated as follows: *** p<0.001, ** p<0.01, * p<0.05, ns: not significant.*

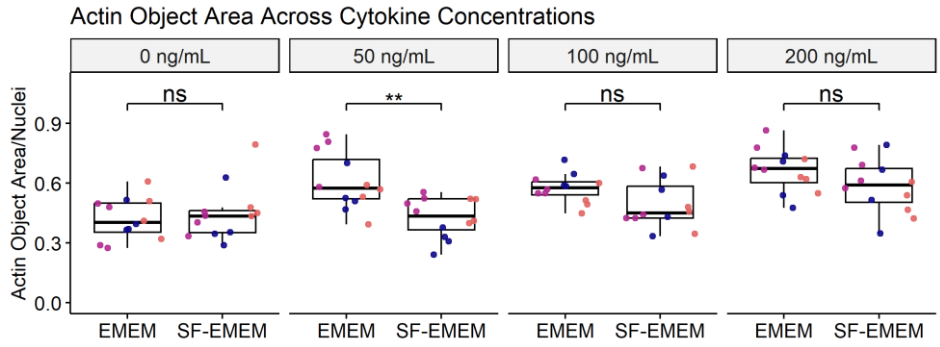

Figure S3. *Average area of actin objects across cytokine concentrations in EMEM and SF-EMEM media. Boxplots show the area of actin objects per nucleus in Caco-2 tubules exposed to increasing cytokine concentrations (0, 50, 100, and 200 ng/mL) in EMEM and SF-EMEM. Each dot represents a measurement from a single chip in the OrganoPlate. Colors indicate independent experiments. Results are derived from three independent experiments (N = 3), with four technical replicates per cytokine concentration (n = 4). Statistical significance between media types at each cytokine concentration was determined using an unpaired t-test, with p-values adjusted for multiple comparisons using the Bonferroni correction. Significance levels are indicated as follows: *** p<0.001, ** p<0.01, * p<0.05, ns: not significant.*

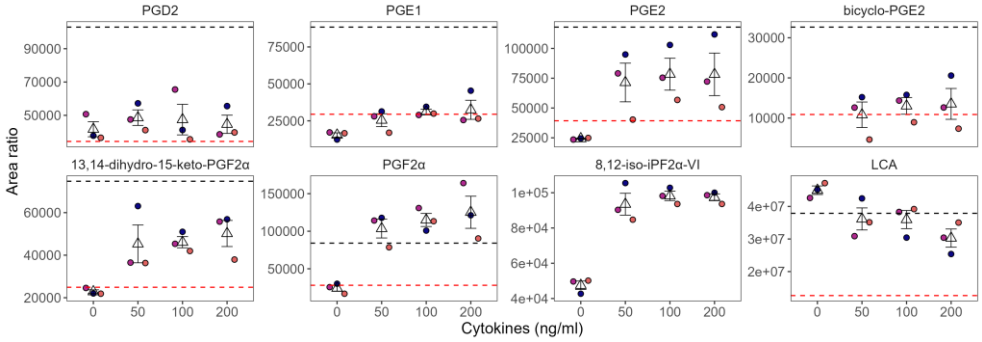

Figure S4. *Impact of cytokine exposure on signaling lipids in EMEM media, apical side that did not meet the quality control criteria (Table S2 in Online Supplementary Materials). Results are derived from three independent experiments (N = 3). Dot colors indicate independent experiments. The triangular marker represents the mean of the three replicates, while the error bars are based on the standard deviation. The red and black dotted lines represent respectively one and three times the median level in blank SF-EMEM.*

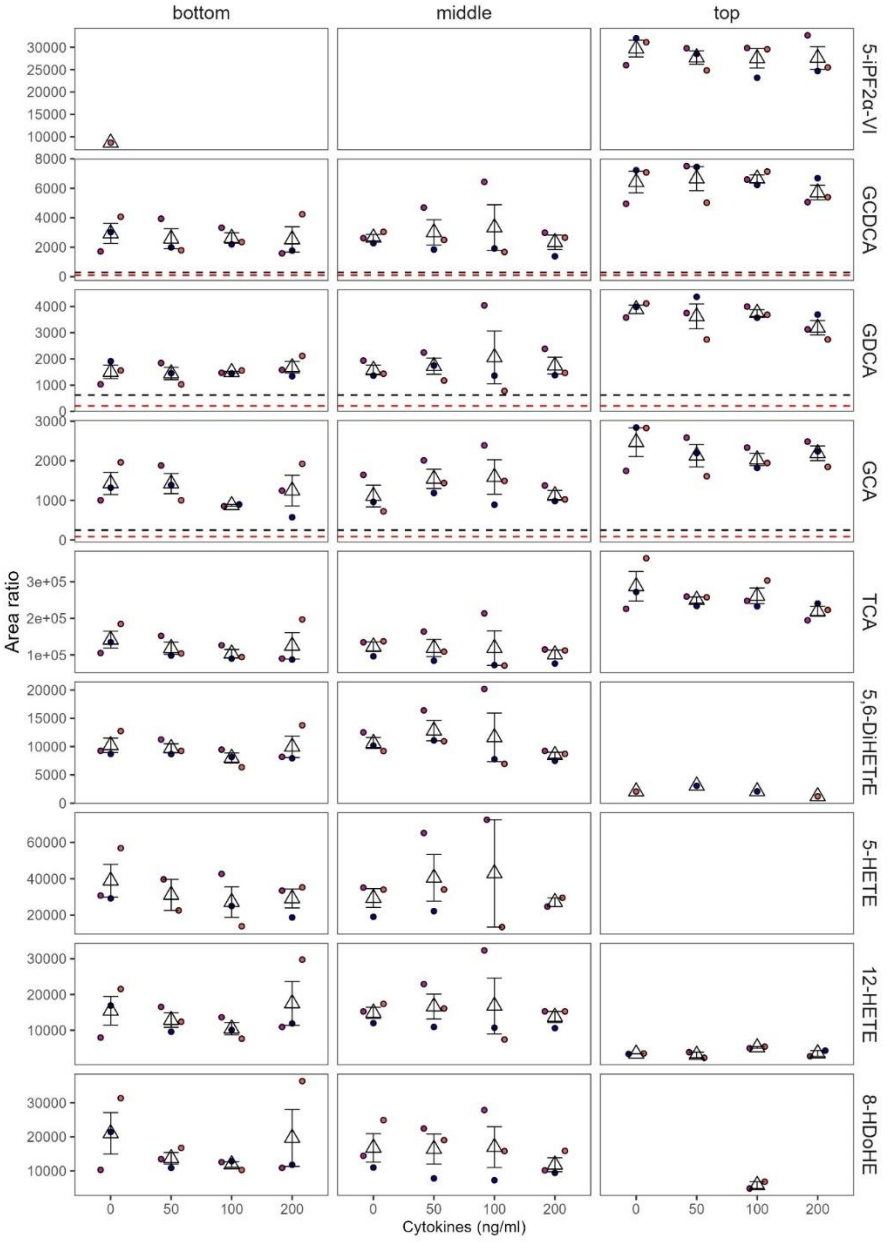

***Figure S5**. Signaling lipids with levels, irrespective of the cytokine concentration, that are lower or higher in the top channel, compared to the middle and bottom channels under SF-EMEM media conditions. Results are derived from three independent experiments (N = 3) except for 5-iPF2α-VI in the bottom channel where only 1 measurement was above the detection limit. Dot colors indicate independent experiments. The triangular marker represents the mean of the three replicates, while the error bars are based on the standard deviation. The red and black dotted lines represent respectively one and three times the median level in blank SF-EMEM.*

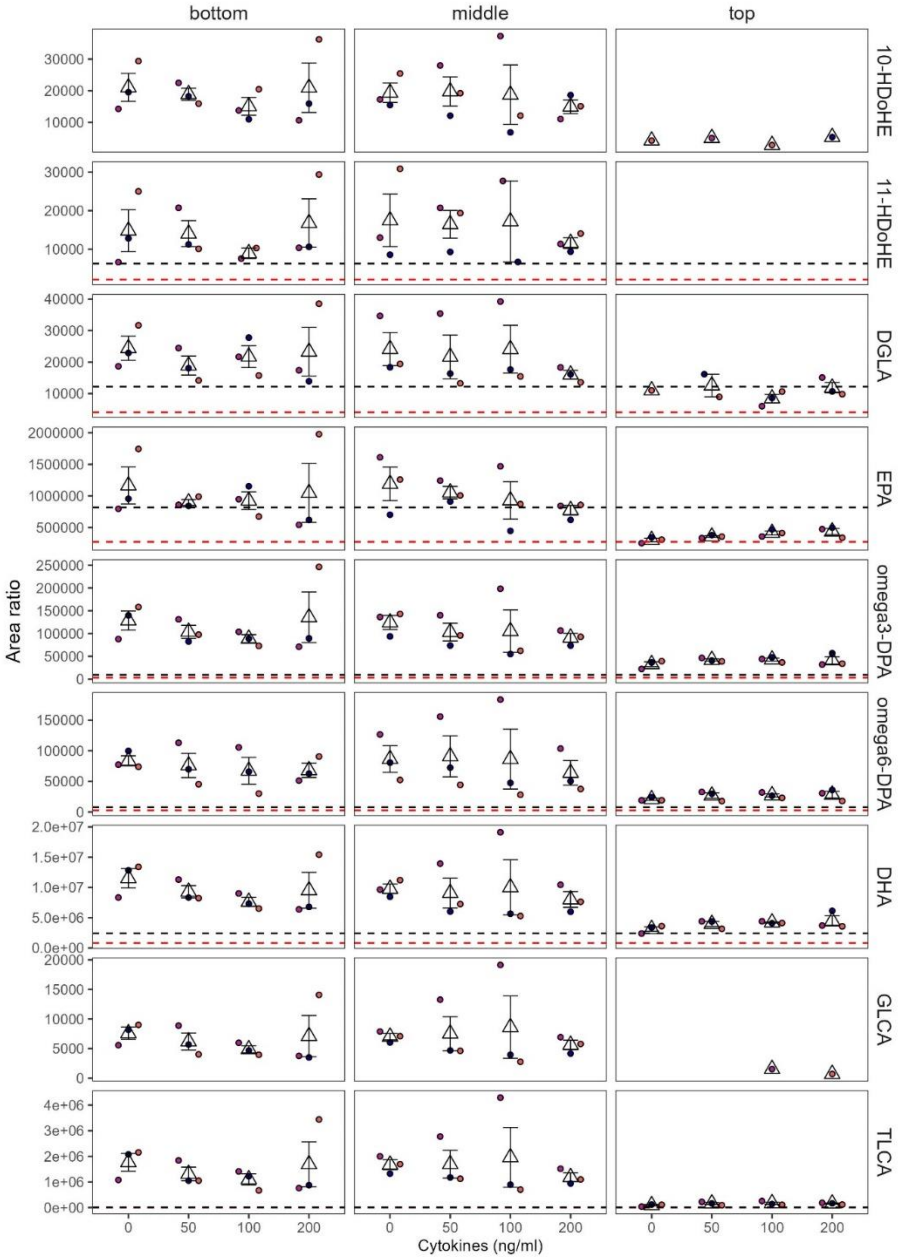

Figure S5 *(continued). Signaling lipids with levels, irrespective of the cytokine concentration, that are lower or higher in the top channel, compared to the middle and bottom channels under SF-EMEM media conditions. Results are derived from three independent experiments (N = 3). Missing measurement points correspond to signals below the detection limit. Dot colors indicate independent experiments. The triangular marker represents the mean of the three replicates, while the error bars are based on the standard deviation. The red and black dotted lines represent respectively one and three times the median level in blank SF-EMEM.*

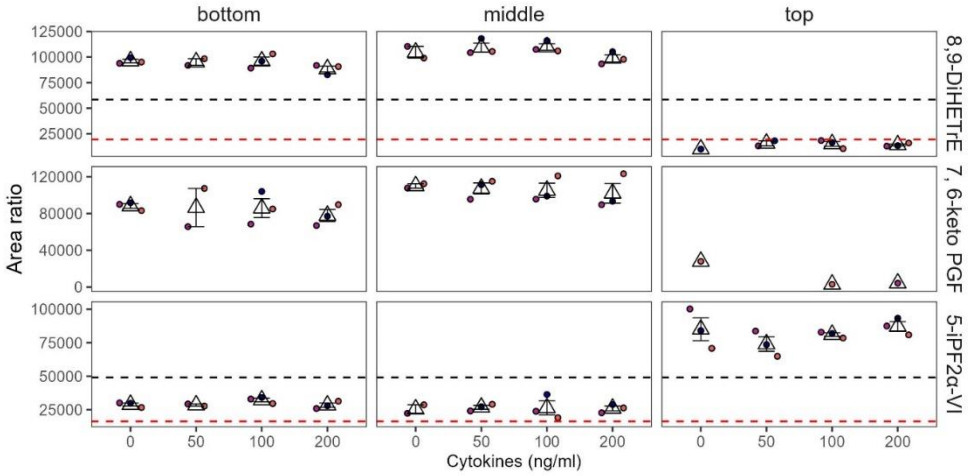

***Figure S6**. Signaling lipids with levels, irrespective of the cytokine concentration, that are lower or higher in the top channel, compared to the middle and bottom channels under EMEM media conditions. Results are derived from three independent experiments (N = 3) except measurements of bottom channel 50 ng/ml and middle channel 0 ng/ml where the replicates were 2. Other missing measurement points correspond to signals below the detection limit. Dot colors indicate independent experiments. The triangular marker represents the mean of the three replicates, while the error bars are based on the standard deviation. The red and black dotted lines represent respectively one and three times the median level in blank EMEM.*

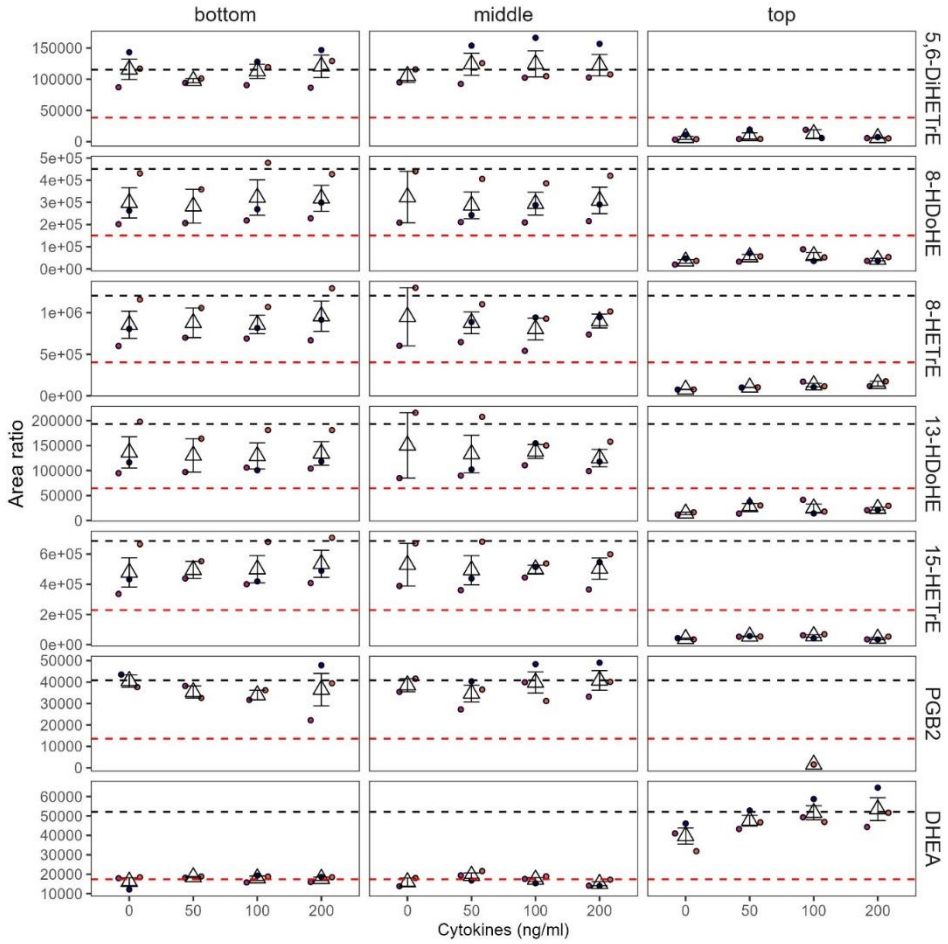

***Figure S7**. Fraction of the signaling lipids with levels, irrespective of the cytokine concentration, that are lower or higher in the top channel, compared to the middle and bottom channels under EMEM media conditions but did not meet out quality control. Results are derived from three independent experiments (N = 3) except measurements of bottom channel 50 ng/ml and middle channel 0 ng/ml where the replicates were 2. Other missing measurement points correspond to signals below the detection limit. Dot colors indicate independent experiments. The triangular marker represents the mean of the three replicates, while the error bars are based on the standard deviation. The red and black dotted lines represent respectively one and three times the median level in blank EMEM.*

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Chapter 6

Conclusion and perspectives

The global rise in allergy incidence is a growing concern, as allergies can significantly diminish quality of life and, in severe cases, be life-threatening. The first 1000 days of life are critical for immune system development and gut microbial colonization. Considering the high immune cell and microbial density in the gut, the microbiome and immune system closely influence each other, making early-life microbiome colonization essential for proper immune development. Disruptions in gut microbiome (GM) composition in early life, particularly reduction in bifidobacteria, have been linked to immune-mediated diseases, including allergies. However, there is a considerable knowledge gap in understanding the link between the GM and allergies, and how microbial-based interventions can be used for allergy management. As the GM impacts host physiology through the production of diverse metabolites, examining these small molecules offers direct mechanistic insights into host–microbiome interactions. The research described in this thesis aimed to study the links between allergy and intestinal health, the GM, and external factors by exploring the metabolome in longitudinal clinical studies and gut barrier functioning in vitro gut-on-a-chip models.

Chapter 2 gave a comprehensive overview of current knowledge on the role of the GM in IgE-mediated CMA and the effect of microbiome-based interventions on the GM in children and animal models. The review showed that IgE-mediated CMA is consistently associated with a reduction in *Bifidobacterium* spp., and that probiotic *Bifidobacterium* interventions effectively induce bifidobacterial growth in the gut. Importantly, the chapter showed that most of the research on the topic is primarily focused on microbiome compositional analysis and that shotgun metagenomics, (meta-)transcriptomics, and (meta-)proteomics research is lacking. What is more, metabolomics studies were found to be greatly limited by the narrow range of metabolites studied, mainly focused on short-chain fatty acids (SFCAs), amino acids, and organic acids. The review highlighted the need for metabolomics studies examining a broader range of gut microbial metabolites to study the interplay between the microbiome and the host. It also greatly emphasized the need for multi-omics studies to get a mechanistic understanding of the link between the GM and CMA in early life.

As stated in the introduction, the studies presented in **Chapter 3** and **Chapter 4** addressed the limited metabolomic scope reported in the literature by covering a wide range of host- and microbiota-derived metabolites, including microbial amino acid metabolites, bile acids, and short-chain fatty acids. In **Chapter 3**, infants at risk of developing allergies, exclusively breastfed for at least 16 weeks, were followed during the first year of their lives, a period of rapid microbial colonization and potential onset of the atopic march. Alteration in the fecal metabolome and key microbiome members were examined in relation to age, diet, delivery mode, and allergy development during this period. The findings revealed significant age-related metabolomic shifts likely driven by concurrent alterations of the host metabolism, feeding practices, and microbiome composition. These included increases in amino acid derivatives, bile acids (BAs), B vitamins, nucleosides, SCFAs, and phenolic acids, along with decreases in long-chain fatty acids and acylcarnitines. We showed that C-section

was significantly associated with fecal metabolome alterations up to six months of age. Owing to the prospective nature of the study, it was possible to identify that infants who developed allergies within the study period had lower levels of *Bifidobacterium* spp. and significantly elevated levels of long-chain fatty acids prior to onset of clinical manifestations. Even though the findings of this research require validation in a larger cohort, the study advanced our understanding of the fecal metabolome development in early life and factors that shape it during this critical period of immune system and microbiome development. Future research should examine larger cohorts and stratify the IgE-mediated and non-IgE-mediated cases, as well as differentiate among various allergy types (skin, food, or respiratory). Differentiation of the allergy subtypes acknowledges the difference in immune mechanisms, which is essential for identifying disease-specific alterations with greater clinical relevance. To elucidate the potential link between elevated long-chain fatty acid levels and allergy development, integrated analyses of the fecal microbiome, plasma metabolome, and maternal breastmilk lipid composition, e.g. free fatty acids, are essential.

Chapter 4 explored the link between IgE-mediated CMA, the GM, and bifidogenic-synbiotic supplementation by means of fecal metabolomics. The longitudinal data analysis revealed minor metabolome alterations associated with tolerance acquisition to cow's milk protein, including alterations to the branched-chain SCFAs, BAs, and amino acid levels. Notably, infants who developed tolerance exhibited significantly elevated citrulline levels, suggesting reduced gut permeability, as well as an insignificantly lower serotonin and 5-hydroxytryptophan, which are involved in inflammation. One of the study's key findings is that the impact of synbiotic supplementation on the fecal metabolome was most pronounced after six months of intervention, with changes largely diminishing by 12 months, suggesting that early intervention is required to maximize the effect of synbiotics. Specifically, synbiotic supplementation led to increased levels of aromatic lactic acids, purine metabolites, long-chain fatty acids, and BAs, reflecting changes in GM activity. Among these, indolelactic acid and 4-hydroxyphenyllactic acid, aromatic amino acid metabolites of infant-type *Bifidobacterium*, were significantly elevated and positively correlated with the abundance of the *Bifidobacterium* genus. These findings complement the microbiome and (meta-)proteomics findings from the same cohort,[1] further supporting the efficacy of the synbiotic intervention in promoting *Bifidobacterium* growth and activity in the gut. While the synbiotic had no statistically significant effect on tolerance acquisition,[2] the observed increase in anti-inflammatory indolelactic acid suggests that synbiotic supplementation may still confer immunological benefits. Larger cohort studies are required to verify these findings and their clinical implication. Additionally, in vitro gut-on-a chip models may be utilized to understand how the tolerance-associated and synbiotic-driven metabolome associations relate to immune response.

Using Caco-2 tubules in a membrane-free microfluidic organ-on-chips platform (OrganoPlate), **Chapter 5** demonstrated how cytokine exposure impacts intestinal barrier integrity and the secretion of signaling lipids under serum-containing and serum-free

medium conditions. Pro-inflammatory cytokine exposure significantly increased intestinal permeability, cellular permeability, and induced actin remodeling under both conditions. While the intestinal permeability and cellular permeability alterations were comparable between media conditions, actin remodeling was significantly lower in serum-free compared to serum conditions. The impaired gut barrier was associated with elevated prostaglandin levels in the apical, but not in the basolateral compartment, with this effect being more pronounced under serum-free conditions. The developed integrated model offers a valuable framework for exploring the interplay between inflammation, barrier integrity, and lipid metabolism in intestinal pathophysiology which is also relevant for allergy research. Future work should refine model systems by co-culturing epithelial cells with immune or mucus-producing cells to better reflect gut physiology.

Perspectives

Research is increasingly revealing the crucial role of the GM in shaping immune function and influencing susceptibility to immune-mediated diseases. Despite that, still the link between immune health, the GM, and nutrition is only slowly being understood. There remains a pressing need for mechanistic studies to clarify how microbial communities influence host's immune response and how nutritional interventions can be utilized as prevention and treatment strategies for immune-mediated diseases, such as allergies. The perspectives presented in this chapter are structured around four key topics: clinical study design considerations, technical considerations, multi-omics data integration, and in vitro modeling.

Clinical study design considerations

To better understand the host-microbiome interaction in the context of allergy in addition to the fecal samples analyzed in this thesis it is also crucial to examine the circulating metabolome. Profiling the plasma metabolome gives insights into which microbial metabolites enter the bloodstream and may influence the host's immune response. The standard sampling of blood is, however, invasive and non-ethical in infants. Thanks to recent developments, blood micro-sampling is emerging as a highly promising, minimally invasive technique, already used for neonates.[3] Since many gut microbial metabolites (e.g., indoxyl sulfate and hippuric acid) get absorbed into the bloodstream and subsequently excreted in urine, profiling the urine metabolome is also relevant. Urine sampling is particularly advantageous in infants, as it is non-invasive and can be achieved using cotton balls as adsorption material.

Immune markers in blood, feces, and saliva are of interest for understanding the relation between host's immune response and the GM. Integrating these markers with metabolomic profiling can enhance the understanding of host-microbiome interactions in allergy as well as following microbiome-targeted interventions. Gastrointestinal motility and transit time, though often overlooked, are important factors affecting the gut microbiome and metabolome[4] and are associated with allergy.[5] Future studies investigating allergy

development and resolution in infants should consider whether observed microbiome and metabolome differences are mediated by variations in intestinal motility. Throughout early life, fecal frequency and consistency may serve as proxies for transit time, with the sweet corn test providing a more direct measure of transit time once solid foods are introduced. Given that certain metabolites are associated with both intestinal immunity and motility, metabolomic data should be interpreted alongside gastrointestinal motility indicators and (fecal) biomarkers of intestinal immune function and barrier integrity.

Considering the dynamic nature of the early-life microbiome and metabolome, future clinical studies in this field should adapt longitudinal designs, as in this thesis. The study design can be further improved by enrolling participants of a narrow age range and doing more frequent sampling throughout the study period. To reduce the variation due to dietary differences, it is recommended that infants receive an age-appropriate standardized diet prior to sampling when feasible. Alternatively, detailed dietary records, particularly after the introduction of complementary foods, can be collected. Such study design would enhance the ability to isolate effects of interest, such as allergy development, and to detect subtle yet potentially important metabolome alterations.

In studies involving breastfed infants, information on the mother's nutrition through dietary records and breastmilk compositional analysis (e.g. HMO, lipid, and metabolomic profiling) would also be valuable in revealing possible associations between maternal diet, breastmilk composition, and infant microbiome and metabolome in the context of allergy development. Such research may guide the design of nutritional interventions for mothers during gestation and breastfeeding periods. Future clinical trials should also examine alternative allergy prevention and treatment strategies for formula-fed infants. Human milk oligosaccharides (HMOs) are structurally diverse components of human breast milk that play a crucial prebiotic role in supporting *Bifidobacterium* growth. The HMOs complexity is not reflected by the galactooligosaccharides and fructooligosaccharides commonly used as prebiotics in infant formula and only a few HMOs have been shown to be both safe and well-tolerated for supplementation in infant formula.[6] Future studies should focus on examining the effect of prebiotic HMOs supplementation, preferably within a synbiotic blend, in the context of allergies.

As highlighted in the introduction of this thesis, *Bifidobacterium* spp. are undoubtedly beneficial for infants' immune system development and maturation. In addition to *B. breve*, used as a probiotic in **Chapter 4** of this thesis, other bifidobacterial species i.e. *B. longum* and *B. bifidum* are also key members of infants' gut and play crucial immunoregulatory roles.[6] Combining those beneficial bifidobacteria as a probiotic blend may thus better reflect the gut community and improve clinical outcomes. Given the variations in HMO degradation capabilities and cross-feeding between bifidobacteria,[8] such probiotic mixtures may be particularly valuable and necessary when combined with prebiotic HMOs blend. Other than bifidobacteria, *Lactobacillus* species are also key infant GM members and have beneficial effects on the immune system[9] and have been used for allergy treatment

(**Chapter 2**). Their probiotic potential as a blend with bifidobacteria species should also be explored in formula-fed infants.

Technical considerations

In this thesis, reverse-phase liquid chromatography coupled with high-resolution mass spectrometry (LC-HRMS) was the primary analytical technique used for fecal metabolomics. Data were acquired in untargeted full-scan mode (MS1 data), while preprocessing was performed in a targeted manner, focusing on known host- and microbially-derived metabolites. Several aspects of data acquisition and processing can be improved to strengthen future exploratory studies. Analyzing the data in an untargeted manner would allow for the detection of both known and novel microbial metabolites potentially involved in the interplay between allergies, the GM, and external factors. However, for untargeted metabolomics, accurate mass and chromatographic retention time information is insufficient for confident metabolite identification. To enhance compound identification and improve biological interpretation, future studies should acquire data using tandem mass spectrometry (MS/MS) using data dependent acquisition or data independent acquisition such as Sequential Windowed Acquisition of All Theoretical Fragment Ion Mass Spectra (SWATH). Additionally, the application of electron activated dissociation (EAD)[10] alongside the commonly used collision induced dissociation (CID) holds promise for enhancing metabolite annotation, as the two techniques are complementary - a potential that remains largely underexplored. Following untargeted metabolomics, targeted quantitative analysis should be performed to validate and confirm key findings.

Future studies should expand analyte profiling to include bioactive lipids like prostanoids and sphingolipids, known for their key role in inflammation and allergic responses.[11] Alterations in the bioactive lipids derived from enzymatic oxidation of polyunsaturated fatty acid such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are expected considering the altered LCFA profiles in infants prior allergy onset observed in **Chapter 3**. Given the role of BAs in regulating immune response[12] and their association with tolerance acquisition observed in **Chapter 4**, future research should also explore the role of recently discovered "microbially conjugated BAs"[13] in immune response and allergy.

Multi-omics data integration

The host–microbiome relationship is inherently complex and multifactorial. While individual omics approaches, such as metabolomics, (meta-)genomics, (meta-) transcriptomics, and (meta-)proteomics, offer valuable insights, none alone can fully capture the complexity of this dynamic interplay. To unravel the mechanistic links between allergies, the GM, and environmental and dietary factors, integrated multi-omics strategies are essential. Ideally, combining all omics levels would provide the most comprehensive picture, however, such an approach is often constrained by high costs, time-limitations and complex data integration. To gain mechanistic insights into the host–microbiome interplay, I envision the integration of metabolomics with whole metagenome sequencing as a

powerful approach, capturing functional microbiome activity along with high-resolution taxonomic profiles and genetic potential. For exploratory research, combining metabolomics with 16S rRNA sequencing still offers valuable, insights into both microbial composition and function. Integrating microbiome and metabolome data remains a significant challenge, necessitating broader metabolite coverage, advanced metabolite annotation, and improved data integration strategies to deepen our understanding of the host–microbiome interplay.

In vitro modeling

By combining epithelial-immune cell co-culture with an anaerobic microbial compartment, gut-on-a-chip models offer powerful platforms for studying intestinal inflammation and host-microbiome interactions.[14] Moving forward, such models should be leveraged to gain mechanistic insights into the link between the GM, intestinal barrier, and host's immune system in the context of allergies.[15] To achieve that, ideally, the microbiome compartment of the gut-on-a-chip model would mimic the complexity of the infant GM. An emerging approach is to culture bacterial isolates from fresh infant fecal samples under anaerobic conditions.[16] A co-culture reflecting key members of the infant GM could be a preferred alternative. Both approaches come with their technical challenges including but not limited to the maintenance of oxygen gradient that supports the anaerobic microbiome and the aerobic human cells. This could be studied in the gut-on-chip platform used in **Chapter 5** using an intestinal epithelium tubule with apical (lumen) channel perfused with an anaerobic bacterial co-culture and a basolateral channel perfused with aerobic medium. To ensure allergen sensitization instead of tolerance acquisition, such models could be developed by firstly skewing the immune compartment toward the T helper cell 2 (Th2) phenotype, followed by exposing the epithelial compartment to the allergen of interest. Such gut-on-a-chip models can be applied following exploratory clinical research, such as that presented in this thesis. Those models have the potential to shed light on how metabolomic alterations associated with allergy development and tolerance acquisition affect the immune response and intestinal barrier and help uncover the underlying immunological mechanisms. Additionally, they can facilitate the evaluation of how intervention-driven metabolomic changes influence the host immune system. The insights gained from the models would be invaluable for designing follow-up clinical trials and guiding prevention and treatment strategies. Gut-on-a-chip models also hold promise in preclinical research, where they can be used to evaluate the effects of novel probiotics and synbiotics on the immune response and intestinal barrier.

Through fecal metabolomics and in vitro modelling, this thesis has contributed to advancing our understanding of the complex interactions between allergy and intestinal health, the GM, and external factors. Future research should prioritize well-designed longitudinal clinical studies, comprehensive metabolomic profiling, immune response assessment, multi-omics integration, and the use of physiologically relevant in vitro models such as gut-on-a-chip systems. In parallel, alternative allergy prevention and treatment strategies that

mimic healthy breastfed infant gut environment should be explored as potential modulators of infant immune development. Achieving these will require multidisciplinary collaboration, ultimately paving the way for microbiome-based nutritional strategies to prevent and treat immune-mediated diseases such as allergies in early life.

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Appendix

Summary

Samenvatting

Curriculum Vitae

List of Publications

Acknowledgements

Summary

The rise in allergy incidence, especially in Western countries, is a growing concern, as allergies can significantly diminish quality of life and, in severe cases, be life-threatening. At the core of allergies is a breakdown in immune tolerance; the inability of the immune system to regulate responses to harmless environmental antigens appropriately. Multiple hypotheses have been proposed to explain the increase in allergies in Western countries with a leading hypothesis proposing that reduced microbial exposure can lead to an ill-trained immune system, incapable of recognizing a friend from a foe, resulting in allergen sensitization and thus allergies.

Our guts harbor a large proportion of our body's immune cells and support one of the largest microbial ecosystems, consisting of trillions of microorganisms. Gut microbial colonization occurs rapidly in early life, particularly during the first year, until the gut microbiome (GM) starts resembling that of an adult by approximately three years of age. This process is shaped by multiple factors, including mode of delivery (vaginal birth versus cesarean section), antibiotic exposure, and diet. Notably, breastfeeding promotes a GM rich in bifidobacteria, which utilize sugars in breastmilk and play a key role in supporting immune system development during early life.

Disruptions in gut microbial composition during early life, particularly reduction in bifidobacteria, have been linked to immune-mediated diseases, including allergies. Because of that, the first three years of life are often seen as a critical period during which GM disturbances can lead to disease, but also as a window of opportunity in which the GM's dynamic nature can be used to prevent or treat diseases such as allergies. However, there is a considerable knowledge gap in understanding the link between the GM and allergies, and how microbial-based interventions can be used for allergy management. As the GM impacts host physiology through the production of diverse metabolites, examining these small molecules offers direct mechanistic insights into host–GM interactions. Those metabolites can be measured non-invasively in fecal samples using a technique called liquid chromatography – mass spectrometry.

The research is based on the hypothesis that the GM in early life influences the development and resolution of allergies via the production of metabolites and that metabolomics can be used to study the function of the GM. The aim of this research is to study the links between allergy and intestinal health, the GM, and external factors by exploring the metabolome in longitudinal clinical studies and in vitro models.

Chapter 2 gives a comprehensive overview of current knowledge on the role of the GM in the most common food allergy in early life: IgE-mediated cow's milk allergy (CMA). The review shows that IgE-mediated CMA is consistently associated with a reduction in *Bifidobacterium* spp., and that probiotic *Bifidobacterium* interventions effectively induce bifidobacterial growth in the gut. Notably, the chapter highlights that research in this area

has predominantly focused on microbial compositional analysis, with a limited number of studies exploring the metabiota and none investigating other omics layers, such as proteomics. Furthermore, metabolomics research has been limited by the narrow range of metabolites examined, primarily short-chain fatty acids (SCFAs), amino acids, and organic acids. The review emphasizes the urgent need for metabolomics studies encompassing a broader range of gut microbial metabolites to better understand the microbiome–host interplay. It also strongly underscores the importance of multi-omics approaches to uncover mechanistic insights into the relationship between the GM and CMA in early life.

Chapter 3 and **Chapter 4** address the limited metabolomic scope reported in the literature by covering a wide range of host- and microbiota-derived metabolites, including microbial amino acid metabolites, bile acids, and SCFAs. In **Chapter 3**, infants at risk of developing allergies, exclusively breastfed for at least 16 weeks, were followed during the first year of their lives, a period of rapid microbial colonization and potential onset of the atopic march. Alteration in the fecal metabolome and key microbial members were examined in relation to age, diet, delivery mode, and allergy development during this period. The findings revealed significant age-related metabolomic shifts likely driven by simultaneous alterations of the host metabolism, feeding practices, and microbial composition. These included increases in amino acid metabolites, bile acids, B vitamins, SCFAs, and phenolic acids, along with decreases in long-chain fatty acids and acylcarnitines. C-section was found to significantly associate with fecal metabolome alterations up to six months of age. Owing to the prospective nature of the study, it was possible to identify that infants who developed allergies within the study period had lower levels of *Bifidobacterium* spp. and significantly higher levels of long-chain fatty acids prior to onset of clinical manifestations. Even though the findings of this research require validation in a larger cohort, the study advanced our understanding of the fecal metabolome development in early life and factors that shape it during this critical period of immune system and microbiome development.

IgE-mediated CMA is typically managed through elimination diets, including the use of amino acid-based formulas (AAF) in formula-fed infants. Given the growing evidence of the beneficial role of *Bifidobacterium* spp. for the immune development and their association with allergy, shown in **Chapter 2** and **Chapter 3**, bifidogenic supplementation of AAF has emerged as a promising strategy in the management of IgE-mediated CMA. **Chapter 4** explores the link between IgE-mediated CMA, the GM, and bifidobacteria-promoting synbiotic supplementation by means of fecal metabolomics. For this, infants diagnosed with IgE-mediated CMA who received either standard AAF or AAF supplemented with synbiotic blend of probiotic *Bifidobacterium breve* M-16 V and prebiotic inulin and oligofructose were followed for a year. The longitudinal data analysis revealed minor metabolome alterations associated with tolerance acquisition to cow's milk protein, including alterations to the branched-chain SCFAs, bile acids, and amino acid levels. Notably, infants who developed tolerance exhibited significantly elevated citrulline levels, suggesting reduced gut permeability, as well as insignificantly lower levels of serotonin and 5-hydroxytryptophan,

which are involved in inflammation. One of the study's key findings is that the impact of synbiotic supplementation on the fecal metabolome was most pronounced after six months of intervention, with changes largely diminishing by 12 months, suggesting that early intervention is required to maximize the effect of synbiotics. Specifically, synbiotic supplementation led to increased levels of aromatic lactic acids, purine metabolites, long-chain fatty acids, and bile acids, reflecting changes in GM activity. Among these, indolelactic acid and 4-hydroxyphenyllactic acid, aromatic amino acid metabolites of infant-type *Bifidobacterium*, were significantly elevated and positively correlated with the abundance of the *Bifidobacterium* genus. These findings complement the microbiome and proteomics findings from the same cohort, further supporting the efficacy of the synbiotic intervention in promoting *Bifidobacterium* growth and activity in the gut. While the synbiotic had no statistically significant effect on tolerance acquisition, the observed increase in anti-inflammatory indolelactic acid suggests that synbiotic supplementation may still confer immunological benefits.

3D gut-on-a-chip platforms are promising in vitro models that replicate key aspects of intestinal physiology. Such models can be used to study the intestinal barrier, the dysfunction of which plays a role in a variety of diseases, such as allergies. Using an intestinal epithelium tubules-on-a-chip model, **Chapter 5** demonstrates how pro-inflammatory cytokines exposure impacts intestinal barrier integrity and the secretion of signaling lipids under serum-containing and serum-free medium conditions. Pro-inflammatory cytokine exposure significantly increased intestinal permeability, cellular permeability, and induced structural changes to the intestinal barrier under both conditions. While the intestinal permeability and cellular permeability alterations were comparable between media conditions, structural damage, assessed by actin remodeling, was significantly lower in serum-free compared to serum conditions. The impaired gut barrier was associated with elevated prostaglandin levels in the apical (luminal), but not in the basolateral compartment, with this effect being more pronounced under serum-free conditions. The developed integrated model offers a valuable framework for exploring the interplay between inflammation, barrier integrity, and lipid metabolism in intestinal pathophysiology which is also relevant for allergy research.

Chapter 6 summarizes the key findings of the thesis and discusses perspectives for future research. Using fecal metabolomics and *in vitro* modelling, this thesis has advanced our understanding of the complex interactions between allergy and intestinal health, the GM, and external factors. The field would benefit from well-designed longitudinal clinical studies integrating multi-omics approaches and advanced physiologically relevant *in vitro* models. In addition, alternative allergy prevention and treatment strategies that mimic healthy breastfed infant gut environment should be explored. Such efforts require multidisciplinary collaboration, ultimately paving the way for microbiome-based nutritional strategies to prevent and treat allergies.

Samenvatting

De toename van de incidentie van allergieën, met name in Westerse landen, is een groeiende zorg, aangezien allergieën de kwaliteit van leven aanzienlijk kunnen verminderen en in ernstige gevallen levensbedreigend kunnen zijn. Aan de basis van allergieën ligt een verstoring van de immuuntolerantie: het onvermogen van het immuunsysteem om adequaat te reageren op onschadelijke omgevingsantigenen. Er zijn verschillende hypothesen voorgesteld om de toename van allergieën in Westerse landen te verklaren, waarvan een leidende hypothese stelt dat verminderde blootstelling aan microben kan leiden tot een slecht getraind immuunsysteem dat vriend en vijand niet goed kan onderscheiden. Dit resulteert in allergische sensibilisatie en dus allergieën.

Onze darmen bevatten een groot deel van de immuuncellen van het lichaam en herbergen een van de grootste microbiële ecosystemen, bestaande uit triljoenen micro-organismen. De kolonisatie van de darmmicrobiota vindt snel plaats in de vroege levensfase, vooral tijdens het eerste levensjaar, waarna het darmmicrobioom (DM) rond de leeftijd van drie jaar begint te lijken op dat van een volwassene. Dit proces wordt beïnvloed door verschillende factoren, waaronder de wijze van geboorte (vaginale geboorte versus keizersnede), blootstelling aan antibiotica en voeding. Borstvoeding bevordert bijvoorbeeld een DM dat rijk is aan bifidobacteriën, dat de suikers in moedermelk benut en een cruciale rol speelt in de ontwikkeling van het immuunsysteem tijdens de vroege levensfase.

Verstoring van de samenstelling van de darmmicrobiota in de vroege levensfase, in het bijzonder een vermindering van bifidobacteriën, is in verband gebracht met immuungemedieerde ziekten, waaronder allergieën. Daardoor worden de eerste drie levensjaren vaak gezien als een kritieke periode waarin verstoringen van het DM kunnen leiden tot ziekte, maar ook als een venster van mogelijkheden waarin de dynamische aard van het DM kan worden benut om ziekten zoals allergieën te voorkomen of te behandelen. Toch bestaat er een aanzienlijke kenniskloof in het begrijpen van de relatie tussen het DM en allergieën, en hoe microbiële interventies kunnen worden ingezet voor allergiemanagement. Aangezien het DM de fysiologie van de gastheer beïnvloedt via de productie van diverse metabolieten, kunnen deze kleine moleculen directe mechanistische inzichten bieden in de interacties tussen gastheer en DM. Deze metabolieten kunnen niet-invasief worden gemeten in fecesmonsters met behulp van vloeistofchromatografie–massaspectrometrie.

Dit onderzoek is gebaseerd op de hypothese dat het DM in de vroege levensfase de ontwikkeling en het verdwijnen van allergieën beïnvloedt via de productie van metabolieten, en dat metabolomics kan worden gebruikt om de functie van het DM te bestuderen. Het doel van dit onderzoek is het bestuderen van de verbanden tussen allergie en darmgezondheid, het DM en externe factoren door het verkennen van het metaboloom in longitudinale klinische studies en in vitro-modellen.

Hoofdstuk 2 geeft een uitgebreid overzicht van de huidige kennis over de rol van het DM in de meest voorkomende voedselallergie in de vroege levensfase: IgE-gemedieerde koemelkallergie (KMA). Het overzichtsartikel toont aan dat IgE-gemedieerde KMA consequent geassocieerd is met een vermindering van *Bifidobacterium*-soorten, en dat probiotische *Bifidobacterium*-interventies effectief de groei van bifidobacteriën in de darm stimuleren. Daarnaast benadrukt het hoofdstuk dat onderzoek in dit veld voornamelijk gericht was op compositieanalyse van de microbiota, terwijl slechts een beperkt aantal studies het metaboloom onderzocht, en geen enkele zich heeft gericht op andere omics-lagen, zoals proteomics. Verder was metabolomics-onderzoek beperkt door de smalle focus op voornamelijk korteketenvetzuren (KVK's), aminozuren en organische zuren. Het overzichtsartikel benadrukt de dringende noodzaak om een breder scala aan door de microbiota geproduceerde metabolieten te analyseren, om de interactie tussen microbioom en gastheer beter te begrijpen. Daarnaast wordt het belang van multi-omicsbenaderingen sterk benadrukt om mechanistische inzichten te verkrijgen in de relatie tussen het DM en KMA in de vroege levensfase.

Hoofdstuk 3 en 4 gaan in op de beperkte metabolomische reikwijdte in de literatuur door een breed spectrum van door de gastheer- en microbiota geproduceerde metabolieten te onderzoeken, waaronder metabolen van microbiële aminozuurmetabolieten, galzuren en KVK's.

In **Hoofdstuk 3** werden zuigelingen met een verhoogd risico op allergieën – die gedurende minstens 16 weken uitsluitend borstvoeding kregen – gevolgd tijdens het eerste levensjaar, een periode van snelle microbiële kolonisatie en mogelijke start van de atopische mars. Veranderingen in het fecale metaboloom en belangrijke leden van de microbiota werden onderzocht in relatie tot leeftijd, voeding, geboortewijze en allergieontwikkeling. De resultaten lieten significante leeftijdsgebonden verschuivingen in het metaboloom zien, waarschijnlijk veroorzaakt door gelijktijdige veranderingen in het metabolisme van de gastheer, voedingspatronen en de microbiotasamenstelling. Deze omvatten stijgingen in aminozuurmetabolieten, galzuren, B-vitaminen, 'KVK's en fenolzuren, evenals dalingen in langeketenvetzuren en acylcarnitines. C-sectie werd geassocieerd met veranderingen in het fecale metaboloom tot zes maanden leeftijd. Door het prospectieve karakter van de studie kon worden vastgesteld dat zuigelingen die een allergie ontwikkelden lagere niveaus van *Bifidobacterium* en aanzienlijk hogere niveaus van langeketenvetzuren hadden, nog vóór het klinisch optreden van symptomen. Hoewel deze bevindingen validatie behoeven in een grotere cohort, vergroot de studie ons begrip van de ontwikkeling van het fecale metaboloom in de vroege levensfase en de factoren die hierop van invloed zijn tijdens deze kritieke periode van immuun- en microbioomontwikkeling.

IgE-gemedieerde KMA wordt meestal behandeld met eliminatiediëten, waaronder het gebruik van aminozuurvoeding bij zuigelingen die flesvoeding krijgen. Gezien het toenemende bewijs voor de gunstige rol van *Bifidobacterium* bij de immuunontwikkeling en hun associatie met allergieën (aangetoond in **Hoofdstukken 2** en **3)**, is bifidogene

suppletie van aminozuurvoeding naar voren gekomen als een veelbelovende strategie in de behandeling van IgE-gemedieerde KMA.

Hoofdstuk 4 onderzoekt de relatie tussen IgE-gemedieerde KMA, het DM en bifidobacterie-bevorderende synbiotische suppletie aan de hand van fecale metabolomics. Zuigelingen met IgE-gemedieerde KMA ontvingen ofwel standaard aminozuurvoeding, of aminozuurvoeding gesupplementeerd met een synbiotische combinatie van *Bifidobacterium breve* M-16V en prebiotische inuline en oligofructose. De longitudinale analyse toonde subtiele metabolomische veranderingen aan die verband hielden met het verkrijgen van tolerantie voor koemelkeiwit, waaronder veranderingen in vertakte KVK's, galzuren en aminozuren. Zuigelingen die tolerantie verwierven vertoonden aanzienlijk hogere citrullinespiegels, wat duidt op een verminderde darmpermeabiliteit, en mogelijk lagere serotonine en 5-hydroxytryptofaanniveaus, beide betrokken bij ontsteking.

Een belangrijke bevinding is dat het effect van synbiotische suppletie op het fecale metaboloom het sterkst was na zes maanden interventie, en grotendeels afnam na twaalf maanden, wat suggereert dat vroege interventie essentieel is om het maximale effect te verkrijgen. Synbiotische suppletie leidde tot verhoogde niveaus van aromatische melkzuren, purinemetabolieten, langeketenvetzuren en galzuren, wat veranderingen in GM-activiteit weerspiegelt. Vooral indoolmelkzuur en 4-hydroxyfenylmelkzuur, aromatische aminozuurmetabolieten die kenmerkend zijn voor zuigelingstype *Bifidobacterium*, waren verhoogd en positief gecorreleerd met *Bifidobacterium*-abundanties. Deze bevindingen sluiten aan bij de microbioom- en proteomicsresultaten uit dezelfde cohort en ondersteunen verder de effectiviteit van de synbiotische interventie in het bevorderen van de groei en activiteit van *Bifidobacterium* in de darm. Hoewel de synbiotische suppletie geen statistisch significant effect had op het verkrijgen van tolerantie, wijst de toename van anti-inflammatoir indoolmelkzuur op mogelijke immunologische voordelen.

Hoofdstuk 5 richt zich op 3D-gut-on-a-chipplatforms, veelbelovende in vitro-modellen die belangrijke aspecten van de darmfysiologie nabootsen. Deze modellen kunnen worden gebruikt om de darmbarrière te bestuderen, waarvan disfunctie een rol speelt in diverse ziekten, waaronder allergieën. Met behulp van een epitheeltubuli-op-een-chipmodel toont dit hoofdstuk aan hoe blootstelling aan pro-inflammatoire cytokinen de integriteit van de darmbarrière en de secretie van signalerende lipiden beïnvloedt onder media met en zonder serum. Pro-inflammatoire cytokinen verhoogden significant de darmpermeabiliteit, celpermeabiliteit en veroorzaakten structurele veranderingen in de darmbarrière in beide mediaomstandigheden. Hoewel de veranderingen in permeabiliteit vergelijkbaar waren tussen beide condities, was de structurele schade — beoordeeld via actineherschikking — aanzienlijk lager in serumvrije omstandigheden. De aangetaste darmbarrière ging gepaard met verhoogde prostaglandinespiegels in het apicale (luminale) compartiment, maar niet in het basolaterale compartiment, waarbij dit effect sterker was in serumvrije media. Het geïntegreerde model biedt een waardevol raamwerk om de interactie tussen ontsteking,

barrièrefunctie en lipidenmetabolisme in intestinale pathofysiologie te bestuderen, wat ook relevant is voor allergieonderzoek.

Hoofdstuk 6 vat de belangrijkste bevindingen van dit proefschrift samen en bespreekt perspectieven voor toekomstig onderzoek. Door middel van fecale metabolomics en *in vitro*-modellen heeft dit proefschrift ons begrip verdiept van de complexe interacties tussen allergie en darmgezondheid, het GM en externe factoren. De onderzoekswereld zou sterk gebaat zijn bij goed opgezette, longitudinale klinische studies die multi-omicsbenaderingen en geavanceerde fysiologisch relevante *in vitro*-modellen combineren. Daarnaast moeten alternatieve strategieën voor allergiepreventie en -behandeling worden onderzocht die het darmmilieu van gezonde, borstgevoede zuigelingen nabootsen. Dergelijke inspanningen vereisen multidisciplinaire samenwerking, en kunnen uiteindelijk de weg vrijmaken voor microbioom-gebaseerde voedingsstrategieën om allergieën te voorkomen en te behandelen.

Curriculum vitae

Mariyana Savova was born on 27th November 1995 in Gorna Oriyahovitsa, Bulgaria. After graduating from high school in 2014, she pursued a 4-years bachelor's degree program in Chemistry in HZ University of Applied Sciences in Vlissingen, the Netherlands. In 2015 she was admitted to the 3-year Analytical Sciences Talent Program organized by Top Institute for Comprehensive Analytical Science and Technology (COAST), where she realized her passion for analytical sciences.

During her studies, she followed a theoretical minor program “Chemistry of Life” in Radboud University in Nijmegen and had two 6-month internships. She worked at Wageningen Food Safety Research in Wageningen on the optimization of liquid chromatography–mass spectrometry (LC-MS) method for the analysis of pesticide in human hair, and later at Unilever Research and Development in Vlaardingen, where she studied lipid oxidation in food emulsions using nuclear magnetic resonance (NMR).

After completing her bachelor's studies with honors in 2018, Mariyana pursued a joint degree master’s program in Chemistry (Analytical Sciences track) of the University of Amsterdam (UvA) and Vrije Universiteit Amsterdam and joined the MSc+ Program offered by COAST. In her second year of studies, she became a member of the Chemistry Program Committee. During her master’s, Mariyana wrote a literature thesis for the company DSM on polyurethane characterization and carried out a research thesis at the Analytical Chemistry group at Van 't Hoff Institute for Molecular Sciences, UvA, in collaboration with AstraZeneca, Macclesfield, the UK. Thesis work on retention-time prediction in supercritical-fluid chromatography was published in *Journal of Chromatography* A (2022), with Mariyana as shared-first author.

After obtaining her Master’s diploma *cum laude*, she started her PhD at Metabolomics and Analytics Centre at Leiden university under the supervision of prof. dr. Thomas Hankemeier, dr. Amy Harms, and dr. Alida Kindt. As part of the EARLYFIT project, Mariyana studied the link between allergies, the gut microbiome, and nutrition in early life by means of fecal metabolomics. During the project she collaborated with scientists from Danone Research & Innovation, Wageningen University, and Radboud University Medical Center. She also collaborated with scientists from Mimetas studying intestinal barrier using their gut-on-a-chip models. During her PhD Mariyana presented her work at multiple conferences, including an oral presentation at ASMS 2024, Anaheim, USA and a poster presentation at Metabolomics 2024, Osaka, Japan.

Mariyana is currently working as a PostDoc in the Nutrition, Microbiome and Metabolomics Research Group headed by Associate Professor Henrik Munch Roager at Department of Nutrition, Exercise and Sports, Faculty of Science at University of Copenhagen. There Mariyana continues to investigate the role of the gut microbiome in human health, with a particular focus on cardiometabolic health, using metabolomics.

Curriculum vitae

Publications related to this thesis

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