Metabolomics insight into the gut microbiome of infants with cow's milk allergy

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Chapter I

General Introduction and Scope

The prevalence of food allergy has risen over recent decades, with early life recognized as a critical window for its development.[1] However, the mechanisms driving the onset and resolution of food allergy remain incompletely understood. Increasing evidence highlights the gut microbiome, which exerts a dynamic impact on the systematic immune system, as an importance player in regulating these processes during early life.[2] The complex interactions between the gut microbiome and host immunity are gradually being deciphered along with the advances in molecular technologies, such as metagenomics, metatranscriptomics, and metabolomics. Among these tools, metabolomics plays a crucial role by capturing microbial activity from a metabolome perspective, offering valuable insights into microbiome-host interactions. Continued advancement in metabolomics techniques contributes to revealing the cross-talk between the gut microbiome and the host, deepening our understanding of how the gut microbiome influences food allergy in early life.

1. LC-ESI-MS based metabolomics

Along with genomics, transcriptomics, and proteomics, metabolomics is one of the omics strategies applied in systems biology, and the combination of these omics strategies provides a general view of how genotype is linked to phenotype (Figure 1).[3] Metabolites, which are the end products of cellular regulatory processes, reflect the ultimate response of a biological system to genetic or environmental changes.[4] The complete set of metabolites in a biological system is described as "metabolome",[5] which was firstly introduced by Oliver et al. in 1998.[6] Metabolomics is an approach to reveal the metabolome of a studied biological system.[3] This trait makes metabolomics a popular and significant strategy for monitoring ongoing biological processes in an organism.[7] In recent decades, metabolomics has been widely applied in biological studies, especially in the diagnosis, treatment, and prognosis of human diseases.[8–11] The popularity of metabolomics has been greatly enhanced by the emergence of advanced analytical techniques, such as nuclear magnetic resonance (NMR) and mass spectrometry (MS). NMR can quantify organic compounds and provide unbiased metabolite profiles for certain biological samples, but it has rather low sensitivity compared to MS.[12] The high sensitivity of MS is largely due to the breakthroughs in MS

technologies, particularly the development of ionization sources.[13]

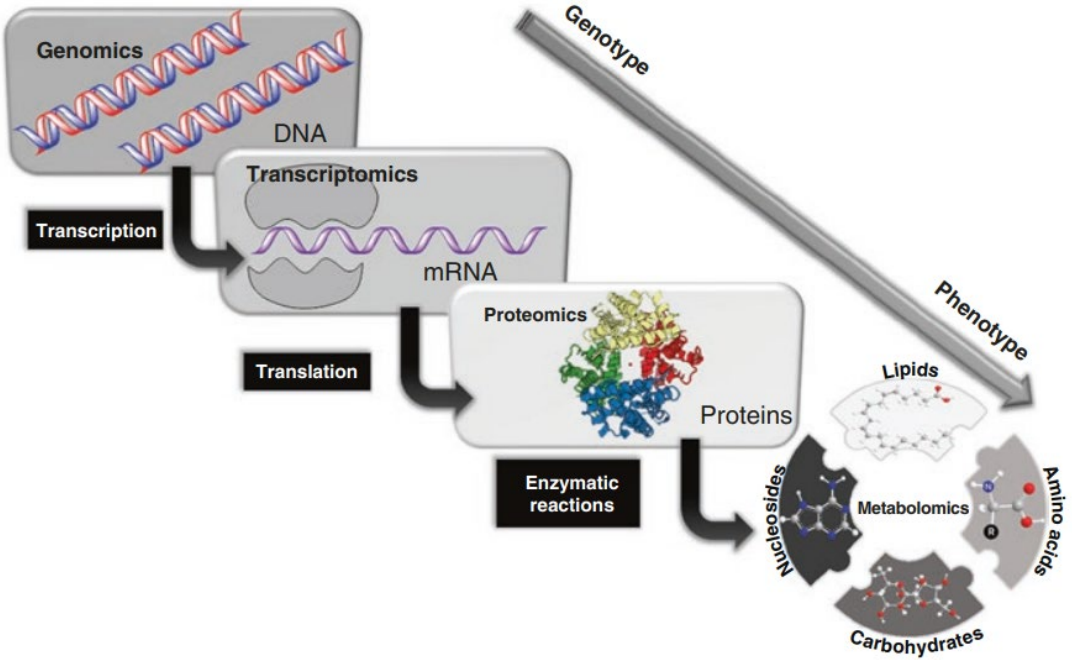

Figure 1. A correlation between the main omics strategies used in systems biology studies. From Klassen *et.al* 2017.[3]

MS is often coupled with various chromatographic separation techniques, such as gas chromatography (GC), liquid chromatography (LC), and capillary electrophoresis (CE). Before the 1980s, coupling GC to MS with an electron ionization (EI) source was the dominant technology for metabolome profiling for decades.[13] However, EI has limitations due to its requirement for high-vacuum and high-temperature conditions, as well as the need for samples to be delivered in gas phase.[14] These constraints restricts its applicability to couple other separation techniques, such as LC and CE, to MS. The exclusive use of EI declined with the development of advanced ionization techniques, such as electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), and atmospheric pressure photoionization (APPI).[13] These ionization sources not only allow the detection of intact molecules as "soft" ionization sources, but are also capable of producing stable, gaseous ionized molecules directly from liquid phase, making them perfectly compatible with LC or CE.[13] The ESI source, initially invented by Dole *et al.* in 1968[15] and further developed by Fenn *et al.*,[16–18] is considered a turning point in advancing the application of LC-MS in life science, including metabolomics. The advantages of the ESI source lie in its versatility, sensitivity, high ionization efficiency, and capability of ionizing molecules over a large mass range.[13,14] However, due to its ionization mechanism, the ESI source is more susceptible to matrix effect, particularly ion

suppression.[13,19]

1.1 Matrix effect in the ESI source

The simplified ionization process in an ESI source is as follows: (1) A liquid sample is delivered from the LC to the spray needle, where an intense electric field is generated at the tip, with an electric potential ranging from hundreds to thousands of volts. (2) The strong electric field at the tip of the spray needle forms a Taylor cone, from which a fine spray of charged droplets is emitted. (3) Droplets evaporate under dry gas and heat, causing them to shrink and the charge density increases on their surface until reaching the Rayleigh limit, where the coulombic repulsion counterbalances the surface tension. (4) Ions are ejected from the droplet or released through coulombic explosion when the coulombic repulsion overcomes the surface tension. Through these steps, the gas-phase ions are generated by the ESI process, allowing for MS analysis.[13,20,21] During this process, matrix components that interfere with any of these ionization steps can impact the ion intensity of analytes.[19,22] Figure 2 illustrates the potential mechanisms of matrix effect during the ESI process: (1) In the desolvation process, matrix components can prevent the analyte from accessing the available charge on the surface of the droplets and/or increase the viscosity and surface tension of the droplet, inhibiting further coulombic explosion. (2) During the coulombic explosion, matrix components can compete with the analytes for charge acquisition. (3) After reaching the gas phase, matrix compounds can neutralize or destabilize the charged ions. (4) The analytes can co-precipitate with nonvolatile matrix compounds, reducing the likelihood of their transfer to the gas phase. As a result, the reproducibility, linearity, selectivity, sensitivity, and accuracy of analyte detection can be significantly affected by matrix effect when using LC-ESI-MS-based methods for quantification.[19,23]

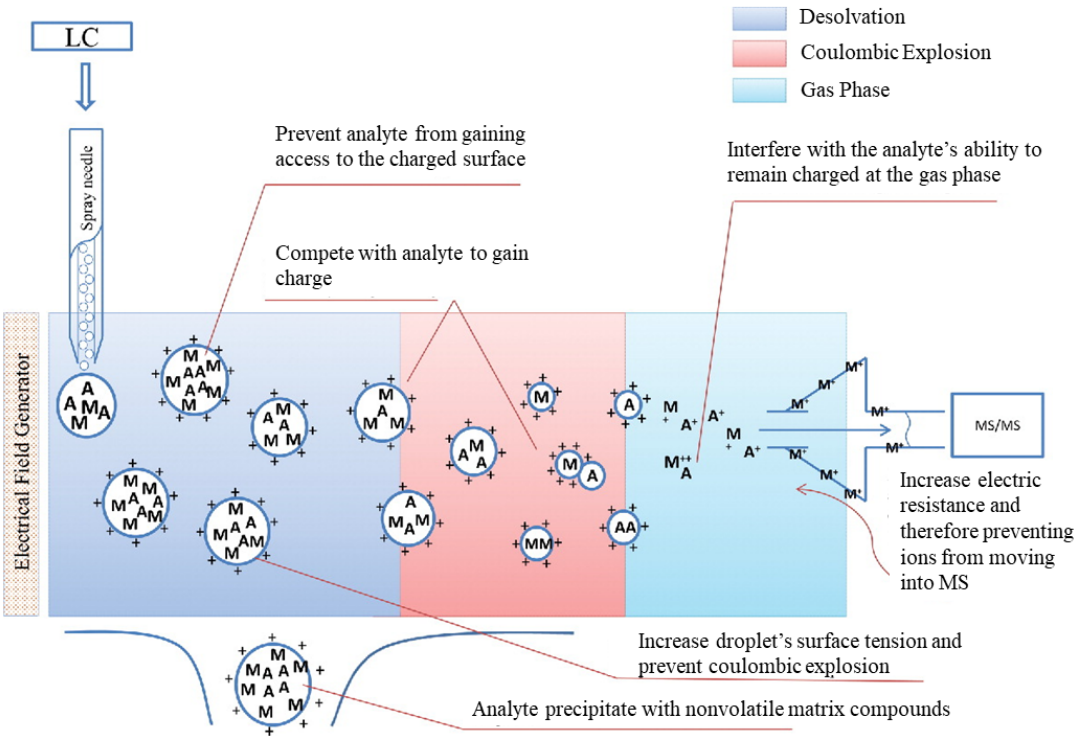

Figure 2. Mechanisms of how matrix components (M) can affect the ionization of analytes (A) in the electrospray ionization (ESI) source. Adapted From Panuwet *et al.* 2016.[19]

1.2 Approaches for addressing matrix effect

Various strategies are employed to minimize and compensate for matrix effect in LC-ESI-MS-based metabolomics studies. In general, matrix effect can be reduced through extensive sample cleanup procedures, tailored LC separation, matrix dilution, and reduced injection volumes.[23,24] Beyond reduction strategies, matrix effect can be assessed using post-extraction spiking (PES) of stable isotopically labeled (SIL) standards and a post-column infusion of standard (PCIS). PES, proposed by Matuszewski *et al.*, is a quantitative approach that evaluates matrix effect by comparing the responses of standards spiked into matrix samples versus matrix-free samples.[25] The other approach, PCIS, introduced by Bonfiglio *et al.* and Choi *et al.* in 1999,[26,27] provides a qualitative assessment of matrix effect by comparing the signals of a post-column infused standard observed with the injections of matrix samples to those of matrix-free samples. As shown in Figure 3, PCIS involves continuously infusing a standard solution via a pump or syringe after separation, then merging it with the LC flow using a T-connector before being injected into the MS. Unlike PES, which assesses

matrix effect at specific retention times,[25] PCIS evaluates it over the entire chromatogram.[26,27] Therefore, PCIS has been recommended as a quality control tool for assessing matrix effect in both targeted and untargeted LC-MS-based metabolomics.[28]

The primary objective of matrix effect evaluation is to identify analytes significantly affected by matrix effect and implement appropriate compensation strategies. In targeted metabolomics, where specific metabolites from known classes are precisely identified and quantified,[3,29] matrix effect compensation is typically achieved by correcting the signal of a target using a surrogate analogue, usually a SIL standard, spiked into the same study sample.[30] Different from targeted metabolomics, untargeted metabolomics aims to profile the metabolome, covering a wide range of metabolites, including unknowns.[3,29] This characteristic makes compensating for matrix effect particularly challenging. Although PCIS, a technique independent of retention time, is a feasible approach for correcting matrix effect in untargeted metabolomics, its application has been rarely reported.[31] Given the importance of untargeted metabolomics in biomarker discovery across diverse fields, such as biomedical research,[32] agriculture,[33] food,[34] and environmental science,[35] addressing the matrix effect in untargeted metabolomics can greatly improve data reliability and expand its applications.

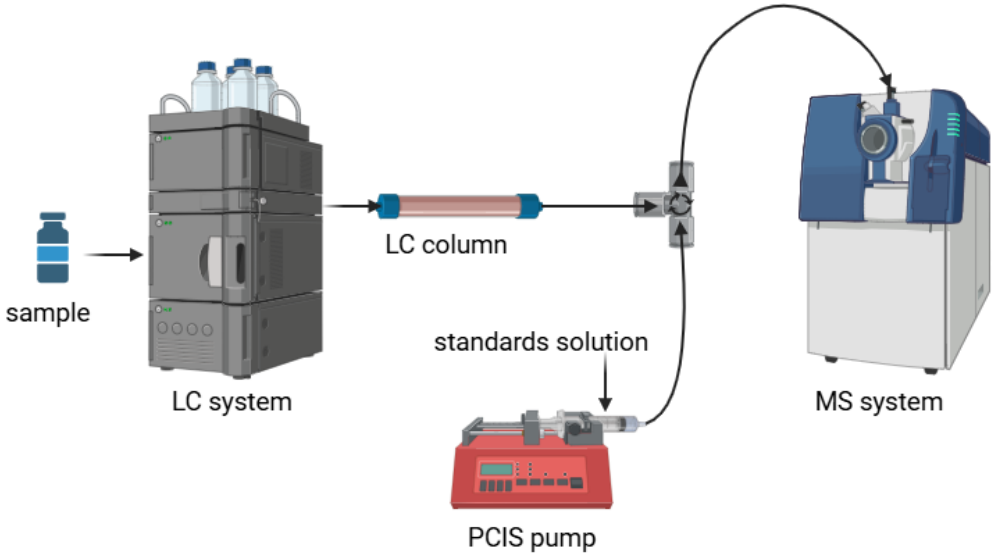

Figure 3. Setup of post-column infusion of standards (PCIS) with LC-MS (Created in https://BioRender.com)

2. Metabolomics and the gut microbiome

The human microbiome was described by Lederberg and McCray in 2001 as "the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space".[36] Our understanding of the human microbiome advanced significantly following the launch of the Human Microbiome Project (HMP) in 2007, an initiative founded by the U.S. National Institutes of Health. The HMP brought together international scientific experts to characterize the human microbial communities and investigate their roles in health and disease.[37] In HMP, biological samples were collected from 15 and 18 body sites in male and female, respectively, across more than 200 donors.[37] Among these sites, the human gut, which harbors the majority of microbes in the body,[38] was found to have an especially diverse microbiota community.[39] This community comprises bacteria, fungi, protists, archaea, and viruses, with bacteria making up around 60% of the dry mass of feces.[40] More than 500 bacterial species colonize the gut of a healthy adult,[38] primarily belonging to the phyla Firmicutes and Bacteroidetes, followed by Proteobacteria, Actinobacteria, Fusobacteria, and Verrucomicrobia.[41]

The co-evolution of the gut microbiome and its human host was initially described as commensal. However, it was later considered more accurate to use the term "mutualistic", which reflects the reciprocal influence and benefits shared between the host and the gut microbiome.[42] The gut microbe is increasingly recognized as a metabolically active "organ" with diverse functions,[43] including fermenting undigested food components, synthesizing essential vitamins, detoxifying harmful compounds, strengthening the intestinal barrier, and regulating the immune system.[44] These functions are tightly interconnected with the host, making gut microbiome a crucial player in human health and disease. Gut microbiota dysbiosis has been observed in many diseases, such as irritable bowel syndrome, inflammatory bowel disease, metabolic dysfunction-associated steatotic liver disease, obesity, diabetes, cardiovascular diseases, colorectal cancer, allergic disease, neurological and psychiatric disorders.[40,45,46] Although the mechanisms underlying the interplay between the gut microbiome and human physiology remain complex, gut microbiome-derived metabolites are believed

to play a critical role in the development and progression of various health conditions.

Figure 4 illustrates examples of well-known gut microbiota-derived metabolites identified over the past decades. The metabolites are primarily generated through three main pathways: (1) digesting dietary compounds (Figure 4a), (2) modifying host-derived metabolites (Figure 4b), and (3) synthesizing them *de novo* (Figure 4c).[47] One major class of gut microbiota-derived metabolites is short chain fatty acids (SCFAs), including formate, acetate, propionate, and butyrate, which are produced via microbial fermentation of undigested carbohydrates in the colon.[48] Another key group involves metabolites derived from an essential amino acid, tryptophan. Microbes in the colon can convert tryptophan into multiple bioactive compounds, including indole, indolepropionic acid (IPA), indole lactic acid (ILA), indoleacetic acid (IAA), indole ethanol (IE), indolealdehyde (IAld), indoleacrylic acid (IA), skatole, and tryptamine.[49]Additionally, gut microbes can metabolize dietary choline, betaine, and L-carnitine to produce trimethylamine (TMA), which can be absorbed in the intestine and subsequently oxidized in the liver to form trimethylamine N-oxide (TMAO).[50] Gut microbiota also play a crucial role in bile acid metabolism. Unconjugated primary bile acids, such as cholic acid (CA) and chenodeoxycholic acid (CDCA), are initially synthesized in the liver from cholesterol and stored in the gallbladder.[51] Upon food intake, they are released into the gut, where certain microbes can convert them into secondary bile acids, primarily deoxycholic acid (DCA) and lithocholic acid (LCA).[51] Apart from metabolizing dietary and host-derived substances, gut microbes are also capable of *de novo* synthesis of important metabolites, such as branched-chain amino acids (BCAAs),[52] polyamines,[53] and vitamins.[54]

Most gut microbiome-derived metabolites play crucial roles in host physiology. For instance, SCFAs are reported to have anti-inflammatory and anti-tumor properties,[48] TMAO has been identified as a predictor of cardiovascular disease pathogenesis,[50,55] and certain secondary bile acids are known as signaling molecules that regulate host endocrine functions.[56] Given the diverse biological functions of these metabolites, integrating metabolomics with other omics approaches, such as metagenomics, metatranscriptomics, and metaproteomics, is essential for gaining deeper insights into

the cross-talk between the gut microbiome and host.

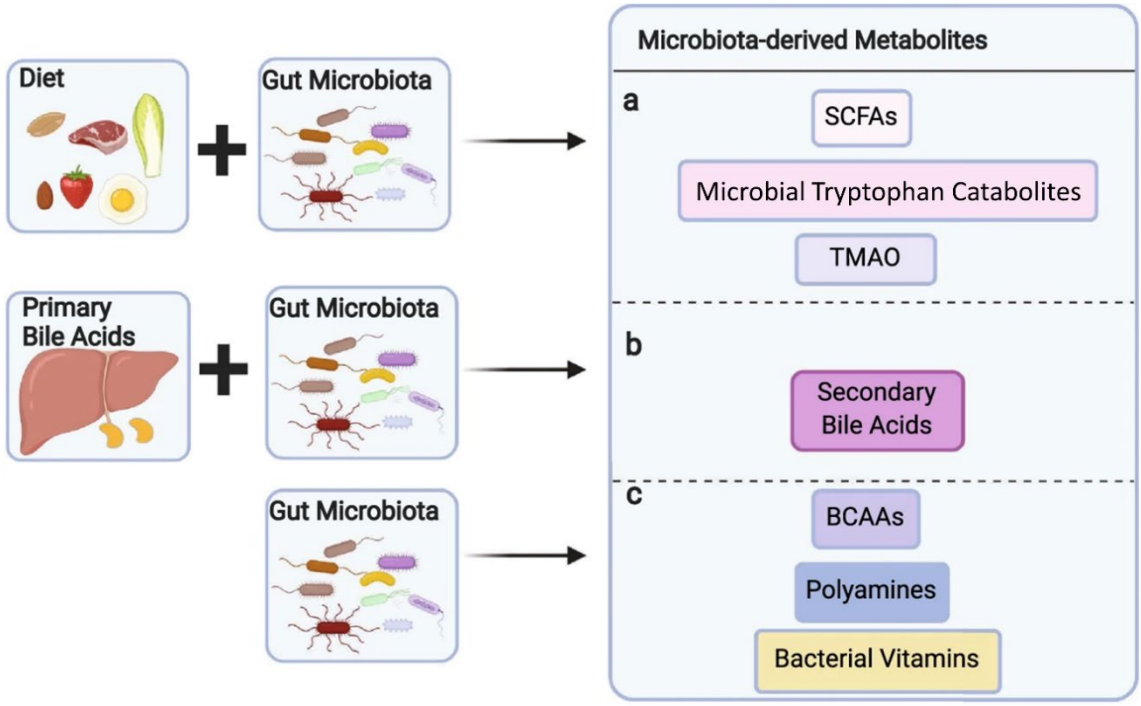

Figure 4. Production of some well-known gut microbiome-derived metabolites. SCFAs: short-chain fatty acids, TMAO: trimethylamine-N-oxide, BCAAs: branched-chain amino acids. From Yang *et al.*[47]

3. The gut microbiome and food allergy in early life

Food allergy is defined as "an adverse health effect arising from a specific immune response that occurs reproducibly on exposure to a given food".[57] It has become a growing global public health concern, particularly in children.[58–61] The estimated prevalence of food allergy is 1-2% in the general population,[62] but rises to 6-8% in children.[63] Most food allergies develop within the first few years of life[1] with major allergens including peanut, tree nuts, fish, shellfish, egg, milk, wheat, soy, fruits, and seeds.[58,61] Among these, cow's milk is the most common food allergen in early childhood.[64,65] It is reported that, in the United States, cow's milk allergy affects approximately 50 % of food-allergic children under one year-old, 40 % of those aged 1-2 years, and 30% of those aged 3-5 years.[59,65]

Numerous factors, including genetics, diet, and environmental influences, contribute to the development and resolution of food allergy.[66,67] Growing evidence also highlights

the gut microbiome as a key player in food allergy of early life. The initial recognition of the role of gut microbiome in allergic diseases dates back to the late 1980s with the proposition of "hygiene hypothesis". It suggests that reduced exposure to infection sources and symbiotic microorganisms may lead to increased rates of allergic diseases.[68] This concept was later extended into the "microflora hypothesis" in 2005, which specifically proposed that the disruptions in the gastrointestinal microbiota during early life impair microbiota-mediated mechanisms of immunological tolerance, thereby increasing the incidence of allergic diseases.[69] Multiple clinical studies have reported altered gut microbiota composition in children with food allergies. For example, Joseph *et al.* observed that children aged 3–5 years with food allergies had significantly lower gut microbiota diversity compared to non-allergic children.[70] Similarly, Japanese children who developed food allergies within their first two years exhibited lower abundances of the bacterial genera *Leuconostoc*, *Weissella*, and *Veillonella* compared to their healthy counterparts.[71] Additionally, reduced levels of *Citrobacter*, *Oscillospira*, *Dorea*, and *Lactococcus* genera in the fecal samples of infants aged 3–6 months have been associated with food allergy development by age three.[72] In contrast, a higher abundance of bacteria from the Firmicutes phyla in infancy (3-6 months) has been linked to the resolution of cow's milk allergy.[73]

As growing evidence supports the role of the gut microbiome in both the development and resolution of food allergy in early life, interest has increased in strategies to modulate its composition and function as a means of preventing and managing food allergy.[74] The gut microbiome can be modified through the administration of probiotics, prebiotics, synbiotics, and fecal microbiome transplantation (FMT).[75] Probiotics, which consist of beneficial live bacteria strains primarily from the *Lactobacillaceae* and *Bifidobacteriaceae* families, aim to directly alter gut microbiota composition and potentially restore microbial balance.[76,77] Prebiotics, on the other hand, are defined as non-digestible food ingredients that can be fermented by gut microbiome, selectively stimulating the growth and/or activity of specific beneficial bacteria.[78] Common prebiotics include fructo-oligosaccharides, galacto-oligosaccharides, and inulin.[74] Synbiotics combine probiotics and prebiotics to enhance the survival and efficacy of probiotic strains.[79] FMT, a more direct approach, is a procedure that transplants fecal

microbiota from a healthy donor to reshape the recipient's gut microbiome.[80] Compared to FMT, probiotics, prebiotics, and synbiotics are more commonly used for food allergy intervention.[76] However, despite their promising potential, clinical evidence supporting the effectiveness of probiotics and/or prebiotics in preventing or treating food allergy remains limited.[76,81,82] Therefore, further research is needed to deepen our understanding of the gut microbiome's role in food allergy and to explore the therapeutic potential of microbiome-targeted interventions.

4. Scope and outline of this thesis

As the intricate relationship between the gut microbiome and food allergy in early life continues to be deciphered, metabolomics offers a powerful tool to explore this cross-communication at the molecular level. Among analytical methods applied in metabolomics, untargeted methods outperform targeted ones in identifying novel metabolites, including those derived from the gut microbiota. One major challenge in untargeted metabolomics is the matrix effect, which can vary between samples, especially those with complex matrices, such as feces. The first hypothesis of this thesis is that the matrix effect in untargeted metabolomics can be monitored and corrected by implementing the PCIS technique with LC-MS methods. The second hypothesis is that the fecal metabolome can provide insights into the cross-talk between the gut microbiome and food allergy in infants with the most prevalent type of food allergy in early life: cow's milk allergy (CMA).

The first hypothesis is examined and verified in **Chapters 2 & 3**. In **chapter 2**, the goal is to develop an untargeted LC-ESI-MS method with PCIS to monitor matrix effect in plasma and fecal samples. To achieve this goal, the first focus is on developing a reverse-phase LC-MS untargeted metabolomics method with PCIS to profile polar to semi-polar metabolites. The development includes injection parameters optimization and validating the method with representative SIL standards. Then, the SIL standards are used to evaluated the capability of PCIS in monitoring the matrix effect of plasma and fecal samples.

As a follow-up, **Chapter 3** aims to investigate the application of PCIS for matrix effect

compensation in untargeted metabolomics. To fulfill this aim, a post-column artificial matrix infusion approach is introduced to the developed LC-PCIS-MS method. This artificial matrix consists of several compounds that are known to disrupt the ionization process of ESI, creating an artificial matrix effect. The artificial matrix effect of a given feature can be determined by comparing its signals obtained with and without artificial matrix infusion. The hypothesis is that the artificial matrix effect can help identify a suitable PCIS for a given feature, and that the selected PCIS can be used to correct the matrix effect of that feature in biological samples. This concept is investigated by comparing the ideal PCISs selected based on compensating artificial and biological matrix effect for diverse SIL standards spiked into plasma, urine, and feces.

In the following two chapters, the focus is on investigating the relationship between the gut microbiome and CMA in early life with the developed untargeted method. To gain insights into this problem, a systematic review is conducted in **Chapter 4**, summarizing existing studies on the microbiome, metabolome, and immune response in CMA children and animal models, including the impacts of interventions with probiotics, prebiotics, and synbiotics. The review highlights a lack of studies on immune responses and metabolomics related to CMA in early life, emphasizing the need for further research in these fields.

The aim of **Chapter 5** is to help address the research gap identified in Chapter 4 concerning CMA in early life. A comprehensive exploration of the fecal metabolome is performed in infants (3-13 months) with CMA by combining the untargeted metabolomics platform developed in Chapter 2 with an additional in-house platform focused on non-polar metabolites. The study includes 39 infants with cow's milk allergy, who were randomized into two intervention groups: one group receiving amino acid-based formula (AFF) and the other group receiving AAF supplemented with synbiotics (inulin, oligofructose, *Bifidobacterium breve* M-16 V) (AAF-S). Fecal samples from all the infants were collected at baseline, as well as six and 12 months after the start of the interventions. By categorizing the infants based on their intervention strategy and cow's milk allergy status after 12-month intervention, the aim is to investigate: (1) the impact of synbiotic supplementation on the fecal metabolome in

infants with cow's milk allergy, and (2) the effect of tolerance acquisition on the fecal metabolome in the infants initially diagnosed with CMA.

This thesis concludes in **Chapter 6** with a general summary and discussion. In this chapter, potential improvements in implementing PCIS to address matrix effect in untargeted metabolomics is discussed, along with recommendations and perspectives on applying metabolomics to investigate the gut microbiome and CMA in early life.

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