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

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Chapter VI

Conclusions and Perspectives

The cross-talk between the gut microbiome and the human host has been increasingly recognized as an important factor influencing human health and disease,[1] including cow's milk allergy (CMA), which is the most common type of food allergy in early life.[2] Although advancements in omics techniques have significantly improved our understanding of this interplay, uncovering the complex mechanisms by which the gut microbiome affects the host remains a challenge. In recent decades, growing evidence suggests that the gut microbiome-derived metabolites serve as important mediators in this interaction.[3] This highlights metabolomics as a key technique for elucidating the gut microbiome's role in human health and disease by providing insights at the molecular level. In metabolomics studies, approaches can be broadly categorized into targeted and untargeted metabolomics, based on hypothesis-driven and hypothesis-generating strategies, respectively.[4] Targeted metabolomics focuses on quantifying a limited number of known metabolites, while untargeted metabolomics aims to profile both known and unknown metabolic features.[4] One of the primary challenges for metabolite quantification in targeted and untargeted metabolomics is matrix effect.[6] Matrix effect is primarily caused by co-eluting matrix components, which can impact the accuracy and reliability of signals detected with liquid chromatography-mass spectrometry (LC-MS), particularly when using an electrospray ionization (ESI) source.[5] In this thesis two hypotheses were investigated. The first hypothesis was that the matric effect in untargeted metabolomics can be monitored and corrected by implementing the PCIS technique with LC-MS methods. The second hypothesis was 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). life.

Matrix effect in untargeted metabolomics

Untargeted metabolomics, a powerful approach for unbiased metabolome profiling, has demonstrated potential for biomarker discovery in diverse fields. However, despite its wide applications, several challenges remain that impact the reliability of untargeted metabolomics. Among these, matrix effect is a major concern, as it can greatly affect the reproducibility, selectivity, and accuracy of metabolome profiling.[6] Stable

isotopically labeled (SIL) standards, the most commonly applied strategy for addressing the matrix effect, are limited to targeted metabolomics due to the requirement of standards spiking. This limitation makes another approach, PCIS, the only applicable method for mitigating matrix effects in untargeted metabolomics, as it is independent of retention time.[6] The effectiveness of PCIS in monitoring and correcting matrix effects has been well demonstrated in targeted metabolomics,[7–10] and it has also been recommended as a quality control tool for matrix effect evaluation in untargeted metabolomics.[11] However, reports on its actual application in untargeted metabolomics remain limited.[12] Therefore, effective strategies to address the matrix effect with PCIS in untargeted metabolomics are still lacking. To tackle this, in **Chapters 2** and **3** of the thesis, we outlined strategies using PCIS to overcome matrix effect in LC-ESI-MS-based untargeted metabolomics, covering matrix effect monitoring and matrix effect compensation.

First, in **Chapter 2**, an untargeted method was developed and applied to evaluated the matrix effect in plasma and fecal samples with PCIS. As part of the method development, the injection amount and reconstitution solvent were first optimized for both plasma and fecal samples. The results showed that optimizing the reconstitution solvent was crucial for balancing the trade-off between peak shape distortion and metabolite solubility, and that proper sample dilution was essential for maximizing metabolites signal intensity while preventing detector saturation in MS. To assess the analytical performance of our untargeted method, the method was validated using a targeted approach with stable isotope-labeled (SIL) standards in plasma and fecal samples. The method exhibited good precision, accuracy, recovery, and repeatability with plasma and fecal samples. By evaluating the matrix effect, it was found that high relative matrix effect (RME) among samples could significantly impact measurement accuracy and reproducibility. However, the SIL standards can only point out the matrix effect at specific retention times. To assess the matrix effect across the entire chromatogram, a PCIS approach was introduced to the developed untargeted metabolomics method. In this approach, xenobiotic compounds were infused post-column during the injection of different plasma and fecal samples, enabling overall

monitoring of absolute matrix effect (AME) and RME by examining the matrix effect profiles of the infused compounds.

The results demonstrated that the PCIS approach effectively identified chromatographic regions exhibiting large AME and RME. Notably, PCIS yielded comparable RME results to those obtained using the traditional post-extraction spiking method, demonstrating its potential as a reliable technique for RME evaluation in untargeted metabolomics. The PCIS approach was applied to predict the RME of over 300 targets covered in our in-house library. The predictions revealed that more targets exhibited RME > 15% in fecal samples compared to plasma. Additionally, for metabolites detectable in both positive and negative ionization modes, most of them experienced larger RME in negative mode than in positive mode. Overall, **Chapter 2** established a comprehensive framework for developing an LC-ESI-MS untargeted metabolomics method using PCIS to monitor the matrix effect in plasma and fecal samples. The findings demonstrated that PCIS is an effective approach for matrix effect monitoring in untargeted metabolomics. This approach has strong potential to improve better data reliability of untargeted metabolomics by identifying regions with severe matrix effect and high matrix effect variation.

The proposed PCIS approach can be further applied to guide the optimization of specific LC parameters, such as the gradient and sample injection amount, to mitigate matrix effects in a reverse-phase (RP) LC-MS untargeted method. A recent study also demonstrated that PCIS contributed to column selection and mobile phase pH optimization for an untargeted hydrophilic interaction liquid chromatography (HILIC)-MS method.[13] Moreover, although both our RPLC-MS and their HILIC-MS methods targeted polar to semi-polar metabolomic features with a mass less than 800 Da, the application of PCIS is not limited by the polarity or mass range of the metabolites. In principle, with careful selection of PCIS candidates, PCIS can serve as a valuable approach for guiding method development to minimize matrix effects in any untargeted metabolomics method.

In addition to matrix effect monitoring, PCIS also has potential for compensating matrix effect in untargeted metabolomics due to its retention time independence. A key challenge in its implementation lies in selecting multiple PCISs for the wide range of metabolic features and determining which one is most effective for correcting the matrix effect specific to each feature. To address this, the application of PCIS from matrix effect monitoring to compensation was investigated in **Chapter 3.**

In this chapter, the workflow for developing a PCIS approach for an LC-ESI-MS-based untargeted metabolomics method was first outlined. Key factors, such as structural diversity, infusion concentration, and room temperature stability, were thoroughly evaluated to select suitable PCIS candidates. The results demonstrated that, at the optimized infusion concentration, the selected PCISs (five standards for positive ionization mode and four for negative ionization mode) exhibited diverse matrix effect profiles, stable infusion signals, and no significant matrix effect interference. Additionally, these compounds remained stable for one week at room temperature, further supporting their long-term usage along with analysis runs. Next, to match a specific feature with its suitable PCIS for matrix effect correction, a novel approach was proposed: post-column infusion of artificial matrices. This matching process was achieved by comparing the ability of a PCIS to compensate for the artificially created matrix effect (ME_{art}).

To ensure that the artificial matrices properly mimicked the biological matrix in inducing matrix effects, multiple artificial matrix compounds were selected based on their relevance to matrix effects mechanisms in an ESI source. L-homoarginine hydrochloride, sodium acetate, and tridodecylmethylammonium chloride were selected as artificial matrix compounds for positive ionization mode, while sodium dodecyl sulphate and sodium acetate were used for negative mode. These compounds can interfere with ESI process of analytes by competing for ionization or increasing the surface tension in droplets, preventing coulombic explosion. Since the presence of ME_{art} was essential for selecting the suitable PCIS, the infused concentrations of these artificial matrix compounds were optimized to obtain 70% artificial absolute matrix effect (AME_{art}) and more than 15% artificial relative matrix effect (RME_{art}). By injecting

samples into the LC-PCIS-MS system with and without artificial matrices infusion, the ME_{art} could be determined across detected features, including both known and unknown metabolites. The selected PCIS could then be used to compensate for biological matrix effects (ME_{bio}).

The effectiveness of ME_{art} was evaluated in selecting PCIS using 19 diverse SIL standards spiked in plasma, urine, and feces. In this evaluation, ME_{bio} and ME_{art} were calculated and used to select the suitable PCIS for each SIL standard in each biological matrix. To incorporate both absolute matrix effect (AME) and relative matrix effect (RME) into the comparison, a matrix effect scoring system was introduced that averaged AME and RME scores as the final ME score. The ME (ME_{art}, ME_{bio}) scores across plasma, urine, and feces were summed to identify the matrix-independent PCIS for each SIL standard. The PCISs selected based on ME_{art} score sums were compared with those identified using ME_{bio} score sums. As a result, 17 out of 19 (89%) SIL standards exhibited consistent PCISs selection based on ME_{art} and ME_{bio} score sums. Considering that ME_{bio} correction is the most commonly applied strategy for PCIS selection in targeted metabolomics,[9,14,15] our results highlight the efficacy of ME_{art} in selecting the suitable PCISs for ME_{bio} compensation.

Subsequently, ME_{art}-selected PCISs were applied to correct for the ME_{bio} in plasma, urine, and feces for the 19 SIL standards. These PCISs improved or maintained the matrix effect scores for 19 (100%) standards in plasma, 16 (84%) in urine, and 18 (95%) in feces. The results demonstrated the efficacy and reliability using ME_{art} to identify suitable PCISs for ME_{bio} correction across various biological matrices. More importantly, since ME_{art} can be determined for any measurable feature by comparing signals acquired with and without artificial matrix infusion, this establishes post-column ME_{art} creation as a feasible approach for selecting PCIS to correct matrix effect in LC-PCIS-MS-based untargeted metabolomics. Ideally, a feature-PCIS-matched library could be constructed using artificial matrix infusion with one or multiple biological matrices, and then applied to compensate for matrix effect in untargeted metabolomics studies.

Following the successful proof-of-concept demonstration of the matrix effect compensation method using artificial matrix-based PCIS selection, further efforts should focus on building the feature-PCIS-matched library to facilitate routine matrix effect correction in untargeted metabolomics. Additionally, comparing significantly altered features before and after PCIS correction in applied studied remains of great interest for further validating this method for matrix effects correction. Although this study included structurally diverse PCISs, over-corrected matrix effects were observed in a few examined SIL standards. This highlights the need to further expand the diversity of PCIS candidates to improve correction those standards and enable more comprehensive matrix effect correction across the metabolome. Furthermore, with well-defined PCIS candidates and a robust ME_{art}-based matching strategy, the LC-PCIS-MS platform can be extended beyond biomedical matrices to applications in food safety, environment science, and other fields where complex matrix effects are commonly encountered.

Fecal metabolome exploration in infants with cow's milk allergy

In **Chapters 4** and **5**, the aim was to deepen our understanding of the interplay between the gut microbiome and CMA in early life through the exploration of the fecal metabolome. To provide a comprehensive overview of current studies on this topic, a systematic review was conducted in **Chapter 4**. This review focused on the modifications and post-treatment alterations in the gut microbiome, metabolome, and immune response in both CMA children (0-12 years) and CMA animal models. By conducting thorough searches in MEDLINE, PubMed, Scopus, and Web of Science, 21 articles published before March 2023 were included, consisting of 13 studies on CMA children and 8 studies on animal models.

In the reviewed studies, no consistent conclusions were drawn regarding the modifications of α- and β-diversity in the gut microbiome in CMA. At the taxonomic level, multiple studies across both CMA children and animal models reported a decrease in the *Bifidobacterium* genus and Lactobacillales order, alongside an increase in the Clostridia class. Regarding CMA management, various intervention approaches,

including different formulas, prebiotics, probiotics, and synbiotics, were applied across several studies. These studies consistently showed increased *Bifidobacterium* levels in both CMA children and animal models following interventions, particularly with *Bifidobacterium* strains-specific treatments. However, the impact of these interventions on other bacterial populations remained inconclusive. In terms of metabolome modifications, decreased short-chain fatty acids (SCFAs), as well as altered amino acid and organic acid profiles, were observed in CMA children. These metabolomic changes appeared to be restored through interventions, with increased SCFAs and balanced amino acid levels. For the immune response, only one study involving CMA children was available, but studies on CMA animal models suggested that interventions could reduce overall cytokine levels, restore the T_h2/T_h1 balance, and induce a regulatory immune response. Additionally, this review highlighted that no study has investigated early-life CMA using multi-omics strategies, such as metagenomics, metatranscriptomics, and metaproteomics. Although several metabolomics studies have been reported, they focused on a limited range of metabolites, emphasizing the need for comprehensive metabolomics studies on CMA in early life.

In **Chapter 5**, a comprehensive investigation of the fecal metabolome in CMA infants undergoing dietary intervention with and without a synbiotic (inulin, oligofructose and *Bifidobacterium breve* M-16 V) was conducted using the untargeted metabolomics method developed in **Chapter 2**, along with an additional in-house platform. Considering the broad metabolite coverage, we primarily focused on known features in this study. By grouping the infants based on CMA status after one year or the type of intervention they received, we explored the distinct impacts of CMA tolerance acquisition and of the synbiotic supplementation on the fecal metabolome of CMA infants. The longitudinal changes in the fecal metabolome across the three time points were analyzed using linear mixed models (LMMs) and repeated measures analysis of variance simultaneous component analysis+ (RM-ASCA+).

By comparing the fecal metabolome of infants with persistent CMA to those who developed CM-tolerance, more pronounced changes in the fecal metabolome related to amino acids, bile acids, and SCFAs were observed in the CM-tolerant group. The CM-

tolerant group exhibited significantly higher levels of lysine and citrulline after one year of intervention compared to the CM-allergic group. Although no significant group differences were found for other metabolites, the metabolome trends along with time indicated a down-regulation of tryptophan-serotonin metabolism, up-regulation of secondary bile acid production, and an increase in butyrate in the CM-tolerant group compared to the CM-allergic group. These alterations might suggest a healthier gut with improved barrier function and a more mature gut microbiome in the CM-tolerant group.

Regarding the impact of the synbiotic, this study demonstrated that the synbiotic significantly altered the fecal levels of aromatic lactic acids, purine metabolites, fatty acids, and bile acids, especially after six months of supplementation. Two aromatic lactic acids (4-hydroxyphenyllactic acid and indolelactic acid), known as infant-type *Bifidobacterium*-derived metabolites, showed a significant increase in the synbiotic group. Moreover, the changes in these metabolites from baseline to later time points were strongly positively correlated with the changed levels of *Bifidobacterium* in the group with synbiotic supplementation. These findings suggested an enhanced abundance and/or activity of infant-type *Bifidobacterium* species, indicating the successful supplementation of the synbiotic. Additionally, the synbiotic supplementation was found to lower the levels of inosine, guanine, and uridine, increase adenine level, and enhance the deconjugation of glycine-conjugated bile acids.

The study in **Chapter 5** contributed to revealing the linkages between early-life CMA, the gut microbiome, and synbiotic intervention. We observed several alterations in fecal metabolomic pathways that may play a role in the outgrowth of CMA in early life. Additionally, Those findings provided evidence for the impact of synbiotic supplementation on modifying the fecal metabolome in CMA infants. This impact was more pronounced after six months of intervention, highlighting the importance of early intervention to maximize the effects of synbiotics. However, no clear conclusions can be drawn regarding the clinical benefits of the synbiotic supplementation on CM-tolerance acquisition, as the tolerance rate observed after one-year synbiotic intervention was consistent with natural outgrowth for infants involved in our study. Despite this, the significant enhancement of metabolites with anti-inflammatory properties, such as

indolelactic acid,[16] suggested a potential beneficial effect of synbiotics in promoting CMA outgrowth. Therefore, it is suggested that further research with larger cohorts is needed to verify our findings and evaluate the therapeutic potential of synbiotics supplementation for CMA in early life.

Although over 300 targets were involved in our study, there are still opportunities for further improvements in metabolomic exploration. First, our study only reported the relative abundance of these targets. Achieving absolute quantification of the targeted metabolites would enhance the accuracy and depth of our interpretation. Second, despite covering a wide range of targets, analyzing the data in an untargeted manner is still necessary to identify other potential metabolomic changes that may not have been captured in our targeted analysis. Moreover, integrating multiple analytical platforms for global profiling, such as HILIC along with RP, could significantly expand the metabolomic coverage and provide a more comprehensive picture. Lastly, this study was conducted with the PCIS setup, providing the opportunity to reanalyze the data and apply matrix effect correction. We believe that matrix effect correction with PCIS holds substantial potential to further enhance the quality of the data presented in this chapter.

Further perspectives

In this thesis aimed to tackle the issue of matrix effect in untargeted metabolomics (**Chapter 2-3**) and expand the understanding of the relationship between the gut microbiome and CMA in early life (**Chapter 4-5**). From a technical perspective, the thesis demonstrated the potential of applying PCIS to address matrix effect in LC-ESI-MS-based untargeted metabolomics methods. The developed PCIS method enabled two primary functions: matrix effect monitoring and matrix effect correction. Matrix effect monitoring is particularly useful during the development phase of an untargeted metabolomics method to help mitigate matrix effect. Matrix effect correction holds great potential to enhance data reliability, advance (semi)quantitative analysis, and ensure more accurate data interpretation in untargeted metabolomics. One direct application of matrix effect correction is solving the problem of matrix dilution when examining the dynamic range using endogenous metabolites with serially diluted quality control (dQC)

samples.[17] This enables the exploration of linearity with endogenous features by correcting matrix effect in a calibration curve constructed using dQC. Additionally, leveraging a dQC series with corrected matrix effect in routine untargeted metabolomics analysis can also improve the data fidelity via advancing the feature filtering with estimated linearity range and response for detected features.[18] Overall, the advances in addressing matrix effect presented in our study will contribute to the broader application of untargeted metabolomics in diverse research fields. However, to expand the implementation of matrix effect correction using PCIS, further efforts are required to develop an automated pipeline that increases throughput for efficiently selecting appropriate PCISs for the hundreds to thousands of features detected in untargeted metabolomics. Meanwhile, incorporating the PCIS pre-processing workflow into existing untargeted data analysis tools could also further promote its application.

With the developed method, **Chapter 5** uncovered several potential metabolomic pathway modifications related to CMA resolution in early life and highlighted significant metabolite changes following the synbiotic intervention. This study contributed to gaining insights into the interplay between the gut microbiome and early-life CMA from a metabolomics perspective. To further reveal the underlying mechanisms regarding the impact of the gut microbiome on early-life CMA, we recommend carrying out more studies with larger-scale cohorts. This will also enable researchers to develop more complex data analysis models to explore how synbiotic interventions can influence CM-tolerance acquisition in early life. Meanwhile, since the fecal metabolome serves as an ideal readout for gut microbial functions,[19] the primary focus of this chapter was on fecal metabolome profiling In the future, combining the fecal metabolome with metabolomes obtained from other biological samples, particularly peripheral blood, could significantly enhance our interpretation of the cross-talk between the gut microbiome and the host. Additionally, comprehensive studies that integrate multi-omics research combining metabolomics, metagenomics, metatranscriptomics, and metaproteomics are still urgently needed to gain a complete understanding of impact of the gut microbiome on the prevention, development, and treatment of CMA in early life. Furthermore, as we enter the era of artificial intelligence,

incorporating techniques like machine learning with integrated multi-omics data holds great promise for advancing our knowledge of the role of the gut microbiome in human health and disease, including CMA in early life.

Final remarks

The rapid expansion of research on human gut microbiome in recent decades has highlighted its role in human metabolism, immune regulation, and behavior.[20] Despite significant progress in deciphering how the gut microbiome affects human health and disease, a long journey lies ahead to fully solve the puzzle. Combining multi-omics analyses has become a trend to unravel the intricate relationship between the gut microbiome and the human host. Among the omics techniques, as a direct readout of phenotypes, metabolomics provides a snapshot reflecting the functional properties of the gut microbiome at the molecular level. This emphasizes the crucial role of metabolomics in revealing this complex relationship and underscores the needs for advances in metabolomics techniques. In this thesis, by proposing strategies to address the matrix effect in LC-ESI-MS-based analytical method, we advanced untargeted metabolomics towards quantitative analysis. The focus then shifted to deepening our understanding of the interactions between the gut microbiome and CMA in early life from a metabolomics perspective. Overall, the research in this thesis suggested that several gut microbiome-involved metabolic pathways may play a role in the acquisition of CM tolerance, and provided evidence that the fecal metabolome can serve as a potential readout to reflect the impact of early synbiotic supplementation in infants. These findings offered valuable insights into the relationship between the gut microbiome and CMA, aiding future research in developing microbiome-targeted strategies for the prevention and management of CMA in early life.

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