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

Zhu, P.

Appendix

Summary

Nederlandse Samenvatting

Curriculum Vitae

List of Publications

Acknowledgements

Summary

The incidence of food allergy has increased over the last few decades, with cow's milk allergy (CMA) being one of the most prevalent food allergies in early life. In recent years, growing research on the gut microbiome has highlighted its crucial role in human health and disease, including its potential impact on CMA in early life. The gut microbiome is thought to exert a dynamic influence on the immune system, thereby potentially regulating the onset and progression of CMA. During this process, gut-derived metabolites have been increasingly recognized as important mediators of the crosstalk between the gut microbiome and the host. This highlights the essential role of metabolomics in deciphering the influence of the gut microbiome on CMA in early life.

In metabolomics, approaches can be broadly categorized into targeted and untargeted metabolomics, with targeted metabolomics focusing on accurate quantification of known metabolites, whereas untargeted metabolomics aims to discover novel biomarkers by performing comprehensive metabolome profiling. Liquid chromatography coupled with mass spectrometry using an electrospray ionization source (LC-ESI-MS) is one of the most applied techniques in metabolomics due to its high sensitivity and robustness. However, because of the ionization mechanism of the ESI source, the LC-ESI-MS method is susceptible to matrix effect, which is caused by co-eluted matrix components and can significantly impact the accuracy and reproducibility of the analysis. The matrix effect remains a major challenge in LC-ESI-MS-based metabolomics, particularly in untargeted metabolomics, where there is lack of effective compensation strategies.

Therefore, the first aim of this thesis is to address the problem of matrix effect in untargeted metabolomics with the technique of post-column infusion of standards (PCIS). The second aim is to gain insights into the crosstalk of the gut-microbiome and food allergy in early life by exploring the fecal metabolome in CMA infants.

Chapter 1 outlines current approaches used to address matrix effect in metabolomics and presents a summary of the interconnections among metabolomics, gut microbiome,

and food allergy in early life. This chapter also provides an overview of the scope of the thesis.

Chapter 2 describes the development of an untargeted metabolomics method incorporating PCIS for matrix effect monitoring in plasma and feces. To assess the analytical performance of this LC-PCIS-MS-based untargeted method, it was validated using diverse stable-isotope labeled (SIL) standards in a targeted manner. The method exhibited good precision, accuracy, recovery, and repeatability. The validation highlighted the issue of matrix effect in the developed method, as it demonstrated that high variation in matrix effect among samples could significantly impact the accuracy and reproductivity of the measurements. To evaluate the matrix effect across the entire chromatogram, the PCIS approach was implemented. This evaluation was performed by post-column infusion of xenobiotic compounds during the injection of blank or diverse matrix samples, enabling the overall monitoring of both absolute matrix effect (AME) and relative matrix effect (RME). The PCIS approach successfully identified chromatographic regions exhibiting severe matrix effect. Moreover, it yielded comparable RME results to those obtained using the traditional post-extraction spiking method, demonstrating its potential as a reliable technique for evaluating RME in untargeted metabolomics.

Chapter 3 extends the application of PCIS from monitoring to matrix effect correction using a novel artificial matrix infusion strategy. The artificial matrix is composed of compounds that interfere with the ESI process of analytes by competing for ionization or increasing the surface tension in droplets, thereby preventing Coulombic explosion. The matrix effect created by the artificial matrix (ME_{art}) for a specific feature can be determined by injecting a matrix sample both with and without the artificial matrix, and subsequently used to select its ideal PCIS for biological matrix effect (ME_{bio}) correction. This approach enabled the matching of metabolic features, including known and unknown ones, to their appropriate PCISs. The concept of this method was validated using diverse SIL standards spiked into plasma, urine, and feces. To incorporate AME and RME into the validation, a matrix effect scoring system was introduced, which calculates the AME and RME scores separately and averages them as the final matrix

effect score. The PCISs selected based on ME_{art} were compared to those selected by biological matrix effect (ME_{bio}), with 17 out of 19 SIL standards (89%) showing consistent PCIS selection, demonstrating the effectiveness of ME_{art} in identifying suitable PCIS. Applying ME_{art}-selected PCISs to correct the ME_{bio} resulted in improved or maintained matrix effect for 100% of SIL standards in plasma, 84% in urine, and 95% in feces. Since ME_{art} can be assessed at any retention time, the study in this chapter suggests that implementing PCIS with artificial matrix infusion shows great potential in identifying suitable PCISs to correct matrix effect for features detected using untargeted metabolomics.

Chapter 4 presents a systematic review of the modifications and post-treatment alterations in the gut microbiome, metabolome, and immune response in children with CMA aged 0-12 years and in CMA animal models. At the taxonomic level, multiple studies consistently reported decreases in *Bifidobacterium* genus and Lactobacillales order, alongside increases in Clostridia class in CMA children. Various intervention approaches, such as different formulas, prebiotics, probiotics, and synbiotics, were applied to manage CMA across several studies. However, a constant increase in *Bifidobacterium* levels was observed only with *Bifidobacterium* strains-specific treatments. Regarding metabolome modification, altered short-chain fatty acids (SCFAs), amino acids, and organic acids were reported in CMA children. These metabolomic changes appeared to be partially restored through interventions, with increased SCFAs levels and balanced amino acid profiles. Limited data were available regarding immune response. Overall, the review highlights that no study has applied multi-omics approaches to investigate the relationship between gut microbiome and CMA in early life. Although several metabolomics studies have been reported, they focused on a limited range of metabolites, emphasizing the need for more comprehensive metabolomics research on CMA in early life.

To fill the gap identified in **Chapter 4**, **Chapter 5** explores the fecal metabolome in CMA infants undergoing dietary intervention with and without synbiotic supplementation (inulin, oligofructose and *Bifidobacterium breve* M-16 V). By grouping the infants based on their CMA status after one year or the type of intervention

they received, the impacts of CM tolerance acquisition and the influence of the synbiotic supplementation on the fecal metabolome of CMA infants were investigated. More pronounced changes in fecal amino acids, bile acids, and SCFAs were observed the infants who acquired tolerance. The tolerant group showed significantly higher levels of lysine and citrulline, and potential evidence of downregulated tryptophan-serotonin metabolism, upregulated secondary bile acid production, and increased butyrate level. 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. The significant increase of the two aromatic lactic acids (4-hydroxyphenyllactic acid and indolelactic acid), known as infant-type *Bifidobacterium*-derived metabolites, suggested an enhanced abundance and/or activity of infant-type *Bifidobacterium* species, indicating the successful supplementation of the synbiotic.

Chapter 6 concludes the thesis with a general summary and discussion. It outlines potential improvements in the implementation of PCIS for addressing matrix effects in untargeted metabolomics and provides recommendations and perspectives on applying metabolomics to study the gut microbiome and CMA in early life.