School of Public Health

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Association of host transcriptomic and microbiome profiles with food allergy

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DECLARATION

I, Khui Hung Lee, certify that:

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made. This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

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The research presented and reported in this thesis was conducted in accordance with the National Health and Medical Research Council National Statement on Ethical Conduct in Human Research (2007) – updated March 2014. The proposed research study received human research ethics approval from the Curtin University Human Research Ethics Committee, Approval Number # HRE2016-0178 & HRE2017-0712 and CAHS Human Research Ethics Committee (HREC), Approval Number # HREC 2016046EP & RGS151 / HREC 2017060EP.

Written patient consent has been received and archived for the research involving patient data reported in this thesis.

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This thesis contains work that has been published and prepared for publication. The contributions of co-authors are listed.

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ABSTRACT

Introduction

The prevalence of food allergy among children is on the rise worldwide and over the last few decades food-related anaphylaxis admissions have increased in developed countries. This has created an urgent need to understand the underlying mechanism of food allergy because this is essential for the development of better diagnostics tools and more effective prevention and treatments.

At present there is a growing appreciation that the host transcriptome and the various host microbiomes play a significant role in allergic diseases, and that the host-microbe interplay is associated with disorders of the immune system. Therefore, elucidation of the specific differences in the host transcriptome and microbiome profiles between food-allergic children and non-food allergic children will advance our understanding of the aetiology of food allergy.

Aim and objectives

The overall aim of this thesis is to systematically elucidate the association of gene expression and microbiome with food allergy. In order to achieve this, this thesis consists of six specific objectives. The first objective is to identify novel genes related to food allergy. The second objective is to identify gene network patterns in children with nut allergy. The third objective is to investigate changes in cellular immune response between children with and without nut allergy. The fourth objective is to identify gut microbiome and pathways associated with food allergy. The fifth objective is to investigate the association between oral microbiome and food allergy. And the last objective is to investigate the house dust microbiome and pathways associated with food allergy.

Methods

Food-allergic children (n = 210) were recruited from the outpatient clinic of the Immunology Department, Perth Children's Hospital while non-food allergic children (n = 69) were recruited with matched age range and gender frequency from the outpatient clinic of the Immunology Department, Perth Children's Hospital and the local community. Peripheral blood, stool samples, saliva samples and house dust samples were collected and questionnaires, which contained demographic and environmental data, were administered.

Transcriptome profiling of whole blood cells was compared between children with (n=23) and without nut allergy (n=7). The expression levels of three upregulated genes with nut allergy were validated on a larger cohort of samples (n=86) by RT-qPCR. The composition of immune cells was inferred from the transcriptomic data using the CIBERSORTx algorithm. Selected genes of the transcriptomic data were then used to construct a co-expression network by using the Weighted Gene Co-expression Networks Analysis (WGCNA) package of R. The gene co-expression modules were subsequently interrogated with pathways analysis tool (InnateDB) and correlated with clinical phenotypes and cellular immune responses.

In parallel, the community composition of gut, oral and house dust were assessed via 16S rRNA gene sequencing of the V3 and V4 variables regions. Microbial alpha and beta diversity as well as relative abundance of the operational taxonomic units (OTUs) were compared between food-allergic children and non-food allergic children using QIIME. OTUs obtained from 16S rRNA gene sequencing were used to construct a co-abundance network via WGCNA and mapped onto Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways using Tax4Fun.

Results

We found for the first time that the *IFIH1*, *DRAM1* and *ZNF512B* genes were novel nut allergy susceptibility genes. These genes were enriched with functions related to type 1 interferon signalling. Our study also demonstrated that the cellular immune response in children with nut allergy was characterised by a significantly lower CD4+ T cells/Treg response and a higher neutrophil response when compared to children without nut allergy.

We also observed a significant difference in the beta diversity of the saliva microbiome although this was absent for the gut microbiome. Our results indicated the enrichment of *Ruminococcaceae UCG-002* in the gut microbiome as well as the depletion of Streptococcus in the oral microbiome of food-allergic children. Further pathway analysis showed the enrichment of methane and glycerolipid metabolism in the gut microbiome of food-allergic children and the enrichment of ubiquinone and other terpenoid-quinone biosynthesis in the gut microbiome of non-food allergic children.

We also found that the house dust microbiome was different for food-allergic and non-food allergic children. Particularly, the house dust microbiome of food-allergic children was shown to be enriched with pathogenic microbial taxa, such as *Streptococcus*, *Gemella*, *Haemophilus*, *Neisseriaceae_unclassified*, *Actinomyces*, *Rothia*, *Alloprevotella* and *Prevotella 7*, and these taxa were involved in the pathways of replication and repair as well as the cofactors and vitamin metabolism. In contrast, the house dust microbiome of non-food allergic children was involved in the amino acid metabolism.

Conclusion

The integration of transcriptional profiling and network analysis identifies several novel genes and associated immune cells that are different between children with and without nut allergy. There is a different microbiome profile in the gut and oral cavity

between food-allergic children and non-food allergic children. A distinct house dust microbiome profile exists in the bedrooms of food-allergic and non-food allergic children. These results of this thesis suggest that the host transcriptome as well as the gut, oral and house dust microbiomes are correlated with food allergy. The transcriptome and microbiomes exhibit co-existence relationships and demonstrate complex correlation patterns, likely resulting to have an association with food allergy. The overall findings of this thesis emphasize that the transcriptome and microbiome profiles should be investigated as a linked entity, i.e. a network, to systematically gain a better understanding of the aetiology of food allergy. These data can assist in the intervention, management, treatment and possible prevention of food allergy.

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DEDICATION

I dedicate this thesis to my family for your constant support and unconditional love. I love you all dearly.

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Song, Y., Khoo, S., **Lee, KH**., Mäkelä, M., Haahtela, T., LeSouëf, P., Zhang, G (2017). Dual responses of CD14 methylation to distinct environments: a role in asthma and allergy. European Respiratory Journal. 50 (1701228).

Lee, KH., Song, Y., O'Sullivan, M., Pereira, G., Loh, R., Zhang, G (2017). The Implications of DNA Methylation on Food Allergy. International Archives of Allergy and Immunology. 173(4): 183-192.

Conference and poster presentations during PhD candidature

Lee, KH., Song, Y., O'Sullivan, M., Metcalfe, J., Loh, R., Zhang, G. RNA sequencing profiling potential biomarkers for nut allergy, presented at the World Allergy Congress (WAC), Lyon, France, 2019.

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Lee, KH., Song, Y., Loh, R., O'Sullivan, M., Zhao, Y., Dykes, G., Zhang, G. Investigating the association between microbiota composition and food allergy in children, presented at the 10th Annual Telethon Kids Institute Student Circle Symposium, Perth, Australia, 2016.

Scholarships

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LIST OF ABBREVIATIONS AND ACRONYMS

16S rRNA	16S ribosomal RNA
Adonis/PERMANOVA	Adonis permutational multivariate analysis
ARG1	*Arginase 1*
CD82	Cluster of Differentiation 82
DNA	Deoxyribonucleic acid
DRAM1	*DNA damage-regulated autophagy modulator 1*
ECHDC3	*Enoyl-CoA Hydratase Domain Containing 3*
FOXO1	*Forkhead box protein O1*
FOXP3	*Forkhead box P3*
GASK1B	*Golgi Associated Kinase 1B*
GNB4	*G Protein Subunit Beta 4*
GO	Gene Ontology
GS	GeneSignificance
GWAS	Genome-wide association studies
HLA	*Human leukocyte antigen*
HREC	Human Research Ethics Committee
IgA	Immunoglobulin A
IFIH1	*Interferon Induced with Helicase C Domain 1*

IgE	Immunoglobulin E
IgG1	Immunoglobulin G1
IgG2a	Immunoglobulin G2a
IgG4	Immunoglobulin G4
IL-1	Interleukin 1
IL1β	Interleukin 1 beta
IL1R	Interleukin 1 receptor
IL1R2	Interleukin 1 receptor, type II
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-17	Interleukin 17
IL18RAP	Interleukin 18 receptor accessory protein
KEGG	Kyoto Encyclopedia of Genes and Genomes
KLHL2	Kelch Like Family Member 2
LDA	Linear discriminant analysis
LEfSe	Linear discriminant analysis effect size
logCPM	log2-counts-per-million
Log2FC	log2(fold change)
LTB4R	LTB4R Leukotriene B4 receptor

LYSMD2	LysM Domain Containing 2
MAPK1	*Mitogen-activate protein kinase 1*
MM	Module Membership
NF-κB	Nuclear factor kappa B
NFKBIA	Nuclear factor kappa B inhibitor alpha
OTUs	Operational taxonomic units
PADI4	Peptidyl Arginine Deiminase 4
PIK3D	Phosphatidylinositol 3-kinase, Catalytic, Delta
PCoA	Principal Coordinate Analysis
PPP1R3D	Protein Phosphatase 1 Regulatory Subunit 3D
QIIME	Quantitative Insights Into Microbial Ecology
RNA	Ribonucleic acid
RNA-Seq	RNA Sequencing
RPTOR	*Regulatory associated protein of MTOR complex 1*
RT-qPCR	Reverse transcription-polymerase chain reaction
SD	Standard deviation
S100A8	S100 Calcium Binding Protein A8
S100A9	S100 Calcium Binding Protein A9
sIgE	Specific immunoglobulin E
SPT	Skin prick test
STAT	Signal transducer and activator of transcription protein

Th1	Type 1 T helper
Th2	Type 2 T helper
Th17	Type 17 T helper
TLR4	Toll Like Receptor 4
TMM	trimmed mean of M values
TNF-α	Tumor necrosis factor alpha
TOM	Topological Overlap Matrix
Tregs	Regulatory T cells
TREM1	Triggering Receptor Expressed On Myeloid Cells 1
WGCNA	Weighted gene co-expression network analysis
ZNF512B	Zinc Finger Protein 512B

1 INTRODUCTION

This chapter evaluates the current state of knowledge on food allergy and is a preface to the contents of this thesis. Specifically, the prevalence, the definition and aetiology, and the risk factors of food allergy are described in sections 1.1, 1.2 and 1.3 respectively. Section 1.3 includes genetic (section 1.3.1) and environmental risk factors (section 1.3.2). This chapter also describes the application of transcriptome analysis (section 1.4) and microbiome analysis (section 1.5) for advancing our understanding of food allergy. The aims and research questions of my PhD research projects are outlined in this chapter. Sections 1.3.2 and 1.5 are reformatted from **Lee, K.H**., Song, Y., Wu, W. et al. The gut microbiota, environmental factors, and links to the development of food allergy. Clin Mol Allergy 18, 5 (2020). https://doi.org/10.1186/s12948-020-00120-x, published under a Creative Commons BY license (http://creativecommons.org/licenses/by/4.0/)

1.1 The prevalence of food allergy

The prevalence of food allergy among children is on the rise worldwide and food-related anaphylaxis admissions in developed countries, including Australia, have increased over the last few decades (1). Such increases are well documented in the United Kingdom (2), United States (3, 4) and Australia (5). For example, the prevalence of food allergy is now thought to affect approximately 7% of children in the United Kingdom (2). In the United States, a population-based survey showed that around 8% of children have a food allergy (3). Additionally, hospital admissions caused by food-related anaphylaxis for children aged up to 18 years old were doubled from 2000 to 2009 in the United States (4). In Australia 11% of 1 year old children and around 4% of 4 year old children are diagnosed with food allergy (2, 5). Hospital admissions caused by food-related anaphylaxis for children up to 14 years old increased at annual rates of more than 10% between 1997 and 2013 (6) in Australia.

At present there is still no cure for food allergy (7). Immunotherapy is the only promising treatment in desensitizing food allergy and is not recommended for clinical practice. Hence, the best management for food allergy is to strictly avoid specific food allergen. The ever increasing prevalence of food allergy and its potential anaphylactic reactions have created an urgent need to understand the underlying mechanism of food allergy. This is necessary to develop better diagnostics tools and more effective prevention or treatment.

1.2 The definition and aetiology of food allergy

Food allergy is defined as an adverse immune reaction to ingested food antigens. These adverse immune reactions are categorized into Immunoglobulin E (IgE) mediated reactions and non-IgE mediated reactions (8, 9). IgE-mediated reactions usually occur immediately upon exposure to the food allergen, and its symptoms typically occur in different organs, such as gastrointestinal, skin, respiratory and cardiovascular system (8). In contrast, non-IgE mediated reactions are usually delayed and occur few hours or even 24 hours following exposure to a food allergen. Its symptoms include eosinophilic oesophagitis, food protein-induced enterocolitis and allergic proctocolitis (9). This thesis primarily focuses on understanding the molecular mechanism of IgE-mediated food allergy.

1.2.1 Epicutaneous exposure to food allergens and food allergy

Besides oral exposure, there is growing evidence that skin is an important route for allergic exposure. For example, studies have shown that food sensitization can occur through the topical application of products containing food allergens to the skin such as peanut oil (10) and wheat (11). In addition, studies have also revealed the coexistence of eczema and food allergy (12-15). A study conducted by Martin et al. showed that infants with diagnosed eczema had a 6 times higher risk of developing hen's egg allergy and 11 times higher risk of developing peanut allergy at the age of 12 months compared to infants without eczema (16).

The importance of epicutaneous exposure to allergens has been well demonstrated in mice studies. Mice with epicutaneous ovalbumin sensitization have a decreased expression of Filaggrin and an increased expression of pro-inflammatory cytokines such as interleukin-17, interleukin-23, interferon-gamma (17), interleukin-6 and interleukin-4 (17-19), which in turn promotes T helper 2 (Th2) cell responses (19) and IgE levels (18), leading to altered mucosal immunity and breakdown of oral tolerance.

1.2.2 Common food allergens and natural history of food allergies

Although food allergy can arise from any food, a large scale population-based cohort study of food allergy has revealed peanut (8.9%), hen's egg (16.5%), sesame (2.5%) and cow's milk (5.6%) are the most common food allergens in Australia (20). Besides these, tree nuts (3.3%) were also found a common food allergen in Australia (21). The clinical onset of food allergy usually occurs in the first year of life. Some children eventually outgrow their food allergies in childhood (22, 23), while others have persistent food allergies (24-26). For instance, studies have reported that 47% of children have outgrown their hen's egg allergy by 2 years of age and 80% by age 4 years of age (22) while 52.6% of children have outgrown their cow's milk allergy by 5 years of age (23). In contrast, peanut, tree nut and sesame allergy generally remain persistent during life. The likelihood for children to outgrow their peanut, tree nut and sesame allergy is 22%, 10% and 20%, respectively (24-26). In addition, peanuts and tree nuts are responsible for most fatal anaphylactic reactions (27, 28).

1.3 The risk factors for food allergy

Until now, the mechanisms that determine why some children develop food allergies whilst others do not are not well understood. It is generally believed that food allergy is caused by complex interactions of genetic and environmental factors (2).

1.3.1 Genetic studies of food allergy

The genetic cause of food allergy has been extensively investigated. Parent(s) with a history of atopy are more likely to have infants with food allergy. For example, a population-based cohort study revealed infants of women with atopic allergy were shown to have greater chances of developing food allergy when compared to infants of women without atopic allergy (29). The genetic predisposition for food allergy was also evidenced by previous familial aggregation studies (30, 31) and more recently, a twin study found that identical twins had higher concordance rates (64.3%) of developing food allergy compared with dizygotic twins (6.8%) (32).

Candidate-gene studies were historically employed to examine the genetics of food allergy. Here a limited number of genetic variants are tested in candidate genes that show association with food allergy based on previous knowledge (33). Now studies have progressed to genome-wide association studies (GWAS) which have become a valuable method to characterize the genetic basis of food allergy. The first GWAS of food allergy identified rs7192 and rs9275596 of the *HLA-DR* and *HLA-DQ* gene regions as determinants of food allergy, particularly peanut allergy (34). The associations of *HLA-DR* and *HLA-DQ* gene regions with food allergy were further

confirmed (35-38) in several ethnical populations. Marenholz et al. conducted a GWAS with children with food allergy, who were identified by their failed oral food challenges, and their matched controls, and identified variants in the *SERPINB* gene cluster that are associated with food allergy (39). A meta-analysis of food allergy for GWAS conducted from Canadian, American, Australian, German, and Dutch populations identified a novel food allergy-susceptible locus in *C11orf30* (rs7936434) (40). Despite the success of GWAS showing which genes are involved in the development of food allergy, these findings only provide limited knowledge on the mechanism of food allergy. It has restricted clinical significance for several reasons. First, the identified single nucleotide polymorphisms by GWAS only represent a small proportion of the overall genetic contribution (41). Second, they are rarely located in protein-coding regions or have known significant impact on gene expression. Most of them are not related to clear causal variants in adjacent genes.

1.3.2 Environmental and other risk factors with food allergy

It is generally agreed that the environment plays a critical role in the development of allergic diseases including food allergy. The hygiene hypothesis (42) states that early childhood exposure to particular microorganisms protects against allergic diseases, and has been one of many prevalent explanations for the significant increase of allergic diseases in modern societies over the past several decades. Recent studies have revisited the hygiene hypothesis to include dietary intake (43-48), daycare attendance

(49-52), pet contact (53, 54), and antibiotic intake (55, 56) as factors that affect the pathogenesis of allergic diseases such as food allergy.

Diet plays an essential role in the development of food allergy. The dietary intake of infants starts with milk, either breast milk or formula (57). Although recent systematic reviews and meta-analysis show no evidence of protection from food allergy by breastmilk (58-60), there are some evidences that breastmilk has the potential to modulate the risk of food allergy depending on its composition, which could influence microbiota composition and oral tolerance induction (61, 62).Breastmilk is suggested to have a role in prevention of allergic diseases through shaping neonatal gut microbiota (61). Breastmilk can directly affect the early microbiota composition by favouring the growth of Bifodobacteria and Lactobacillus and affecting the metabolic function of microbiota, which in turn affect immune development and maturation. Additionally, the duration of breastfeeding influences the development of food allergy as infants that receive breastfeeding for a brief period have a higher risk of developing cow's milk allergy (47).

Other than the feeding method and the duration of breastfeeding, the timing of solid food introduction is also seen as a key factor that may influence the development of food allergy. Several studies, including randomized controlled trials (RCT), have demonstrated that early introduction of allergenic foods such as peanuts and eggs may actually be beneficial in preventing food allergy and a delayed introduction of allergenic food may contribute to allergic disease(65-68). The most compelling evidence to date comes from the Learning Early About Peanut (LEAP) study, which

randomized 640 children with severe eczema and/or egg allergy to either early (age 4 to 11 months) or delayed (age 5 years) introduction of peanut (65). The LEAP study found that the early introduction of peanut was associated with a 86% reduction of developing peanut allergy by 5 years of age. Similar to this, Enquiring about Tolerance (EAT) trial also found that early introduction of peanut and hen's egg would significantly lower the relative risks of developing peanut allergy and hen's egg allergy (66). In contrast, two randomized controlled trials (67, 69) found no significant association between early introduction of hen's egg and reduced risk of developing hen's egg allergy. Although the results of hen's egg allergy studies have been inconclusive, a recent systematic review of randomized controlled trials investigating the timing of allergenic food introduction and the risk of developing food allergy found moderate-certainty evidence that early introduction of cooked egg (age 6 to 9 months) reduced the risk of egg allergy (59). This systematic review also found moderate-certainty evidence that early introduction of peanut (age 4 to 11 months) reduced the risk of peanut allergy by 5 years of age.

Daycare attendance is also proposed to be protective against food allergies. Several cross-sectional studies demonstrated that early childhood attendance is inversely associated with atopy (49, 50) and IgE levels (51). When compared with infants who did not attend daycare, infants who attended daycare showed an increase in gut microbiome diversity, species richness values as well as butyrate producers, including *Clostridiales* and *Lactobacillus* (Firmicutes phylum), which in turn reduced allergic inflammation and increased oral tolerance (70). In contrast, another study revealed a

positive association between early childhood daycare attendance and the development of food allergy (52). The discrepancy between these studies might be caused by the different allergy phenotypes or the heterogeneity of study design including study methodology.

Pet contact is another factor contributing to the protection of food allergy. For example, a study conducted by Koplin et al showed that children with a pet dog at home have a reduced risk of developing hen's egg allergy by one year of age (54). Another study also found that pet exposure during the first three months of life reduces the odds of developing food allergy (53). This protective effect might be due to increased microbial exposure from the pet (71). Pets increase exposure to endotoxin, which is commonly "found on the outer membrane of Gram-negative bacteria" (72). Some studies have reported an inverse association between high-endotoxin environments and allergic diseases (73, 74), while other studies have reported a positive association (75-77). The host response to environmental endotoxin appears to depend on the pre-existence of allergic disease and genetic inheritance (atopy condition) of the host.

Emerging data suggests a positive association between antibiotic intake in early life and food allergy (55, 56). Antibiotic administration can reduce the microbiota diversity and alter the microbiota composition (55, 78, 79), resulting in failure of the signal transmission via Toll-like receptor 4 (78). The inability to signal via Toll-like receptor 4 resulted in markedly increased peanut-specific IgE and Th2 cytokine responses. Nevertheless, the association of antibiotic intake in early life and the development of

food allergy is debated (14). A large family cohort study did not find an association between antibiotic intake in the first year of life and food allergy (14).

In summary, there is clear evidence that the development of food allergy involves a complex multifactorial process and heterogeneous aetiology. Both genetic and environmental factors, as well as the human microbiome, are involved in the development of food allergy. With the recent advances in molecular biological techniques, unbiased or hypothesis-generating multi-omics approaches have been proposed to characterize the underlying molecular mechanism of food allergy. Next generation high-throughput technologies are being used to systematically and comprehensively characterize biological systems at multiple levels (80-82). This allows the exploration of the underlying physiology and pathophysiology mechanisms of diseases including food allergy. The following sections (1.4 and 1.5) review the application of transcriptomic and microbiome analysis in the studies of allergic diseases, particularly food allergy.

1.4 Transcriptome analysis

Transcriptome analysis is used to predict and understand the pathogenesis of allergic diseases. The host transcriptome consists of all RNA molecules that are transcribed from the host genome (83), and includes coding RNA, such as messenger RNA, and noncoding RNA (84). Gene expression profiling is a quantitative measurement of messenger RNA for thousands of genes at once and has been used to identify gene signatures and pathways related to diseases (83). Microarrays first made the

transcriptomic analysis available. Microarrays are still in use but they can only quantify predefined transcripts or genes (85, 86). More recently, RNA sequencing has emerged as a powerful method for characterizing the transcriptional profiles. It allows for full sequencing of the transcriptome in the organism, detection of low-expressed genes and improves gene-level quantification accuracy in phenotypes (85, 86).

Transcriptome analysis in food allergy generally uses peripheral blood and peripheral blood mononuclear cells as blood is easily accessible. Several studies have investigated the transcriptome of peripheral blood mononuclear cells in patients with food allergy. They have identified gene signatures, associated immune cells and pathways related to food allergy (87-90). For example, Kosoy et al (90) performed blood transcriptome profiles using microarray and found that children with baked egg allergy showed an increased expression of interleukin 5, interleukin 9 and tumor necrosis factor alpha when compared to children without baked egg allergy. By comparing epigenome and transcriptomic profiling analysis of CD4+ T-cells in children with and without hen's egg allergy, Martino et al. identified a disrupted expression of metabolic (*RPTOR*, *PIK3D*, *MAPK1*, *FOXO1*) and inflammatory genes (*IL1R*, *IL18RAP*, *CD82*) in children with hen's egg allergy (88). This study also found that the upregulated genes from CD4+ T-cells in children with hen's egg allergy were involved in contractile response (myogenesis, apical junction, epithelial transition), signal transduction (STAT and WNT signalling), and fatty acid metabolism (cholesterol homeostasis). Watson et al (87) conducted a RNA sequencing analysis of transcriptomic profiles from a cohort of peanut allergic children before, during, and

after randomized, double-blind, placebo-controlled oral peanut challenges. This study found six genes (*LTB4R*, *PADI4*, *IL1R2*, *PPP1R3D*, *KLHL2,* and *ECHDC3*) associated with severe peanut responses through the regulation of the Rel/ Nuclear factor kappa B (NF-κB) transcription factor family. They also found increased proportions of macrophages and neutrophils, and reduced proportions of naive CD4+ T-cells upon peanut challenges. Another study conducted by Do et al identified *NFKBIA* and *ARG1* as the key genes responsible for peanut severity through integrative analysis of whole-blood transcriptomic and epigenomic (89). The authors also found these upregulated genes were associated with neutrophil activation and neutrophil-mediated immunity.

Overall, these studies suggest the involvement of multiple genes in multiple cell populations, such as CD4+ T-cells and neutrophils, in the pathogenesis of food allergy. CD4+ T-cells play a crucial role in the initiation and regulation of IgE-mediated food reactions (88, 91, 92). The induction of regulatory T cells, a subset of CD4+ T-cells, is known to express transcription factor forkhead box P3 in response to foreign antigens (92) and these cells perform a suppressive function by secreting anti-inflammatory cytokines (93). Hence, reduced CD4+ T cells, especially regulatory T cells, could result in the failure of oral tolerance.

Neutrophils are the first responders of the immune system to the site of inflammation and react to invading pathogens (94). Other than infection and inflammation, neutrophils have also been linked to allergic diseases (95-98). Mice models demonstrated that FcγRIIIA and FcγRIV (96), which are expressed by neutrophils,

increase the reaction severity while the depletion of neutrophils inhibits acute allergic reactions. In addition, human studies also identified several markers of neutrophil activation to be associated with acute anaphylaxis, such as *S100A8*, *S100A9*, *TLR4*, *TREM1*, *S100A9* (97) as well as *interleukin 17A* (99).

1.5 Microbiome Analysis

The human microbiome plays a critical role for the health or disease of its host. The mucosal surfaces in the human body are native to complex communities of microorganisms. The human body is estimated to consist of 10-100 trillion microbes, and more than 1,000 bacterial species (100). These microbes do not only interact with each other but also interact with the host and its environment, shaping the local immune system and maintaining homeostasis (101).

The microbiome was first suggested to be associated with the development of food allergy through an epidemiological study, which found children from a smaller household size were associated with a higher rate of developing allergic diseases compared to children from a larger household size (102). The application of next-generation high-throughput sequencing, such as 16S rRNA gene sequencing, has advanced our understanding of host-microbe interactions. 16S rRNA gene sequencing has highly conserved regions for primer design and hypervariable regions (103) which allows for identifying thousands of new microorganisms that were not identified previously.

1.5.1 Gut microbiome and food allergy

The human gut contains the highest number of microbes (104) of which 90% are Bacteroidetes and Firmicutes (105). This gut microbiome is known to affect the course of food allergies. This is particularly evidenced in human studies (106-109). For example, a study conducted by Ling et al. (106) found a reduced abundance of Bacteroidetes and an increased abundance of Firmicutes in infants with food allergy when compared to healthy controls. Another study found enrichment of Firmicutes in children with egg allergy when compared to controls (107). Yet another study also found enrichment of Firmicutes in food-allergic children compared to non-allergic siblings and healthy controls (108). In contrast, a large observational cohort study in the United States showed that children with persistent cow's milk allergy had a higher abundance of Bacteroidetes and a lower abundance of Firmicutes than control subjects (109). These data seem to be inconsistent due to heterogeneity in study design, such as different techniques to characterize the gut microbiota, different sampling time points, and different allergic phenotypes. Hence, the microbe(s) associated with food allergy require further investigation.

The role of the gut microbiome has been demonstrated in both human studies (110) and animal models (111-115). The gut microbiome may modulate the immune system and oral tolerance by affecting host metabolism (111, 112) and the alteration of adaptive immunity (113).

Major attention has been directed to the possible role of short-chain fatty acids, such as butyrate, propionate, and acetate in affecting the immune system (70, 116-118),

since short-chain fatty acids are the main product of the digestive action of the gut microbiota [29, 30]. Production of short-chain fatty acids, particularly butyrate, is able to enhance the Vitamin A metabolism, in turns inducing the activity of ALDH in CD103+MLN DCs, and increasing the percentages of T regulatory (Tregs) cells and increasing IgA production (114). Meanwhile, short-chain fatty acids are able to inhibit histone deacetylases activity, resulting in regulation of *aldh1a1* expression, which contributes to immune tolerance. Other than this, short-chain fatty acids can bind metabolite-sensing G-protein coupled receptors, GPR43 or GPR109A (119), in turn promoting the tolerogenic CD103+ DC function and protecting against food allergies (120). Moreover, short-chain fatty acids can reduce the production of pro-inflammatory cytokines including IL-1β, IL-6, IL-17 (110), and meanwhile increase the production of anti-inflammatory mediators including IL-10 (110, 121). Thus, short-chain fatty acids are viewed as a key factor in promoting immunological tolerance towards harmless antigen and preventing inflammation.

Adaptive immune responses are divided into two types: humoral immunity, regulated by B cells (122, 123), and cell-mediated immunity, regulated by T cells. The role of Tregs, subset of CD4+ T cells in oral tolerance development to food allergen, have been confirmed in animal models (124, 125) as well as human studies (126, 127) in which the induction of allergen-specific Treg cells is highly associated with a favourable allergy outcome. Microbiota, especially Clostridia species, in this case, were shown to be able to induce the production of Tregs (115, 128), which helps to inhibit allergic inflammation and promote oral tolerance (109, 112, 129, 130).

1.5.2 Oral microbiome and food allergy

In addition to the gut microbiome, the oral microbiome is also relevant for the health and disease of its host. This was first proposed by oral microbiologist W. D. Miller in the 1890s (131). According to Miller, oral microbes and their products can profoundly affect the development of diseases. Pathogenic bacteria enter into the blood circulation through the oral mucosal barrier which can later result in chronic gut inflammation (132) through the reduction of Th17 cells and faecal IgA levels as well as an induction in the ratio of M1 to M2 macrophages (133).

Since the oral microbiome contributes to host immune responses and inflammation, it is reasonable to propose that the oral microbiome may also affect the development of food allergy. A murine model of food allergy identified a reduced diversity of oral microbiome in ovalbumin sensitized mice compared with controls (134). This study identified a reduced diversity of oral microbiome in ovalbumin sensitized mice compared with controls. It also showed that the oral microbiome is dominated by *Streptococci* spp., including *Streptococcus sanguinis* and *Streptococcus gordonii*, as well as *Lactobacillus* spp. In addition, significantly higher IgA levels in ovalbumin sensitized mice were reported. This association between oral dysbiosis and food allergy (134) encourages further understanding of oral microbiome modulation and its impact on the pathogenesis of food allergy, as this is still in its infancy stage.

1.5.3 House dust microbiome and food allergy

Indoor environments contribute significantly to the human exposure to environmental microbes, as people spend most of their time indoors (135). One of the most common

generated indoor pollutants is house dust. House dust contains an average of 9,000 different species of microbes (135). To be specific, the average household has more than 2,000 different types of fungi and 7,000 different types of bacteria. Therefore, transmission of house dust microbes, either through inhalation, ingestion or cutaneous, may be contributing to the onset of allergic diseases.

To understand the association between house dust microbiome exposure and allergy outcomes, researchers have initially compared the endotoxin concentrations in house dust of children with and without allergic diseases (73, 136). These studies reveal that exposure to a high level of house dust endotoxin has a negative association with development of allergic diseases. Contradictory results are also reported, where exposure to high levels of house dust endotoxin shows an increased rate of developing allergic diseases (76, 77). Despite these discordant results, house dust endotoxin may under circumstances protect against allergic diseases and the development of allergic diseases.

Recent studies using 16S rRNA gene sequencing have found that several taxa from house dust microbiome are associated with allergic diseases in children (72, 137, 138). For example, a case-control study of 104 children showed that reduced exposure to house dust microbiome, particularly Firmicutes and Bacteriodetes, in the first year of life may have a higher chance of developing atopy developing atopy and atopic wheeze (137). Another study identified *Lactococcus* genus as a risk factor for asthma and twelve bacterial genera (mostly from the Actinomycetales order) as a protective factor towards asthma (138). Loo et al. identified the indoor microbes of children with

allergic diseases are enriched with *Anaplasmataceae*, *Bacteroidaceae*, and *Leptospiraceae* (72). Although these studies have reported an association between house dust microbiome and allergy, there is very limited data examining the association between house dust microbiome and food allergy.

1.6 Overall aim and objectives

This thesis explores the molecular mechanism of food allergy among children in Perth, Australia. The overall aim of this thesis is to systematically elucidate the association of gene expression and microbiome with food allergy. Food-allergic children are those with a clinical history of allergic reactions towards at least one type of food, while non-food allergic children are those without any clinical history of food allergic reactions.

The specific objectives of this thesis are to:

1. identify novel genes related to food allergy;
2. identify gene network patterns in children with nut allergy;
3. investigate changes in cellular immune response between children with and without nut allergy;
4. identify gut microbiome and pathways associated with food allergy;
5. investigate the association between oral microbiome and food allergy;
6. investigate the house dust microbiome and pathways associated with food allergy.

1.7 Public Health Relevance

Food allergy affects 1 in 11 infants and 1 in 4 children in Australia (2, 5). Our aim is to systematically elucidate the association of gene expression and microbiome with food allergy. We compare the profiles of host transcriptome, gut microbiome, saliva microbiome and house dust microbiome between food-allergic children and non-food allergic children. The results of this thesis contribute towards a greater understanding of mechanisms underlying food allergy and may have utility in providing targets or biomarkers for better prediction and early identification of food allergy, thus improving management and prevention strategies for this unpleasant, sometimes fatal sickness in children.

2 STUDY DESIGN AND METHODOLOGY

This chapter briefly describes the study design and methodology, which underpinning the conduct of the whole Ph.D. project.

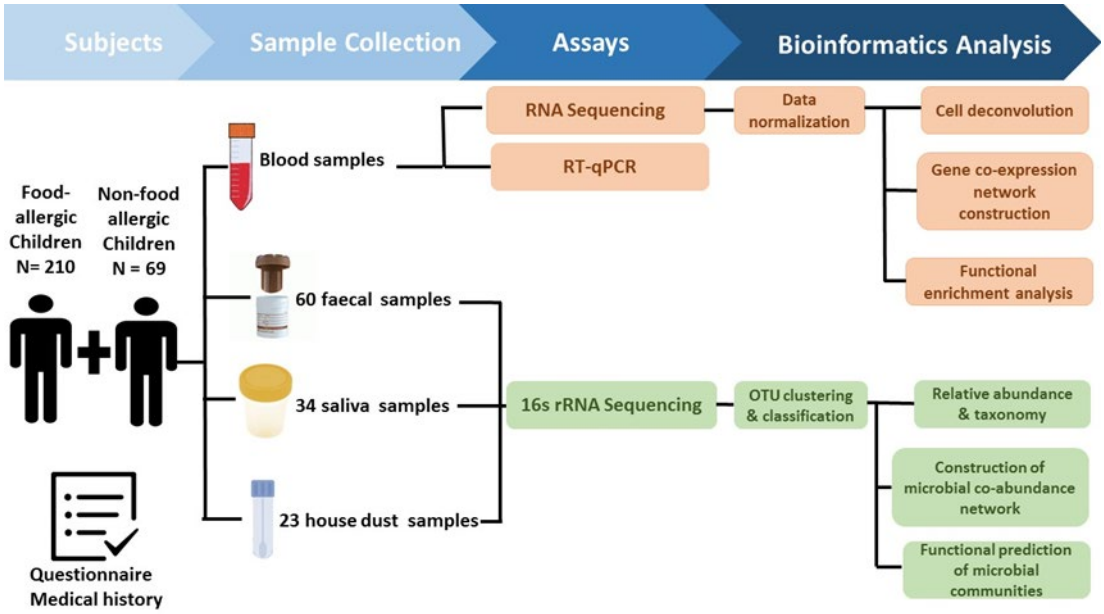


Figure 2-1 Study Overview

2.1 Study Population

A total of 279 individuals were recruited from the outpatient clinic of the Immunology Department, Perth Children's Hospital (previously known as Princess Margaret Hospital for Children) and local communities.

There were two different groups of controls being used as comparison. For chapter 4, children with suspected or diagnosed allergies to nuts were recruited from the outpatient clinic of the Immunology Department, Perth Children's Hospital and they were grouped into children without nut allergy and children with nut allergy based on the diagnoses outcome of the patients on the day of recruitment. Food allergy was defined by immediate symptoms (1–2 h) after food allergen ingestion combined with either failed oral food challenge or with positive skin prick test wheal diameter ⩾

3 mm to nuts, hen's egg and cow's milk. In contrast, a negative skin prick test and/or a passed oral food challenge defined the subject as children without a food allergy. While for chapters 5,6,7, gut, oral and house dust microbiome were compared between children who currently diagnosed to have an allergic reaction to nuts, hen's egg and cow's milk and those who never have any allergic reaction to any food allergens (Figure 2-1).

2.2 Sample collection and processing

2.2.1 Blood Sample Collection and RNA extraction

4 ml peripheral whole blood was collected into PAXgene RNA blood tubes (Qiagen, Mississauga, Ontario, Canada) and serum tube by trained phlebotomists in the Pathology Department at Perth Children's Hospital. Total RNA samples were extracted using the PAXgene Blood RNA Kit in accordance with the manufacturer's instructions (Qiagen). The concentration and purity of total RNA were measured by determining the A260/280 and A260/230 ratios, respectively (NanoDrop, Thermo Scientific, Wilmington, DE, USA).

2.2.2 Faecal sample collection and processing

Faecal sample was collected from participating child by the parent(s) using a provided faecal collection kit, which included a screw cap faecal container (SARSTEDT, Nümbrecht, Germany), an underpad sheet, a pair of disposable gloves, a white paper bag and a sealed plastic bag with labels. The faecal sample was then transported back to the laboratory in an esky cooler and stored at -80°C freezers by a researcher within

2 hours of collection. DNA was later extracted using the QIAamp DNA Stool Mini Kit (Qiagen) following the manufacturer's instructions. The eluted DNA was stored at -80 ˚C until analysis.

2.2.3 Saliva sample collection and processing

Saliva sample was collected from participating children by the parent(s) using a provided saliva collection kit, which included a sterile plastic container with yellow lid, a pair of disposable gloves and a sealed plastic bag with labels. The saliva sample was then transported on ice to the laboratory -80°C freezers for storage by a researcher within 2 hours of collection. DNA was later purified using a PureLink™ Microbiome DNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA) in accordance with the manufacturer's instructions. The eluted DNA was kept refrigerated at -80 ˚C freezers until further analysis.

2.2.4 House dust sample collection and processing

House dust sample was collected from the hard surface of the participant's bedroom, mainly on a cupboard by a researcher using a sterile cotton swab moistened with sterile saline. The cotton swab was then placed immediately back to its sterile tube and transported on ice to the laboratory -80°C freezers for storage by the researcher within 2 hours of collection. DNA was later purified using a PureLink™ Microbiome DNA Purification Kit (Thermo Fisher Scientific) in accordance with the manufacturer's instructions. The eluted DNA was kept refrigerated at -80 ˚C freezers until analysis.

2.3 Quantitative reverse transcription-polymerase chain reactions (RT-qPCR)

Whole blood RNA was reverse transcribed to cDNA with the QuantiTect Reverse Transcription Kit (Qiagen) in accordance with manufacturer's instruction. RNA-specific primers for quantitative reverse transcription-polymerase chain reactions (RT-PCR) were designed on the specific region of the genes in house and tested on AMPLIFY Version 1.0 (Bill Engels, University of Wisconsin, Genetics, USA). RT-qPCR was performed on a ViiA7 ™ Real-Time PCR System (Thermo Fisher Scientific).

2.4 Library preparation and sequencing

30 RNA samples were sent to Australian Genome Research Facility to perform RNA sequencing. The library of RNA sequencing was prepared using Illumina's TruSeq stranded RNA Library Prep Kit and then sequenced on Illumina HiSeq 2500 platform (Illumina, San Diego, CA, USA).

60 stool samples, 34 saliva samples and 23 house dust samples were sent to Novogene Bioinformatics Technology Co., Ltd (Beijing, China) for library preparation and sequencing. The libraries, which targeted the V3-V4 region of the 16S rRNA gene, were prepared using Ion Plus Fragment Library Kit (Thermo Fisher Scientific) and sequenced on the Ion S5 XL platform (Thermo Fisher Scientific).

2.5 Bioinformatics analysis

2.5.1 Quantification of immune cells

CIBERSORTx (139), a deconvolution algorithm, was then used to estimate the abundance of different cell types from RNA-Seq data based on cell type specific reference gene expression profiles. CIBERSORTx provides 22 types of functionally defined human immune cells (LM22 signature matrix) as a reference, and we focused on proportions of 12 human immune cells. Cell type proportions in children with and without nut allergy were compared by employing an independent t-test and were correlated with co-expression modules derived from WGCNA analysis.

2.5.2 Gene expression profile of RNA sequencing

The gene count data were imported, organised, filtered and later normalized based on trimmed mean of M values (TMM) method using edgeR package of R. Counts were then converted to log2-counts-per-million (logCPM) with precision weights, based on voom method using Limma package of R prior to statistical analysis. Gene expression profile was then identified by comparing between children with nut allergy and children without nut allergy.

2.5.3 Quantitative Insights into Microbial Ecology (QIIME)

The raw sequences of microbiome data were demultiplexed and quality filtered using Quantitative Insights into Microbial Ecology (QIIME) (1). Operational taxonomic units (OTUs) were clustered at 97% similarity level against the SILVA reference database (release 128) (2). Alpha diversity, which included Chao1, observed OTUs,

and the Shannon index, were estimated using the alpha_rarefaction.py script in QIIME. Beta diversity (weighted and unweighted UniFrac distance matrices) was measured using the beta_diversity.py script in QIIME. Principal Coordinate Analysis (PCoA) was obtained to visualise unweighted and weighted Unifrac distances in a two-dimensional structure. A comparison of the relative abundance of OTUs between groups (phylum to genus levels) was computed using the summarize_taxa_through_plots.py script in QIIME.

2.5.4 Construction of co-expression and co-abundance network

Genes with high coefficient of variation from RNA sequencing data were used to construct a co-expression network and OTU count data with 97% identity, which undergone Hellinger transformation, were used to construct a co-abundance network by using the WGCNA (15). Highly co-expressed genes and co-occurred microbial taxa were then assigned into several module memberships. After that, module trait association analysis was used to calculate the correlation between the modules and the phenotype to detect the module(s) with the greatest susceptibility to food allergy. Next, hub genes and hub taxa were determined based on the high absolute value of the GeneSignificance and Module Membership obtained from intramodular analysis. These hub genes and hub taxa of the significant modules were then visualized using Cytoscape v3.8.0 (140).

2.5.5 Functional enrichment analysis

Biological function and associated pathways enriched in each module modules were also characterized employing Gene Ontology (GO) and InnateDB(141).

In parallel, the generated OTUs table and OTUs taxonomy was mapped onto Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways using R package, Tax4Fun. Linear discriminant analysis (LDA) effect size (LEfSe) analysis (http://huttenhower.sph.harvard.edu/lefse/) was performed to detect biomarkers of the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways that differed significantly between non-food allergic children and food-allergic children.

2.6 Administration of the questionnaires

A standard questionnaire was administered to parents or guardians of children to collect general information such as age and gender, children and family history of allergic diseases as well as infant feeding regime and other environmental exposure such as pet exposure.

A separate set of questionnaire on dietary intake and antibiotic intake was administered to those parents or guardians, who provided faecal, saliva and house dust samples of their children.

3 CHARACTERISTICS OF THE STUDY POPULATION WITH FOOD ALLERGY AND RISK FACTORS

This chapter described the demographics characteristics and risk factors of children and without food allergy living in Perth, Australia.

3.1 Characteristics of the study population

A total of 279 children were recruited in the study, of which 164 blood samples, 69 faecal samples, 47 saliva samples and 46 house dust samples were collected and 279 questionnaires were administered.

Sixty-one percent of the subjects were boys, with the median age for non-food allergic children and food-allergic children of 6.0 years and 8.5 years, respectively. Two hundred and ten of the 279 children had physician-diagnosed food allergies. One hundred and forty-six children were diagnosed with a nut allergy, 14 children were diagnosed with a hen's egg allergy, 3 children were diagnosed with a cow's milk allergy, and 47 children were diagnosed with multiple food allergies. Multiple food allergies are defined as the presence of food allergies from two different food categories; for example, allergies to hen's egg and nuts meet the criterion, whereas a child who is allergic to only several tree nuts would not meet the criterion for multiple food allergies. Detailed characteristics of the study population are listed in Table 3-1.

Table 3-1 Characteristics of the study population

Characteristic	**Non-food allergic children (n=69)**	**Food-allergic children (n=210)**
Male, n (%)	40.0 (58.0)	129.0 (61.4)
Female, n (%)	29.0 (42.0)	81.0 (38.6)
Age, (mean ± SD)	6.9±4.3	8.6±4.3*
Type of food allergies, n		
Nut allergy	-	146
Multiple food allergies	-	47
Hen's egg allergy	-	14
Cow's milk allergy	-	3

*p less than 0.05

3.2 Results of skin prick test (SPT) and specific IgE (sIgE)

Of the 210 food-allergic children, 61 (21.9%) children had positive reactions to one food allergen (either SPT ≥ 3mm or sIgE ≥ 0.35 IU/ml) and 118 (42.3%) had positive reactions to multiple food allergens. The positive results of SPT and sIgE for food-allergic children were shown in Table 3-2.

Table 3-2 Positive results of SPT and sIgE for food-allergic children

	Positive SPT result n (%)	**Positive sIgE n (%)**
At least one food allergen (n)	169	67
Egg yolk	22.0 (13.0)	9.0 (13.4)
Egg white	35.0 (20.7)	17.0 (25.4)
Egg	2.0 (1.2)	1.0 (1.5)
Almond	17.0 (10.1)	19.0 (28.4)
Brazil Nut	15.0 (8.9)	15.0 (22.4)
Cashew	74.0 (43.8)	27.0 (40.3)
Hazelnut	17.0 (10.1)	19.0 (28.4)
Macadamia	10.0 (5.9)	14.0 (20.9)
Pecan	13.0 (7.7)	17.0 (25.4)
Pistachio	60.0 (35.5)	23.0 (34.3)

Walnut	33.0 (19.5)	27.0 (40.3)
Pinenut	16.0 (9.5)	15.0 (22.4)
Coconut	3.0 (1.8)	2.0 (3.0)
Peanut	112.0 (66.3)	42.0 (62.7)
Cow's milk	12.0 (7.1)	3.0 (4.5)

3.3 Result of oral food challenge

Although oral food challenge is “the gold standard to diagnose food allergy”, it is not common practice in the daily clinical setting due to its anaphylactic reactions (142). Considering peanuts and tree nuts are responsible for most fatal anaphylactic reactions, hence those children with a history of recent anaphylaxis (within 12 months) and/or high levels of IgE specific to nut(s) are excluded from oral food challenge. The decision was made by clinical doctors as common clinical practice. Therefore, only 54 (25.7%) children had the OFC tested. The results of the positive oral food challenge were shown in Table 3-3.

Table 3-3 Positive oral food challenge in the study population

	Food-allergic children (n=54)
Positive oral food challenge result, n (%)	
Egg	13.0 (24.1)
Brazil Nut	1.0 (1.9)

Cashew	3.0 (5.6)
Pecan	1.0 (1.9)
Walnut	3.0 (5.6)
Peanut	13.0 (24.1)

3.4 Atopic history of children and parent(s)

Eczema history was known for 165 children, 140 (66.7%) of whom were diagnosed with food allergy (Table 3-4). Eczema ($p = 0.001$) was significantly associated with food allergy. In contrast, there were no significant differences found in parental atopy history between non-food allergic children and food-allergic children.

Table 3-4 Atopic history of child and parent(s)

	Non-food allergic children (n=69)		**Food-allergic children (n=210)**		
	N	**%**	**N**	**%**	***P***
Personal eczema history	25.0	42.4	140.0	66.7	**0.001**
Parental atopy history	50.0	83.3	182.0	91.5	0.071

3.5 Infant dietary intake and childhood dietary intake

Among the infant dietary intake and childhood dietary intake, colostrum feeding ($p <0.001$) and the duration of breastfeeding period ($p =0.015$) were found to be significantly associated with food allergy (Table 3-5). In contrast, there were no significant differences found in another infant's dietary intake and childhood dietary

intake, including the type of first feed, breastfeeding and timing of solid food introduction between non-food allergic children and food-allergic children.

Table 3-5 Infant dietary intake and childhood dietary intake

	Non-food allergic children (n=69)		**Food-allergic children (n=210)**		
	N	**%**	**N**	**%**	***P***
First feed					0.960
Breastmilk	56.0	86.2	183.0	87.1	
Formula	4.0	6.2	11.0	5.2	
Other	5.0	7.7	16.0	7.6	
Colostrum	28.0	46.7	163.0	82.7	**< 0.001**
Breastfeed	54.0	98.2	183.0	94.3	0.239
Breastfeeding period (months): mean (SD)	9.5	6.9	12.6	8.3	**0.015**
Timing of solid food introduction: mean (SD)	6.3	2.9	5.9	4.1	0.526

3.6 Living lifestyle

Pet contact when born (p = 0.006) showed a significant association with food allergy (Table 3-6). In contrast, pet contact regularly, attending daycare and kindergarten were not associated with food allergy.

Table 3-6 Other living characteristics

	Non-food allergic children (n=69)		**Food-allergic children (n=210)**		
	N	**%**	**N**	**%**	***P***
Day care attendance	37.0	61.7	110.0	54.7	0.342
Kindergarten attendance	42.0	70.0	156.0	79.2	0.138
Pet contact when born	18.0	30.0	99.0	50.0	**0.006**
Pet contact regularly	36.0	60.0	144.0	72.7	0.060

3.7 Dietary intake and antibiotics intake

There were no significant differences observed in fruit daily intake, vegetable daily intake and antibiotic intake between non-food allergic children and food-allergic children (Table 3-7). However, non-food allergic children were found to have significantly higher daily consumption of yogurt ($p = 0.033$) compared to food-allergic children.

Table 3-7 Dietary intake and antibiotics intake

	Non-food allergic children (n=37)		**Food-allergic children (n=38)**		
	N	**%**	**N**	**%**	***P***
Fruit daily intake					0.659

0~2 kinds	23.0	76.7	30.0	81.1	
>= 3 kinds	7.0	23.3	7.0	18.9	
Vegetable daily intake					0.463
0~2 kinds	24.0	80.0	26.0	72.2	
>= 3 kinds	6.0	20.0	10.0	27.8	
Yogurt daily intake	17.0	56.7	11.0	30.6	**0.033**
Antibiotics intake last 2 weeks	0.0	0	2.0	5.4	0.228

3.8 Multivariate model

In a multivariate model, personal eczema history and pet contact when born were associated with an increased risk of food allergy after adjusted for age and gender (Table 3-8). Cow's milk allergy was additionally included as a potential confounder for colostrum and breastfeed. Colostrum remains statistically significantly associated with food allergy after adjustment.

In the unadjusted analysis, yogurt daily intake was inversely associated with food allergy. However, this factor was no longer associated with food allergy in the adjusted model.

Table 3-8 Logistic regression of factors associated with food allergy

	Unadjusted			**Adjusted†**		
	OR	**(95% CI)**	***P***	**OR**	**95% CI**	***p***

Personal eczema history	2.72	(1.51-4.91)	**0.001**	3.19	(1.72-5.91)	**< 0.001**
Parental atopy history	2.14	(0.92-4.97)	0.076	2.01	(0.86-4.72)	0.107
Colostrum	5.48	(2.93-10.26)	**< 0.001**	5.10	(2.57-10.11)	**< 0.001**^
Breastfeed	0.31	(0.04-2.44)	0.265	0.32	(0.04-2.62)	0.287^
Day care attendance	0.75	(0.42-1.36)	0.342	0.71	(0.39-1.30)	0.269
Kindergarten attendance	1.63	(0.85-3.13)	0.141	1.11	(0.51-2.44)	0.794
Pet contact when born	2.33	(1.26-4.33)	**0.007**	2.30	(1.23-4.31)	**0.009**
Pet contact regularly	1.63	(0.85-3.13)	0.141	1.61	(0.87-2.98)	0.129
Fruit daily intake			0.659			0.661
0~2 kinds	1			1		
>= 3 kinds	0.77	(0.24-2.50)		0.76	(0.22-2.59)	
Vegetable daily intake			0.464			0.711
0~2 kinds	1			1		
>= 3 kinds	1.54	(0.49-4.88)		1.27	(0.36-4.48)	
Yogurt daily intake	0.34	(0.12-0.93)	**0.035**	0.35	(0.12-1.01)	0.052

† adjusted with age and gender only

^ Colostrum and breastfeed were additionally adjusted for cow's milk allergy.

3.9 Discussion

This chapter described the clinical and demographics characteristics of children living in Perth, Australia and evaluated the impact of previously identified risk factors on the onset of food allergy in this study population, including the personal and parental

history of atopy, infant dietary intake and childhood dietary intake, living lifestyle and dietary intake in Perth, Australia.

Our study showed that eczema was highly associated with food allergy. This finding was consistent with previous studies, which revealed infants with early onset of eczema had a higher risk of developing food allergies (12-14, 16). One possible mechanism leading to this association could be mutations in the *filament aggregating protein (filaggrin)* gene (143, 144). The barrier defect caused by filaggrin deficiency makes the study subjects more likely to have cutaneous sensitization via antigen-presenting cells and systemic atopic response (145). Therefore, direct skin contact with food allergens in those with impaired skin barriers, caused by *filaggrin* mutations or eczema, could facilitate sensitization, leading to the onset of a food allergy.

Existing evidence suggests that colostrum (146) and pet contact (54, 147, 148) have protective effects on food allergy. Colostrum, which is the first human milk produced by mothers after delivery, is rich in immunologic components such as secretory IgA (149). Colostrum is shown to increase IgA levels, which in turn induces expression of tolerogenic cytokines such as transforming growth factor and Interleukin 10 (150) as well as promote gut-microbiota symbiosis and immune homeostasis (151), leading to oral tolerance. Pet exposure during the first three months of life was suggested to have a lower odd of developing food allergy (53). In contrast, our data indicated that colostrum and pet contact when born might be associated with an increased risk of food allergy. With 91.7% of food-allergic children in our study were found to have at

least an atopic parent, the observed positive association between these two factors and food allergies in our study might be caused by reverse causation.

The timing of solid food introduction was previously investigated as one of the environmental factors that could influence the development of food allergy. The results are conflicting with a study that revealed a significant association between the timing of solid food introduction and food allergy (48), while other studies found no significant association (12, 152, 153). This discrepancy could be explained by the difference in types of food allergy being investigated. The frontier study focused on peanut allergy only while the latter studies covered various types of food allergy. Consistent with latter studies, our findings also did not find any association between the timing of solid food introduction and food allergy.

In addition, there are incongruent results for daycare attendance as a possible environmental factor in increasing the risk of food allergies, with some studies showing a negative association between early daycare attendance and allergic disease, particularly food allergy (49, 50, 154) while other study showing a positive association between the daycare attendance and food allergy (52). Our findings also provided supporting evidence to the latter study that there was no association between daycare attendance and food allergy.

Growing evidence proposed a fundamental role for yogurt consumption in protecting against food allergy (155). Yogurt, which contains *Lactobacillus* species, is suggested to regulate intestinal microbiota abundance and immune response as well as improve intestinal barrier function (155). *Lactobacillus* species such as *Lactobacillus*

rhamnosus GG can increase the expression of proinflammatory cytokines (Tumor necrosis factor alpha and Interleukin 6) or anti-inflammatory cytokines (Interleukin 10) (156) as well as increase colonic regulatory T cells in the intestine (157). In our study, univariate analysis revealed that non-food allergic children were likely to have a higher daily intake of yogurt. However, after adjusting for age and gender, there was no association between yogurt daily intake and food allergy.

Notably, this study has three limitations. First, the study subjects were recruited from the Department of Immunology, Perth Children and local communities. They were not randomly selected from the whole population; therefore, they are not well representative of the population. A convenience sampling strategy was used for the subject recruitment with the aim to collect blood and microbiome samples for further transcriptomic and microbiome investigations. Although we analysed several common risk factors for the association with food allergy in the population as above-mentioned in the chapter this analysis is not the aim of my thesis. I acknowledge that the convenience sampling method can have selection bias for this association analysis. Second, this is a cross-sectional cohort and not a birth cohort; therefore, there may be recall bias inherent in the study design. Third, this is a high-risk cohort with 80-90% of the parents having a history of atopy. This genetic background may influence risk factors. Hence, the associations between these risk factors and food allergy should be interpreted in light of these limitations.

4 IDENTIFYING GENE NETWORK PATTERNS AND ASSOCIATED CELLULAR IMMUNE RESPONSES IN CHILDREN WITH OR WITHOUT NUT ALLERGY

This chapter described Aims1, 2 and 3, the aims of which were to identify novel genes related to food allergy, identify gene network patterns in children with nut allergy and investigate changes in cellular immune response between children with and without nut allergy. This chapter is presented in the manuscript format that has been submitted to the journal of Scientific Reports. The manuscript is still under peer review at the time of submission of this thesis. Some of the contents of this chapter have been presented at the World Allergy Congress 2019, held in Lyon, France, December 2019. It was also presented at a poster session at the Mark Liveris Symposium held in Perth, Australia, October 2018.

4.1 Abstract

Although evidence suggests that the immune system plays a key role in the pathophysiology of nut allergy, the precise immunological mechanisms of nut allergy have not been systematically investigated. The aim of the present study was to identify gene network patterns and associated cellular immune responses in children with or without nut allergy. Transcriptome profiling (n=30) of whole blood cells was compared between children with and without nut allergy. Three genes were selected to be validated on a larger cohort of samples (n=86) by RT-qPCR. The composition of immune cells was inferred from the transcriptomic data using the CIBERSORTx algorithm. A co-expression network was constructed employing WGCNA on the top 5000 most variable transcripts. The modules were interrogated with pathway analysis tool (InnateDB) and correlated with clinical phenotypes and cellular immune responses. Proportions of neutrophils were positively correlated and CD4+ T-cells and regulatory T-cells (Tregs) were negatively correlated with modules of nut allergy. We also identified two upregulated genes, namely *IFIH1*, *DRAM1* and a downregulated gene *ZNF512B* as hub genes for nut allergy. Further pathway analysis showed upregulation of type 1 interferon signalling in nut allergy. Our findings suggest that upregulation of type 1 interferon signalling and neutrophil responses and downregulation of CD4+ T-cells and Tregs are a hallmark of the pathogenesis of nut allergy.

4.2 Introduction

Today, food allergy affects up to 11% of infants and 3.8% of children, with progressively increasing prevalence over the last few decades (5). Children with food allergy are at risk for potentially life-threatening allergic reactions including breathing difficulties, swollen tongue, throat tightness and wheezing, particularly when exposed to nut allergens (158). Moreover, some food allergies such as nut allergy tend to be persistent over the full duration of a person's lifetime and can cause anaphylactic reactions (158, 159). With the exception of treatment of peanut allergy (160), immunotherapy for desensitizing in food allergy is still not recommended for clinical practice (7). Hence, the best management for food allergies is to strictly avoid specific food allergens. As such, it is crucially essential to elucidate the fundamental mechanisms of food allergy to help develop effective prevention and treatment for this condition in children.

Food allergy is mechanistically characterized by the development of an overactive immune response to an otherwise harmless allergen, resulting in a T helper 2 (Th2) polarized cytokine response to the allergen and the production of an Immunoglobulin E (IgE) antibody response. Upon recognition of food allergens by antigen presenting cells, mainly dendritic cells (DCs), naïve T-cells are instructed to differentiate into allergen-specific Th2 cells (161), which are characterized by the expression of type-2 cytokines including interleukin (IL)-4, IL-5, and IL-13 (162). These cytokines then mediate immune responses to food allergens by supporting B-cell proliferation (163-165), promoting IgE isotype switching, and inducing the activation of mast cells and

basophils (166-169). Given that immune responses to food allergens are complex and involve multiple cell populations, we reasoned that an unbiased systems biology approach could reveal a unique level of insight into the underlying immunological mechanisms. RNA sequencing has been widely used for investigating the pathogenesis of complex human diseases (170), because it enables the systematic study of the molecular states that underpin pathogenic states (171). RNA sequencing data can be analysed with systems biology methods such as weighted gene co-expression network analysis (WGCNA), to elucidate the global architecture of the gene expression program and unmask systems-level properties of the biological systems under study (172). In the current study, we applied an RNA sequencing approach coupled with cell deconvolution and weighted gene co-expression network analysis to identify gene network patterns and associated cellular immune responses in children with or without nut allergy, in order to better understand immunological mechanisms of nut allergy.

4.3 Methods

This study was approved by the Curtin Human Research Ethics Committee (Curtin HREC 10718) and Child and Adolescent Health Service (CAHS) Human Research Ethics Committee (CAHS HREC 2016046EP) and conducted in accordance with the National Health and Medical Research Council National Statement on Ethical Conduct in Human Research. Written informed consent was obtained from all parents on behalf of participants. Our study included a total of 86 individuals (79 children with a nut allergy and 7 children without a nut allergy). Children (aged 1-16 years old) with

suspected or diagnosed allergies to nuts were recruited from the outpatient clinic of the Immunology Department, Perth Children's Hospital. Diagnoses of food allergy were determined by an immunologist on the day of recruitment based on the clinical outcomes of the patients. Venous blood was collected into PAXgene RNA blood tubes (PreAnalytiX, Qiagen, Hilden, Germany) by a trained phlebotomist.

4.3.1 RNA extraction

Total RNA samples were extracted using PAXgene Blood RNA Kit according to the manufacturer's instructions (Qiagen). Total RNA concentration and purity were assessed by determining the A260/280 and A260/230 ratios, respectively (NanoDrop).

4.3.2 Library preparation, RNA sequencing and Quality control (QC)

The library preparation, sequencing read, QC and read alignment of 30 samples were performed at the Australian Genome Research Facility (AGRF). Briefly, the library was prepared using Illumina's TruSeq stranded RNA Library Prep Kit as per the manufacturer's instructions. The library was then sequenced on Illumina HiSeq 2500 platform. The raw reads were processed by the Illumina bcl2fastq 2.20.0.422 pipeline. The sequence reads from all the samples were analysed according to AGRF quality control measures. The cleaned sequence reads were aligned against the Homo sapiens genome (Build version HG38) by the Tophat aligner (v2.1.1). The reads corresponding to each gene were summarized using the featureCounts v1.5.3 utility of the subread package (http://subread.sourceforge.net/).

4.3.3 Gene expression profile of RNA sequencing

The count data were imported, organised, filtered and later normalized based on trimmed mean of M values (TMM) method using edgeR package. Counts were then converted to log2-counts-per-million (logCPM) with precision weights, based on voom method using Limma package of R prior to statistical analysis (173). Empirical Bayes statistics were applied to compute model statistics and calculate log-fold change (log2FC) of differential expressed genes. The cut-off criterion for significant analysis was: $|\log2FC| > 0.3$ and $p < 0.05$.

4.3.4 RT-qPCR

RNA was reverse transcribed to cDNA using the QuantiTect Reverse Transcription Kit (Qiagen, Germany). One μg of the total RNA from each sample was mixed with gDNA wipeout buffer and RNase-free water to prepare annealing mix, followed by incubation at 42 °C for 2 mins. The annealing mix was stored on ice until reverse transcription master mix was added. The final mixture was incubated for 30 min at 42°C and 3 min at 95°C to inactivate reverse transcription reaction before storing at −80°C.

RNA-specific primers for reverse transcription-polymerase chain reactions (RT-qPCR) were designed on the specific region of the genes. Quantitative PCR was performed on a ViiA7 ™ Real-Time PCR System (Thermo Fisher Scientific). qPCR thermal cycling was set as follows: initial denaturation at 95°C for 5min, followed by 40 cycles at 95°C for 10s and 55°C for 30s and a final extension at 95°C for 15s, 60°C for 1min and 95°C for 15s. 18s rRNA was measured as the housekeeping gene for qPCR. The

fold change in the expression of targeted genes was computed using the ΔΔCt method (174).

4.3.5 Quantification of immune cells

The RNA-Seq data was then used to estimate the cellular composition of the whole blood samples using CIBERSORTx (139). CIBERSORTx is a deconvolution algorithm, which can estimate the abundance of different cell types from RNA-Seq data based on cell type specific reference gene expression profiles. CIBERSORTx provides 22 types of functionally defined human immune cells (LM22 signature matrix) as a reference, and we focused on proportions of 12 human immune cells (B cells, CD8+ T-cells, CD4+ T-cells, follicular helper T-cells, Tregs, gamma delta T-cells, NK cells, monocytes, dendritic cells, mast cells, eosinophils and neutrophils). The cell type proportions in children with and without nut allergy were compared employing an independent t-test, and were correlated with coexpression modules derived from WGCNA.

4.3.6 Gene co-expression network construction

Genes with low variation from RNA sequencing data were then filtered out using a coefficient of variation cutoff (CV>0.05). This resulted in the identification of 9462 stably expressed genes in all the samples. Top 5000 genes with high coefficient of variation values were selected for additional analysis.

The selected genes were then used to construct a co-expression network by using the WGCNA (140). First, the soft-thresholding power was calculated by using a scale-free

topology and the soft-thresholding power was selected based on a scale-free topology index (R_2) of 0.85 (Supplemental figure S 4-1). Next, the selected soft-thresholding power of 8 was used to calculate the adjacency matrix, which was then transformed into a Topological Overlap Matrix (TOM). TOM was then used to perform average linkage hierarchical clustering in order to identify modules of highly coexpressed genes. Network modules were subsequently identified using a dynamic tree cut algorithm with a minimum cluster size of 30 and merge cut height of 0.25 (default) and later assigned to clusters of highly co-occurred genes with different colours for visualization.

4.3.7 Module trait relationship construction

Module eigengenes were used to perform principal component analysis of the expression matrix from each module. The correlation between module eigengenes (ME), proportions of immune cells, phenotype as well as demographics traits such as age, gender, and batch effect were calculated using Pearson correlation coefficient. Modules, which have p-value <0.05, were identified to have significant correlations with nut allergy and these modules were selected for further analysis.

4.3.8 Hub Genes Selection and Visualization

Next, an intramodular analysis was performed to determine the hub genes in the selected modules by summing the connection strengths with other module genes. Hub genes were defined based on the standard cut off of module membership (MM) > 0.8

and gene significance (GS) > 0.5. Genes of the significant modules were then visualized using Cytoscape v3.8.0 (175).

4.3.9 Functional enrichment analysis

The biological function and associated pathways enriched in each module was characterized by employing gene ontology (GO) and using InnateDB (141).

4.4 Results

4.4.1 Study Population

A total of 79 whole blood samples (23 whole blood samples for RNA sequencing and 79 whole blood samples for RT-qPCR) were obtained from children with nut allergy, and 7 whole blood samples were obtained from children without nut allergy. Sixty four percent of the subjects were boys, with the median age for children without nut allergy and children with nut allergy of 9.1 years and 9.6 years, respectively. The groups did not significantly differ from each other with regard to age (p=0.794) and gender (p=0.667). Several participants in children with and without nut allergy had self-reported eczema. Clinical diagnoses for environmental allergens were not sought for this study.

4.4.2 Gene expression profiling of whole blood in children with or without nut allergy

We utilized RNA sequencing to assess gene expression patterns on 30 whole blood samples of children with and without nut allergy. RNA sequencing produced a total of 520 million reads with an average of 17 million mapped reads per sample. A total of

12,523 genes were detected in both samples with and without nut allergy. Differential expression analysis adjusted for age, sex, and batch effect identified 184 upregulated genes (log2FC >0.3) and 490 downregulated genes (log2FC <-0.3) (Figure 4-1). However, it is noteworthy that these results were not significant after adjusting for multiple testing.

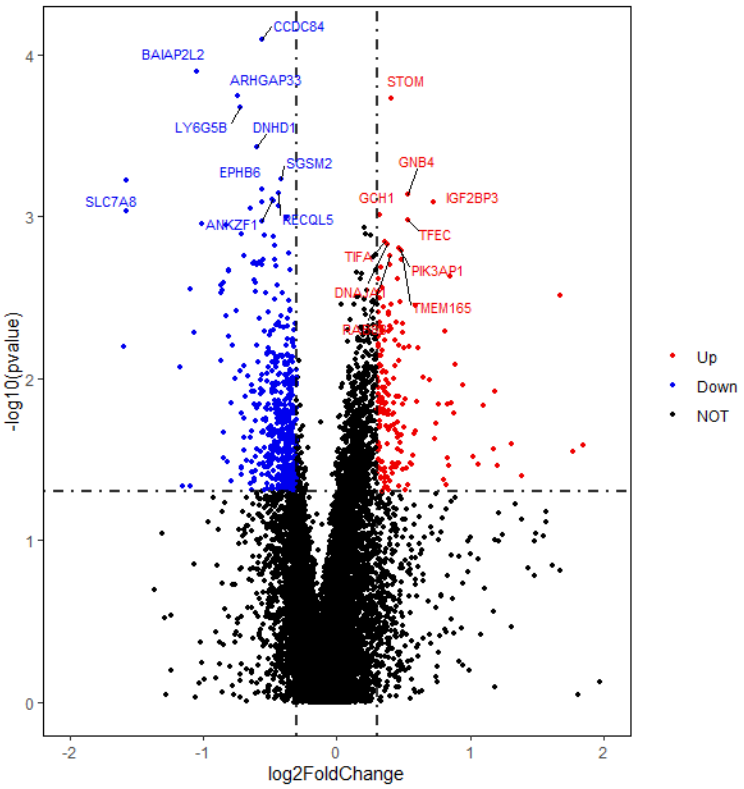


Figure 4-1 The volcano plot for differentially expressed genes. The horizontal axis represents the log2 fold change, and the vertical axis represents the -log10(pvalue). Red denotes upregulated genes with log2FC > 0.3 and p-value < 0.05 while blue denotes downregulated genes with log2FC < -0.3 and p-value < 0.05. The top 15 differentially expressed genes are labelled.

4.4.3 Validation of RNA sequencing results with RT‑qPCR

We selected three genes for validation (*GNB4*, *GASK1B* and *LYSMD2*) by RT-qPCR on a larger cohort of samples according to the following criteria: 1) dysregulated genes based on RNA-seq; 2) High abundance based on logCPM of RNA-seq data; 3)These genes are particularly interesting, associating with infection and inflammation based on literature. We observed the results of RT-qPCR were significantly correlated with RNA sequencing (p<0.01) (Supplemental figure S 4-2). These results suggested that our RNA-Seq data was reliable.

4.4.4 Gene co-expression network construction

We constructed a coexpression network on the top 5000 most variable genes as described in the methods. Through WGCNA, we identified 12 modules of co-expressed genes, and each module comprised between 71 to 1807 genes (Table 4-1). Among the highly variable genes, only 536 genes (10%) were not assigned to any module, and these genes were clustered into the grey module as per default. Pathways analysis of the modules with InnateDb demonstrated that the modules were significantly enriched for coherent biological functions (Table 4-1).

Table 4-1 Modules and associated biological processes

Module colours	**No. of genes**	**Associated biological processes**	**Adjusted p-value**
Black	190	malonyl-CoA biosynthetic process	**0.020**

Blue	643	innate immune response	**<0.001**
Brown	384	negative regulation of dendritic cell differentiation	**0.032**
Green	266	positive regulation of humoral immune response	0.122
Greenyellow	90	chronic inflammatory response to antigenic stimulus	**0.003**
Magenta	132	establishment of T-cell polarity	**0.037**
Pink	187	gene expression	**0.001**
Purple	116	regulation of cytokine secretion	**0.015**
Red	229	IMP biosynthetic process	0.174
Tan	71	type 1 interferon signaling pathway	**<0.001**
Turquoise	1807	transcription, DNA-templated	**<0.001**
Yellow	349	protein import into peroxisome matrix, translocation	0.211

4.4.5 Module trait relationship construction

The module eigengenes were further compared between children with and without nut allergy using module trait association analysis to identify the nut allergy-associated modules. Three modules out of 12 modules were identified to be significantly associated with nut allergy (Figure 4-2), which included tan module (r = 0.43, p = 0.03), purple module (r = 0.4, p = 0.04) and green module (r = -0.48, p = 0.01). The upregulated modules (tan and purple modules), were negatively correlated to CD4+ T-cells and positively correlated to neutrophils. In addition, the purple module was also negatively correlated to Tregs. In contrast, the downregulated module (green module)

was positively correlated to CD4+ T-cells and Tregs as well as negatively correlated to neutrophils. However, two modules that were not significantly associated with nut allergy (red and greenyellow modules) were also observed to be strongly correlated with neutrophils.

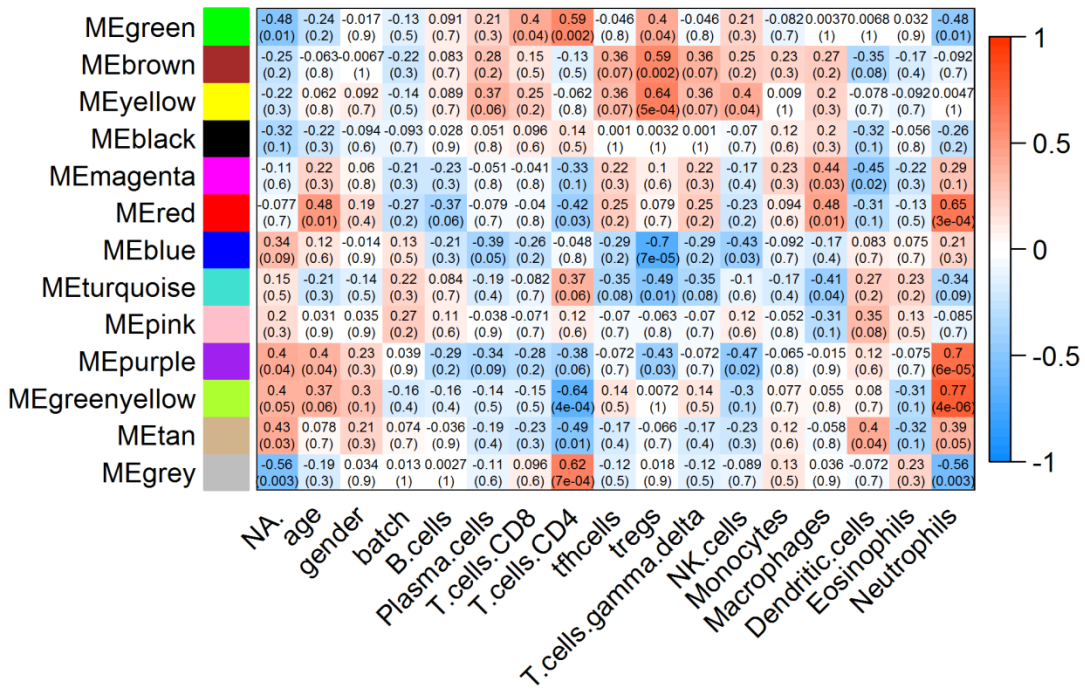


Figure 4-2 Module-trait associations. Each row corresponds to a module eigengene (ME) while each column corresponds to either phenotype (NA: nut allergy) or demographic traits such as age and gender. Each cell contains the corresponding correlation coefficient (display at the top of the cell) and corresponding p-values for each module (display at the bottom of the cells within parentheses). Blue and red colours of the spectrum on the right denote low and high correlation, respectively.

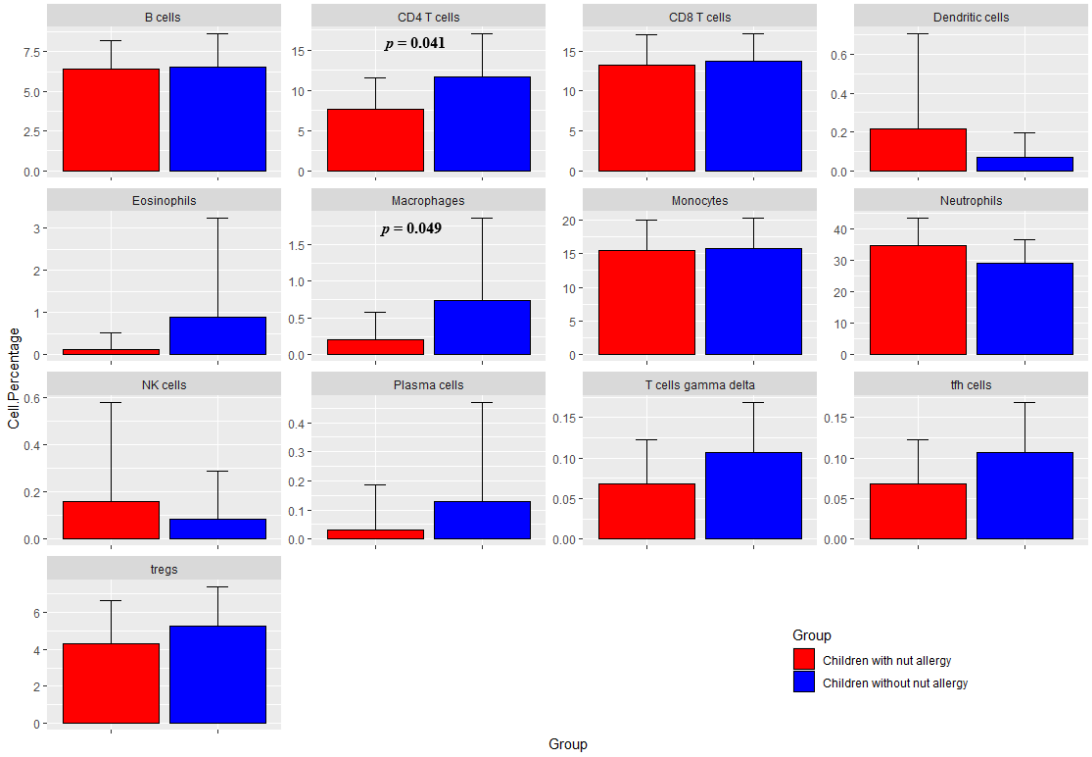


Figure 4-3 Differential immune cell type expression was observed between children with and without nut allergy. Blue colour represents children without nut allergy, while red colour represents children with nut allergy.

The proportions of the 12 human immune cells in children with or without nut allergy are shown in Figure 4-3. The cellular composition was dominated by neutrophils, Monocytes, CD8+ T- cells, CD4+ T-cells, B cells and Tregs. Among all the cell types, CD4+ T-cells and macrophages were observed to have a significant difference between children with and without nut allergy.

4.4.6 Significant modules and associated biological process

The Tan module was revealed to have the highest positive correlation with nut allergy. In the tan module, 71 genes were identified to be correlated with food allergy and these genes were found to be involved in type 1 interferon signalling pathway. Particularly, *IFIH1* was identified as a hub gene of the tan module (GeneSignificance > 0.5 and Module Membership > 0.8, Figure 4-4).

The purple module was also positively correlated with nut allergy. In the purple module, 116 genes were identified to be correlated with food allergy and these genes were found to be involved in the regulation of cytokine secretion. Particularly, *DRAM1* was identified as a hub gene within the purple module (GeneSignificance > 0.5 and Module Membership > 0.8, Figure 4-4).

In contrast, the green module was found to have a negative correlation with nut allergy. In the green module, 266 genes were identified to be correlated with food allergy and these genes were found to be involved in the positive regulation of humoral immune response. Particularly, *ZNF512B* was identified as a hub gene of the green module (GeneSignificance > 0.5 and Module Membership > 0.8, Figure 4-4).

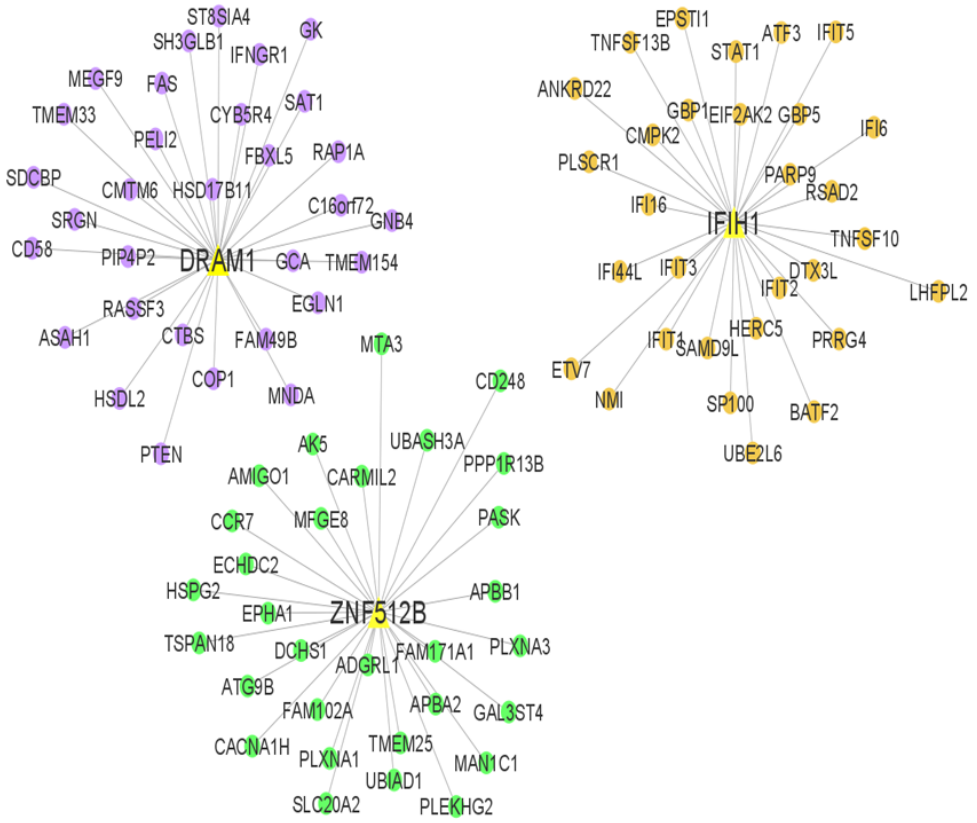


Figure 4-4 Co-expression network of top 30 genes in 3 distinct modules (purple, tan and green), hub gene is indicated with yellow triangle shapes and other linked genes are in round shapes and colour coded according to the module colour.

4.5 Discussion

Nut allergy can cause severe, life-threatening anaphylactic reactions. Here, we profiled the transcriptome of immune cells to provide a window into the regulation of immune function in the context of food allergy and anaphylaxis. The application of WGCNA identifies the global connectivity structure of the gene expression program, and unmasks systems-level features of the underlying biology (176, 177). Herein, we

analysed gene expression profiles obtained from whole blood transcriptome of children with and without nut allergy using WGCNA to identify molecular and cellular immune features associated with nut allergy**.** In the present study, we identified upregulation of the type I interferon production (tan) and cytokine production (purple) modules and downregulation of the humoral immune responses (green) module in children with nut allergy. These changes were positively correlated with proportions of neutrophils and negatively correlated with proportions of CD4 T cell/Treg. The upregulated modules were characterized by hub genes, namely *IFIH1*, *DRAM1*, which were involved in type 1 interferon signalling pathway and the regulation of cytokine secretion while the downregulated module was characterized by a hub gene, namely *ZNF512B*, which was involved in the positive regulation of humoral immune response. Together, our data suggest that the nut allergy is associated with the upregulation of type 1 interferon and neutrophil responses, and downregulation of CD4 T cell/Treg responses. Consistent with our studies, previous studies have also identified the enrichment of type 1 interferons in subjects with allergic diseases compared to control (90, 178). The exposure to allergens activates toll like receptors, which in turn mediates the production of type 1 interferons, inducing phosphorylation of STAT1 and inducing expression of pro-inflammatory cytokines (178).

A limitation of gene expression profiles obtained from whole blood transcriptome is that the data are potentially confounded by variations in cellular composition. The integration of the co-expression network and cell deconvolution approaches allowed us to link co-expression patterns within each module with specific immune cells.

CD4+ T-cells and Tregs are already the focus of investigation in studies of food allergy (88, 91, 92). Mouse models of food allergy demonstrated a critical role for CD4+Foxp3+ Treg cells in suppressing food allergy. These studies observed a significant increase in the percentage of CD4+Foxp3+ Treg cells in ovalbumin sensitized mice with mucosal tolerance induction compared to the intolerant group (126, 179, 180). In addition, CD4+Foxp3+ Treg cells were found to inhibit the activation of dendritic cells, mast cells, basophils, and eosinophils, suppress the production of allergen-specific IgE, inhibit Th1, Th2, and Th17 migration patterns and effector functions as well as promote the secretion of IgG4 (181). Consistent with these studies, we also observed a downregulation of CD4+ T-cell and Treg responses in the pathogenesis of nut allergy.

Our analysis also pointed to a possible contribution of neutrophils to the pathogenesis of nut allergy. However, we also observed a significant association of neutrophils with other modules that were not associated with nut allergy. These contrasting results could be due to the heterogeneity of neutrophils as previous studies have revealed variations in phenotype and functions of neutrophils in the development of allergic diseases. On the one hand, neutrophil Fc gamma receptors, FcγRIIIA and FcγRIV as well as several markers of neutrophil activation, *S100A8*, *S100A9*, *TLR4*, *TREM1*, *S100A9* (97) and *interleukin 17A* (99), are shown to induce acute anaphylaxis (95, 96). On the other hand, neutrophils are revealed to be a source of anti-inflammatory and immunomodulatory cytokines, such as interleukin-10 (182). Moreover, neutrophils can suppress NF-kB activation in the macrophage, which in turn reduces the

expression of pro-inflammatory cytokines (tumor necrosis factor, chemokine ligand 8 and interleukin-6), leading to the resolution of inflammation (183). Considering the heterogeneity of neutrophils and only limited information is available on the roles of neutrophils, our findings require further research on neutrophils-related gene networks with nut allergy.

Our study adds to the growing body of literature describing the association of upregulation of type 1 interferon and neutrophil responses, and downregulation of CD4 T cell/Treg responses with food allergy, particularly nut allergy by suggesting these immune cells responses were not driven by single genes, but networks of co-regulated genes via direct/ indirect interactions with *IFIH1*, *DRAM1* and *ZNF512B*. However, our results should be interpreted with caution due to the following limitations: First, the sample sizes used for RNA sequencing are small (n=30), but gene expression levels of three selected genes are validated using RT-qPCR in a larger sample (n=86). Second, it is not known if the observed patterns of gene expression are related to the mechanisms that drive nut allergy or alternatively are reacting to nut allergy due to the cross-sectional nature of our study. Third, our study only focused on nut allergy, and therefore it is not known if the mechanisms we identified are relevant to other food allergies. Fourth, flow cytometry-based assays to target multiple cell populations were not feasible due to the volume restriction on blood collection from children. However, we have applied an unbiased deconvolution approach to infer the proportions of 12 human cells in whole blood transcriptome. In addition, our study does not define whether changes in cellular proportions precede or follow transcriptomic changes.

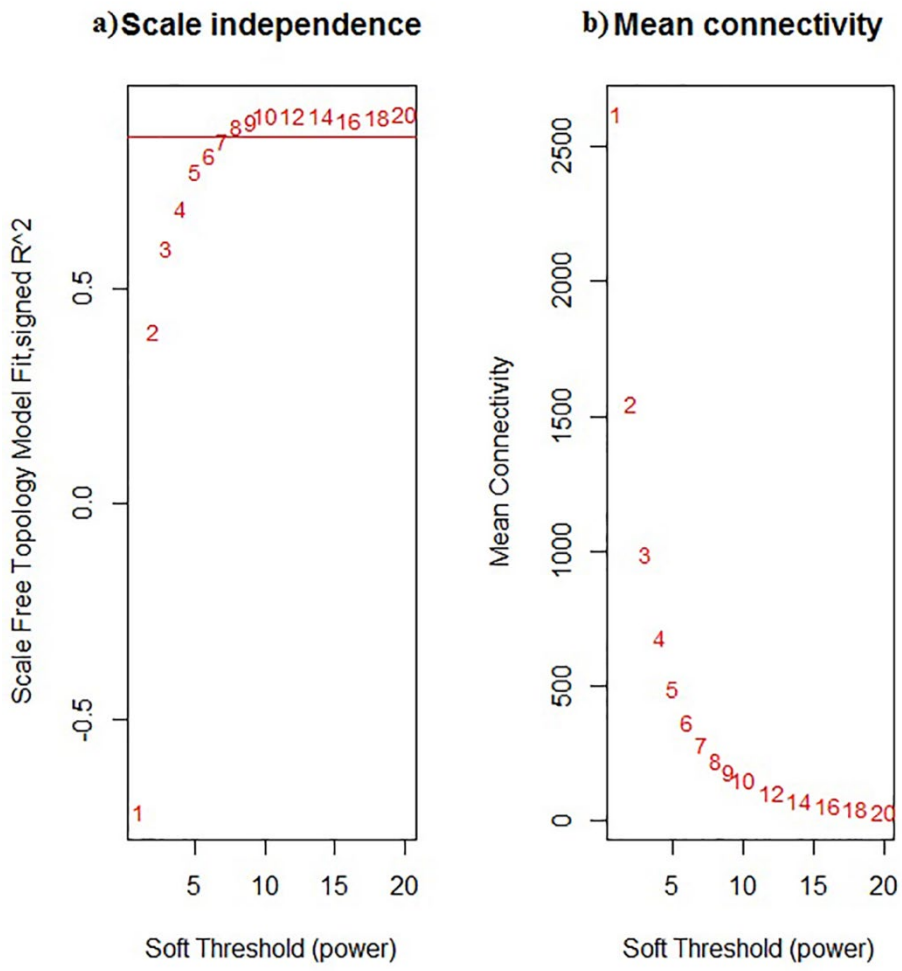
Further experiments are needed to identify the cellular origin of the gene expression signals associated with nut allergy.

4.6 Conclusion

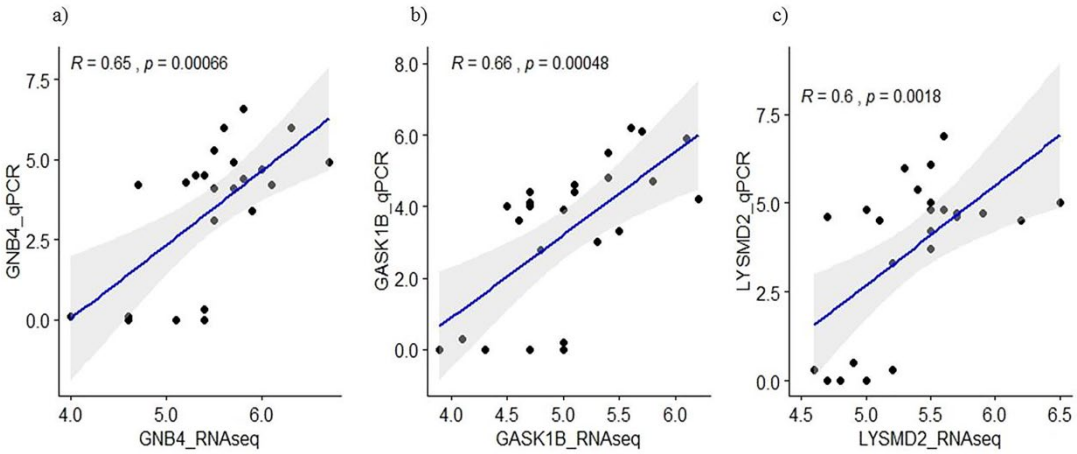
To conclude, our study identified upregulation of two gene network modules and downregulation of one gene network module in children with nut allergy. We identified neutrophils, CD4+ T-cells and Tregs to be highly correlated with nut allergy. We also identified *IFIH1*, *DRAM1* and *ZNF512B* as hub genes for nut allergy and these hub genes were related to type 1 interferon signaling pathways, cytokine signaling, and positive regulation of humoral immune responses, respectively. Our findings may provide a valuable reference for further elucidation of the mechanisms of nut allergy. Furthermore, these hub genes and immune-related cells may become important therapeutic targets for treating nut allergy.

4.7 Supplemental Information

4.7.1 Supplemental figures



Supplemental figure S 4-1 Determination of soft-thresholding power in the WGCNA. (a) The plot shows the scale-free topology fit index (y-axis) for different soft-thresholding powers (β) (x-axis). The higher the RΛ2 value, the closer to scale-free topology. Scale-free topology at β=8 (b) Analysis of the mean connectivity (degree, y-axis) for various soft-thresholding powers (x-axis).



Supplemental figure S 4-2 Pearson correlation between expressions of RNA Sequencing and RT-qPCR in a) GNB4, b) GASK1B, c) LYSMD2 (*P*<0.01).

5 DYSFUNCTIONAL GUT MICROBIOME NETWORKS IN CHILDHOOD IGE-MEDIATED FOOD ALLERGY

This chapter described Aim4, the aim of which was to identify gut microbiome and pathways associated with food allergy. This Chapter is reformatted from Lee, K.H., Guo, J., Song, Y. et al. Dysfunctional Gut Microbiome Networks in Childhood IgE-Mediated Food Allergy. Int J Mol Sci 22, 4 (2021). https://doi.org/10.3390/ijms22042079, published under a Creative Commons BY license (http://creativecommons.org/licenses/by/4.0/)

5.1 Abstract

The development of food allergy has been reported to be related with the changes in the gut microbiome, however the specific microbe(s) associated with food allergy remains elusive. This study aimed to comprehensively characterize the gut microbiome and identify individual or group gut microbes relating to food-allergy using 16S rRNA gene sequencing with network analysis. Faecal samples were collected from children with IgE-mediated food allergies (n=33) and without food allergy (n=27). Gut microbiome was profiled by 16S rRNA gene sequencing. OTUs obtained from 16S rRNA gene sequencing were then used to construct a co-abundance network using Weighted Gene Co-expression Network Analysis (WGCNA) and mapped onto Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. We identified a co-abundance network module to be positively correlated with IgE-mediated food allergy and this module was characterized by a hub taxon, namely *Ruminococcaceae UCG-002* (phylum Firmicutes). Functional pathway analysis of all the gut microbiome showed enrichment of methane metabolism and glycerolipid metabolism in the gut microbiome of food-allergic children and enrichment of ubiquinone and other terpenoid-quinone biosynthesis in the gut microbiome of non-food allergic children. We concluded that *Ruminococcaceae UCG-002* may play determinant roles in gut microbial community structure and function leading to IgE-mediated food allergy.

5.2 Introduction

Emerging evidences has pointed towards the critical role of microbial communities in human health and disease, including regulation of the mucosal barrier function (184-187), metabolism (188-190) and host immune responses (186, 187, 191). This is particularly evident in the gastrointestinal (GI) tract, where the diversity and richness of microorganisms are highest (192). Gut dysbiosis, which is defined by a lack of short-chain fatty acids/butyrate-producing bacteria, in particular members of the phylum Firmicutes, is commonly associated with low levels of short-chain fatty acids. The low levels of short-chain fatty acids may reduce the tolerogenic CD103+ DC function (114), decrease the percentage of T regulatory cells (Tregs) (114), decrease the production of IgA (114), increase histone deacetylases activity (193), increase the production of pro-inflammatory cytokines (IL-1β, IL-6, IL-17) (110), and decrease the secretion of anti-inflammatory cytokines (IL-10) (110), all leading to the development of food allergy.

Previous studies have started to unveil an association between the gut microbiome and food allergy. A large observational cohort study in the United States showed that food-allergic children had a higher abundance of Bacteroidetes and a lower abundance of Firmicutes than children with resolved food allergy (109), while some studies showed the opposite results (106, 107).

Considering the complexity of structure, function and compositional variability, the gut microbiome can be modelled and expressed as networks to infer the dynamic nature of the host–microbe interactions (101). One approach to construct co-

abundance network modules is to apply weighted gene co-expression network analysis (WGCNA) to quantify the co-abundance of operational taxonomic units (OTUs) across multiple samples. Developed by Horvath and colleagues, WGCNA was initially used to construct gene networks based on their similar biological functions and identify the hub gene that may associated with phenotypic traits (140). We used WGCNA in this study to analyse the association between gut microbiome and disease phenotype by forming the complex microbial communities into different co-abundance network modules in order to identify hub taxa, the centralities of these co-abundance modules. Through this, we expect that WGCNA will identify potential target microbes, which may play a key role in regulating/ influencing the microbe-microbe interactions, leading to the onset of food allergy.

5.3 Methods

5.3.1 Study subject

From January 2018 to March 2019, children with immunologist-diagnosed food allergy were recruited from Immunology Outpatient Clinic, Perth Children's Hospital. Children from 1 year old to 7 years of age with immunologist-diagnosed food allergy were eligible for participation. Non-food allergic children, with age and gender matched were recruited from the local community.

All parents of the subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the National Health and Medical Research Council National Statement on Ethical Conduct in

Human Research, and the protocol was approved by the Human Research Ethics Committee (HREC), Perth Children's Hospital (RGS151 / HREC 2017060EP) and Curtin University (HRE2017-0712).

5.3.2 Faecal sample collection and processing

Parents/ guardians of the participants were provided a faecal collection kit, which included a protocol of faecal collection, a screw cap faecal container (Sarstedt, Germany), an underpad sheet, a pair of disposable gloves, a white paper bag and a sealed plastic bag with labels. Once collected, the faecal sample would then be transported on ice by a researcher within 2 hours of collection to the laboratory -80°C freezers for storage.

DNA was then extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Germany) in accordance with the manufacturer's instructions. The PCR amplication and sequencing of sixty stool samples were performed by Novogene Bioinformatics Technology Co., Ltd (Beijing, China). Briefly, PCR was carried out using Phusion® High-Fidelity PCR Master Mix and GC Buffer (New England Biolabs, Beijing, China) in accordance with the manufacturer's instruction. PCR thermal cycling was set as follows: initial denaturation at 98°C for 1min, followed by 35 cycles at 98°C for 10 s, 50°C for 30 s and 72°C for 90 s, and a final extension at 72°C for 5 min. The samples were then subjected to electrophoresis on a 2% agarose gel for detection. Samples with a bright main strip between 400 and 450 bp were chosen for further analysis. The PCR products were purified using the Gene JET Gel Extraction kit (Thermo Scientific), and

the sequencing libraries were constructed using Ion Plus Fragment Library Kit (Thermo Fisher Scientific, USA) in accordance with the manufacturer's instruction. The library quality was monitored using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, St. Louis, MO, USA) and a Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA). Lastly, the library, which targeted the V3-V4 region of the 16S rRNA gene was sequenced on the Ion S5 XL platform (Thermo Fisher). A total of 4,858,507 sequences reads that passed the quality check (>Q20, error rate < 1%) were generated.

5.3.3 Quantitative Insights into Microbial Ecology (QIIME)

The raw sequences were then demultiplexed and quality filtered using QIIME (194) by removing those raw sequences with read-quality score less than 19, setting length fall below 3bp and consecutive quality base below 75%. The filtered sequences were then screened for chimeras using the usearch61 algorithm (195) and putative chimeric sequences were removed from the data set. Sequences were clustered into operational taxonomic units (OTUs) at a 97% similarity level against the SILVA reference database (release 128) (196). The OTUs with low relative abundance (less than 0.005%) were removed. All further analyses were performed at a rarefied depth of 22178 sequences per sample to correct for differences in the read depth across samples.

Alpha diversity and beta diversity of microbial communities were analysed using QIIME. Alpha diversity was estimated using two different indices: 1) Chao1, which takes into accounts only the abundance; 2) observed OTUs, which takes into accounts

only the observed species; 3) the Shannon index, which takes into accounts the abundance and evenness of OTUs. Beta diversity was measured using the weighted and unweighted UniFrac distance matrices. Principal Coordinate Analysis (PCoA) was obtained to visualise unweighted and weighted Unifrac distances in a two-dimensional structure. The Adonis permutational multivariate analysis (Adonis/PERMANOVA) was performed to compare beta diversity dissimilarity matrices. A comparison of the relative abundance of OTUs between groups was computed using the Mann Whitney test. A probability value of $p< 0.05$ was considered statistically significant.

5.3.4 Construction of microbial co-abundance network

In order to have a better understanding of the co-abundance network of the microbial taxa, Weighted Gene Correlation Network Analysis (WGCNA) package of R (140) was then performed to conduct network analysis by using OTU count data (with 97% identity threshold), which has undergone Hellinger transformation, by transforming OTU count data from absolute to relative abundance that gives low weights to variables with low counts and many zeros (197).

Taking into account that the use of correlation analysis in analysing the microbiome data can lead to a spurious association, WGCNA applied few steps to reduce the number of false positive connections introduced by spurious associations. A soft thresholding power β was determined based on scale-free topology index (R2) of 0.85. The most appropriate soft thresholding power was then used to construct a weighted adjacency matrix to which the co-abundance similarity has been raised. By raising the

absolute value of the correlation to a soft thresholding power ($\beta \geqslant 1$), this step emphasized a strong correlation coefficient. Then, to further minimize the effects of noise and spurious associations, the adjacency matrix was transformed into a topological overlap matrix and the corresponding dissimilarity was calculated. This topological overlap matrix was particularly useful when the original adjacency matrix was sparse or susceptible to noise by replacing the isolated connections with weighted neighbourhood overlaps, thus, reducing the effects of spurious associations leading to a more robust network. The modules were subsequently identified using a dynamic tree cut algorithm with a minimum cluster size of 30 and merge cut height of 0.25 and later assigned the clusters of highly co-occurred taxa to different colours for visualization.

After that, module trait association analysis was used to calculate the correlation coefficient between modules and food allergy as well as demographics traits such as age and gender. Modules with P values < 0.05 were regarded significant food allergy-related modules.

5.3.5 Hub taxa selection and visualization

Next, an intramodular analysis was performed to determine the hub taxa by summing the connection strengths with other module taxa. Moreover, the hub taxa have to meet the absolute value of the TaxaSignificance > 0.2 and Module Membership (MM) > 0.8. Taxa of the significant modules were then visualized using Cytoscape v3.8.0 (198).

5.3.6 Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Analysis

All OTUs table and OTUs taxonomy were mapped onto Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways using R package, Tax4Fun. Linear discriminant analysis (LDA) effect size (LEfSe) analysis (http://huttenhower.sph.harvard.edu/lefse/) was performed to detect biomarkers of the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways that differed significantly between non-food allergic children and food-allergic children. Default settings (alpha = 0.05, effect-size threshold of 2) were applied.

5.4 Result

5.4.1 Gut microbial alpha diversity

A total of 60 samples were included in our final analysis (33 food-allergic children and 27 non-food allergic children). Thirty-nine percent of the subjects were boys, with the median age for non-food allergic children and food-allergic children of 5.9 years and 5.0 years, respectively. The groups did not significantly differ from each other with regard to age (p=0.200) and gender (p=0.525). The food allergies noted in the food-allergic children included nuts (n = 23), egg (n = 4) and mixed allergies (n = 6).

To determine the average species diversity in a habitat or specific area, alpha diversity was evaluated using Chao1, Shannon index and observed OTUs matrices. Chao1 showed that non-food allergic children had lower species richness compared to food-allergic children, while Shannon index and observed OTUs showed that non-food allergic children and food-allergic children had similar gut microbial community richness and evenness (Table 5-1).

Table 5-1 Comparison of gut microbial alpha diversity between food-allergic children and non-food allergic children. Values represent mean ± SD.

	Non-food allergic children	**Food-allergic children**	***p***
Chao1	565.7±91.7	622.3±87.4	**0.020**
Observed OTUs	458.9±86.0	502.8±83.9	0.058
Shannon diversity index	5.3±0.7	5.5±0.7	0.395

5.4.2 Gut microbial beta diversity

To determine the degree of inter-group dissimilarity, beta diversity was evaluated using unweighted and weighted UniFrac distance matrices. Beta diversity did not show a significant difference between food-allergic children and non-food allergic children (Supplemental figure S 5-1).

5.4.3 Gut microbial composition

OTU dataset for food-allergic children and non-food allergic children consisted of 7 phyla, 14 classes, 16 orders, 28 families and 105 genera. At the phyla level, the gut microbiota was dominated by Firmicutes and Bacteroidetes, with lower abundance of Proteobacteria, Verrucomicrobia, Actinobacteria, Tenericutes and Cyanobacteria (Supplemental figure S 5-2) in children with and without food allergy. There was no significant difference in the phylum level between food-allergic children and non-food allergic children (Supplemental Table S 5-1).

One hundred and five genera were identified, and only 18 genera were accounted for more than 1% across all samples (Supplemental Table S 5-2). There was no significant difference in the genera level between food-allergic children and non-food allergic children.

5.4.4 Microbial co-abundance network

To better characterize gut microbial taxa in food-allergic children, we applied WGCNA to identify clusters of microbial taxa whose differential representation was correlated with food allergy. Each cluster was represented as a colour module.

Through WGCNA, we were able to identify 14 modules of co-abundant taxa and the number of taxa within modules ranged from 32 to 167 (Table 5-2). Among all the taxa, only 167 taxa (17%) were not included in any colour module, and these taxa were grouped into the grey module as per default.

Table 5-2 The number of taxa in the 14 modules

Module colours	**Freq**
Black	54
Blue	88
Brown	88
Green	67
green-yellow	34
Grey	167

Magenta	48
Pink	51
Purple	47
Red	66
Salmon	32
Tan	33
Turquoise	114
Yellow	82

5.4.5 Hub taxa associated with food allergy

The module eigengenes between children with and without food allergy were further compared with using module trait association analysis to identify the food allergy-associated modules.

Our results showed that a co-abundance network module (turquoise) was positively correlated with food allergy (r = 0.27 p = 0.04) (Figure 5-1). Particularly, *Ruminococcaceae UCG-002* was identified as the hub taxa (TaxaSignificance > 0.2 and Module Membership > 0.8) (Figure 5-2) for this module. In addition, 10 dominant taxa (> 1% relative abundance across all samples) were also identified in the module. The majority of the dominant taxa came from Firmicutes phylum, including the genera of *Ruminococcaceae UCG-002*, *Eubacterium oxidoreducens group*, *Eubacterium coprostanoligenes group* and *Lachnospiraceae* (*NK4A136* and *UCG-008*). Other than this, the dominant taxa also included genera taxa from the phyla of Bacteroidetes

(*Bacteroides*, *Alistipes*, *Parabacteroides* and *Prevotella 2*) as well as Proteobacteria (*Rhodospirillaceae*).

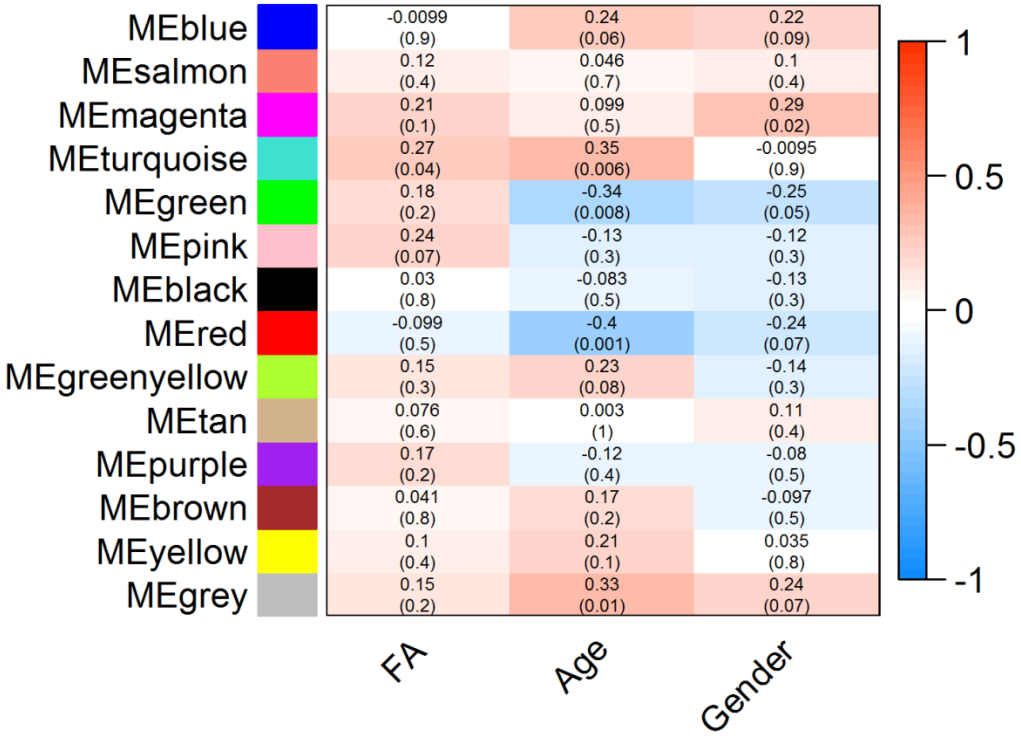


Figure 5-1 Module-trait associations. Each row corresponds to a module eigengene (ME) while each column corresponds to either phenotype (FA: food allergy) or demographic traits such as age and gender. Each cell contains the corresponding correlation coefficient (display at the top of the cell) and corresponding p-values for each module (display at the bottom of the cells within parentheses). Blue and red colours of the spectrum on the right denote low and high correlation, respectively.

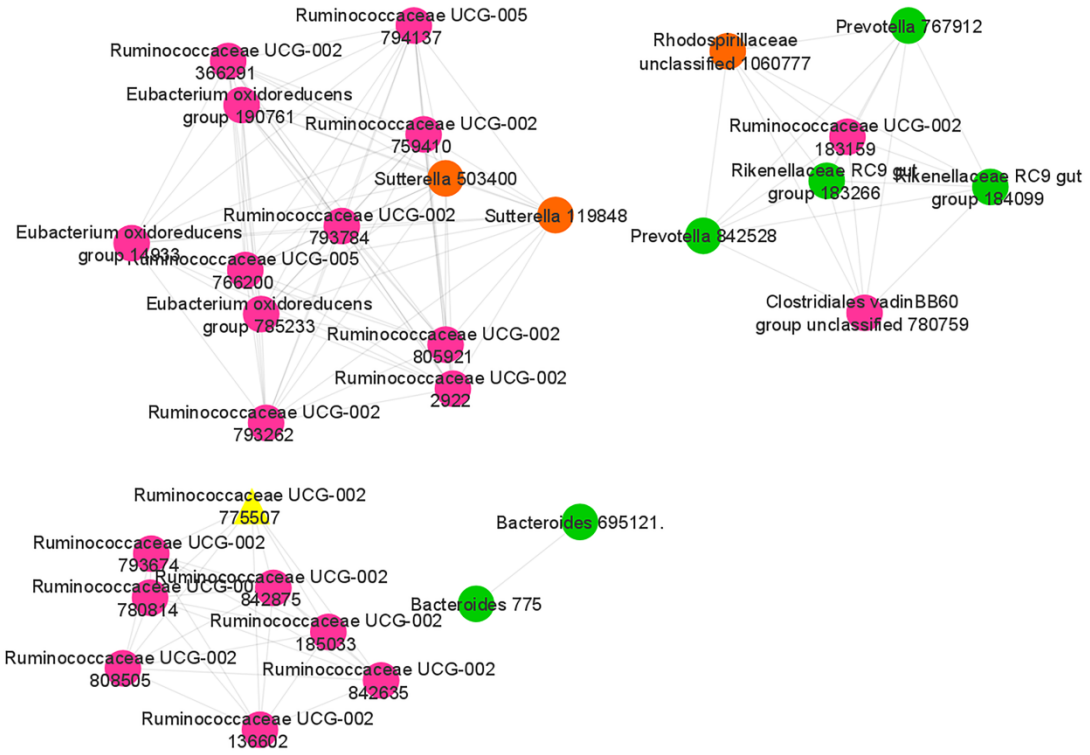


Figure 5-2 Network analysis identifies a distinct module of co-associated taxa. The highly correlated taxa in the comparisons of food allergic children and non-food allergic children are indicated and colour coded according to the phylum. Green colour represents Bacteroidetes phylum, pink colour represents Firmicutes phylum while orange colour represents Proteobacteria phylum. Hub taxon is indicated with yellow triangle shape and other connector taxa are in round shapes.

5.4.6 Predicted functional pathway of gut microbial taxa associated with food allergy

In order to have a better understanding of the functional pathway of gut microbial taxa that are associated with food allergy, linear discriminant analysis effect size (LEfSe) was performed by using the Tax4fun output. Using the threshold values (LDA > 2.0, $p < 0.05$), LEfSe revealed distinct KEGG pathway differences between gut microbiota of food-allergic children and non-food allergic children (Figure 5-3). Specifically, methane metabolism and glycerolipid metabolism were found to be enriched in food-allergic children. In contrast, ubiquinone and other terpenoid-quinone biosynthesis, as well as *Vibrio cholerae* pathogenic cycle were found to be enriched in non-food allergic children.

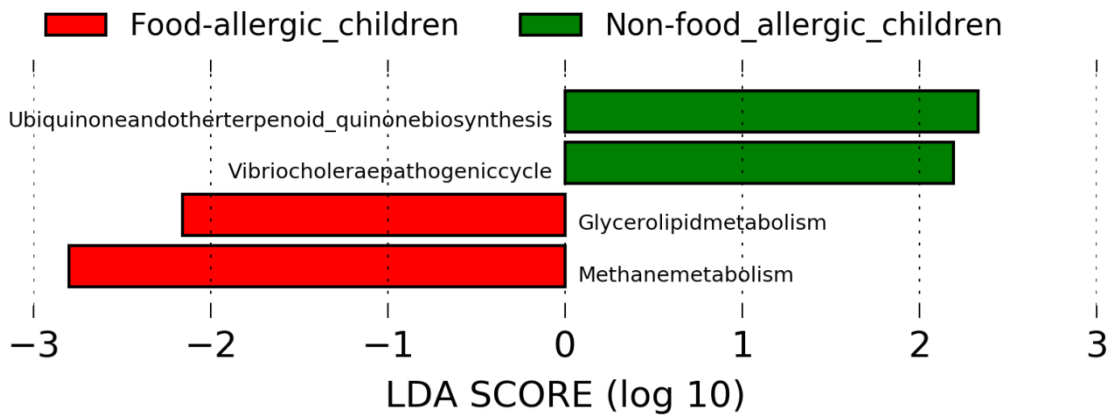


Figure 5-3 LEfSe analysis revealed distinct KEGG pathway differences in gut microbiota between food-allergic children and non-food allergic children. KEGG pathway enriched in food-allergic children was indicated with red while the KEGG pathway enriched in non-food allergic children was indicated with green. Only the taxa that met a LDA significant threshold of >2 are displayed. LEfSe:

Linear discriminant analysis effect size. LDA: Linear discriminant analysis. KEGG: Kyoto Encyclopedia of Genes and Genomes.

5.5 Discussion

There is increasing evidence that alterations in the gut microbiome are related to the development of food allergy (106, 107, 109, 199), although the specific microbe associated with food allergy remains elusive. Our objective for this study was to perform 16S rRNA gene sequencing in integration with network analysis to characterize the gut microbiome and identify individual gut microbes or network modules of them that differ between food-allergic children and non-food allergic children. To our knowledge, this is the first study to characterize the gut microbiome of food-allergic children by applying network analysis.

Through network analysis, we identified a co-abundance network module (turquoise) to be positively correlated with food allergy and this module was characterized by a hub taxa, *Ruminococcaceae UCG-002* (Firmicutes phylum). Our finding is consistent with the results of previous studies demonstrating that a high relative abundance of *Ruminococcaceae* is associated with both food allergies (107), and high fat diet in murine models (200-202), a factor which is known for its association with food allergy. Taken together, these findings suggest that the high relative abundance of *Ruminococcaceae,* induced by a high fat diet, may produce acetic and propionic acid that promote the synthesis of lipogenesis and cholesterol (203), which in turn cause

disruption of intestinal effector mast cell responses as well as induction of intestinal permeability and gut dysbiosis (204), leading to exacerbations of allergic responses.

We also identified a number of dominant taxa in this co-abundance network module that were highly related with food allergy, with the majority of them coming from phylum Firmicutes. Firmicutes has been suggested to play a role in modulating the immune system and subsequent development of allergic diseases (106, 205). A case-control study was conducted to investigate the association of gut microbiome and food allergy by comparing the gut microbiota composition between 34 infants with food allergy and 45 healthy controls (106). The data revealed that the relative abundance of Firmicutes in food-allergic subjects was higher than that of the control subjects. Another study conducted by Chen et al. (205) also showed that Firmicutes was enriched in food–sensitized children.

The enrichment of pathways related to methane metabolism and glycerolipid metabolism (a subcategory of lipid metabolism) in the gut microbiome of food-allergic children was observed. However, KEGG pathways related to metabolism of cofactors and vitamins (ubiquinone and other terpenoid-quinone biosynthesis) was significantly enriched in the gut microbiome of non-food allergic children. Methane is the anaerobic fermentation product of endogenous and exogenous carbohydrates through intestinal microbiota (206). The increase production of methane caused by high fat diet (207) may cause gastrointestinal disorders (206, 208). Our finding of enriched glycerolipid metabolism in food-allergic children was consistent with recognized roles of dietary lipid in regulating inflammation and food allergy (204, 209). A high-fat diet has been

previously shown to change gut microbiota composition, leading to inflammation and food-allergic reactions. In contrast, the key role of ubiquinone in protecting against food allergy has been gaining attention lately. The deficiency of coenzyme Q10, which is a kind of ubiquinone, may develop and worsen the progress of food allergy in children (210).

Our finding of increased gut microbiota diversity in food-allergic children when compared with non-food allergic children appears contrary to several other food allergy studies, in which gut microbiota diversity was higher in healthy controls than food-allergic subjects. However, a study conducted by Fazlollahi et al (107) has also shown that gut microbiota diversity could be higher in children with egg allergy compared to controls. Some other studies reported no association between gut microbiota diversity and food allergy (106, 211). This has indicated a subtle relationship between gut microbiota diversity and food allergy. Hence, the role of microbiome in food allergy was suggested to be considered along with the interplay between different taxa and their metabolic effects rather than only examining a single dimension, bacterial diversity.

Taken together, we speculate that that increased abundance of *Ruminococcaceae* along with other dominant microbial taxa, may remodel the normal gut microbial ecosystem into a state of dysbiosis through the pathways of methane metabolism and glycerolipid metabolism, which in turn may elicit a host IgE-mediated allergic response. Our findings highlight the usefulness of network analysis in disentangling the hub taxa, *Ruminococcaceae* that may play determinant roles in gut microbial community

structure and functions leading to IgE-mediated food allergy. The differences in the co-abundance patterns of gut microbiome between children with and without food allergy may help us to understand the complex interrelationships between gut microbiome and food allergies. This information potentially aids targeted dietary or probiotic strategies for clinical practice to improve food allergy outcomes. Although our study revealed there was an association between gut microbiome network and food allergy, there were several limitations in the study. Firstly, the sample size was small. However, the application of network analysis in our study has deciphered key microbial populations that may be associated with food allergy, including those with low relative abundance but highly relevant to the onset of food allergy through characterizing the interactions of microbes at the community scale. Secondly, 16S rRNA gene sequencing is only sensitive to the genus level, but not species and strains. Thirdly, as this was a cross-sectional study, our results could not indicate a causal relationship between the gut microbiome and food allergy. Finally, as our study aimed to construct a microbial network through 16S rRNA gene sequencing and weighted correlation network analysis, the actual roles of these taxa predicted to be related to food allergy have not yet been evaluated. Therefore, further studies utilizing metagenomic analysis or real-time PCR in larger cohorts are required to confirm our results.

5.6 Conclusion

Our study provides a better understanding of the gut microbiome with respect to the presence of *Ruminococcaceae UCG-002* interacting with other dominant taxa including *Eubacterium oxidoreducens group*, *Eubacterium coprostanoligenes group*, *Lachnospiraceae* (*NK4A136* and *UCG-008*), *Bacteroides*, *Alistipes*, *Parabacteroides*, *Prevotella 2* as well as *Rhodospirillaceae* and these interactions are associated with food allergy. Integrative view of gut microbial ecology in our study may help to understand the microbial interactions associated with IgE-mediated food allergy.

5.7 Supplemental Information

5.7.1 Supplemental tables

Supplemental Table S 5-1 The comparison of gut microbiota at the phyla level between food-allergic children and non-food allergic children.

Phylum	**Non-food allergic children**	**Food-allergic children**	***p***
Proteobacteria	4.55	3.08	0.071
Tenericutes	0.01	0.17	0.127
Firmicutes	38.9	44.84	0.169
Actinobacteria	0.35	0.26	0.231
Bacteroidetes	55.77	51.19	0.274
Verrucomicrobia	0.38	0.41	0.312

Cyanobacteria	0.01	0.02	0.51

Values expressed as relative abundance (%).

p: p-value.

Supplemental Table S 5-2 Relative abundance of predominant genera in gut microbiota between food-allergic children and non-food allergic children (⩾ 1% across all samples)

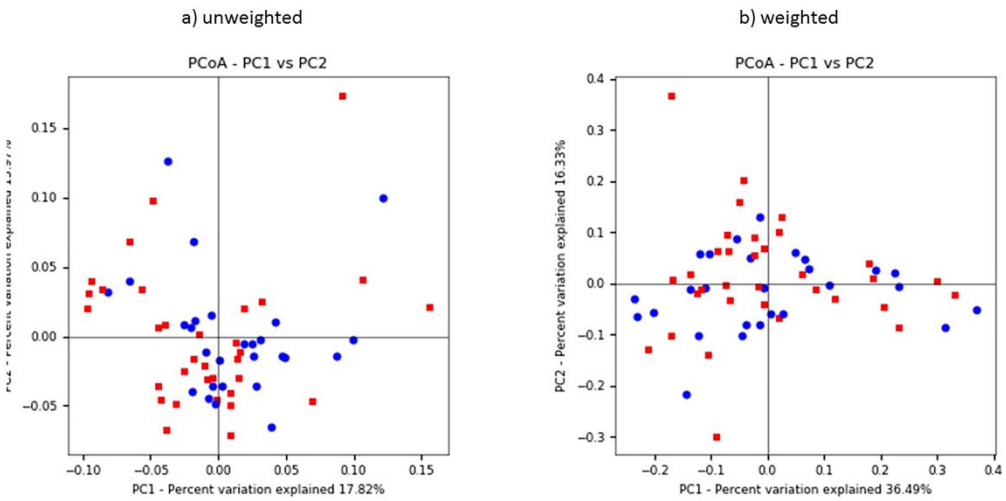
Genus	**Non-food allergic children**	**Food-allergic children**	***p***
Ruminococcaceae UCG-002	1.33	3.41	0.071
Eubacterium oxidoreducens group	0.74	1.15	0.073
Prevotella 9	7.74	5.84	0.155
Eubacterium eligens group	1.20	1.63	0.193
Alistipes	6.45	5.58	0.209
Subdoligranulum	1.70	2.69	0.243
Faecalibacterium	11.56	10.26	0.268
Barnesiella	1.19	1.41	0.323
Lachnospiraceae NK4A136 group	0.83	1.47	0.353
Ruminococcus 1	1.39	1.11	0.368
Lachnospiraceae UCG-008	5.91	5.28	0.409
Roseburia	2.11	3.34	0.444
Lachnospira	0.65	1.32	0.518

Eubacterium coprostanoligenes group	1.40	1.46	0.682
Sutterella	1.26	0.86	0.783
Bacteroides	35.13	34.05	0.899
Parasutterella	1.87	1.35	0.958
Parabacteroides	3.80	2.88	0.994

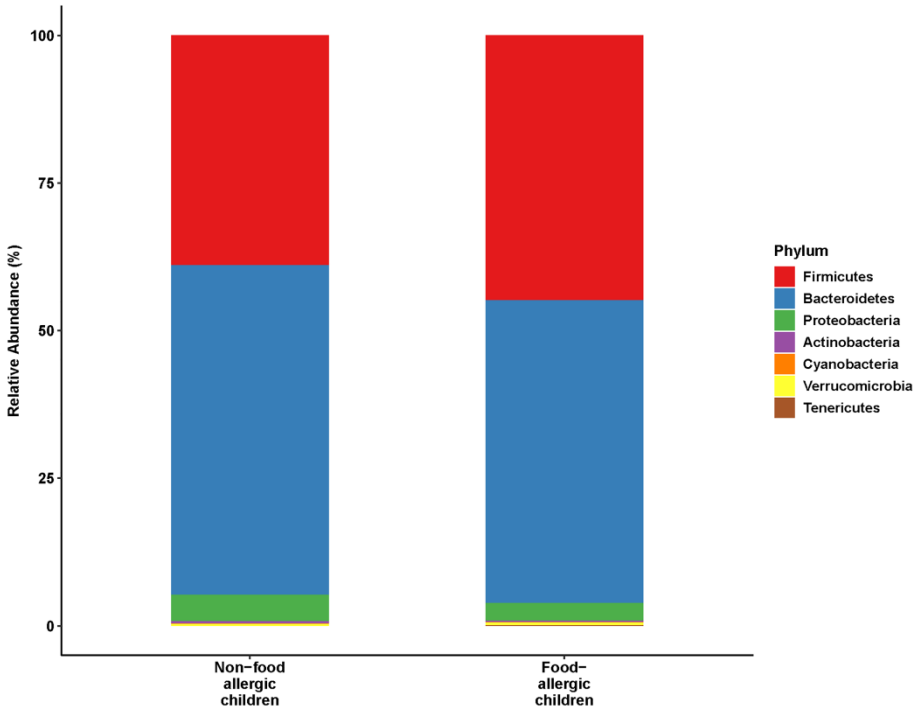
Values expressed as relative abundance (%).

p: p-value.

5.7.2 Supplemental figures



Supplemental figure S 5-1 PCoA plots of individual gut microbiota in food-allergic children (red) and non-food allergic children (blue) derived from (a) unweighted and (b) weighted UniFrac distances. Each symbol represents a sample. PCoA: Principal Coordinate Analysis.



Supplemental figure S 5-2 Relative abundance of gut microbial phyla

6 CHARACTERIZATION OF ORAL MICROBIOME IN FOOD-ALLERGIC CHILDREN

This chapter described Aim5, the aim of which was to investigate the association between oral microbiome and food allergy. This chapter is presented in the manuscript format.

6.1 Abstract

Introduction: Oral microbiome has been previously shown to influence predisposition to allergic disease but not food allergy. The aim of this study was to perform 16S rRNA gene sequencing in integration with network analysis to compare the oral microbial compositions in food-allergic and non-food allergic children.

Method: Saliva samples (n=34) were collected from children with IgE-mediated food allergies and non-food allergic controls. The characterization of saliva samples was performed via 16S rRNA gene sequencing of the V3 and V4 variables regions. Microbial alpha and beta diversity as well as relative abundance of the operational taxonomic units (OTUs) were compared between the two groups using QIIME. OTUs obtained from 16S rRNA gene sequencing were then used to construct a co-expression network using WGCNA and mapped onto Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways using Tax4Fun.

Results: Our study revealed food-allergic children have a distinct oral microbiome compared to non-food allergic children. The co-abundance module of food-allergic children was dominated by *Prevotella 7* and *Haemophilus* while the co-abundance module of non-food allergic children was dominated by *Abiotrophia*, *Gemella*, *Granulicatella*, *Bergeyella*, *Porphyromonas*, *Prevotella*, *Neisseria*, *Rothia* and *Leptotrichia*. Furthermore, our data suggested that *Streptococcus* was the hub taxa of the co-abundance network module for non-food allergic children.

Conclusion: Dysbiotic oral microbiome network may play a role in food allergy. This study advanced our understanding of the dynamic oral microbiome network and its correlation to food allergy.

6.2 Introduction

Oral cavity, which is revealed as the first site of encounter between the immune system and foreign antigens (212), has received attention for its association with health and disease (186, 187, 191). Oral microbiome harbours complex and diverse microbial communities, which comprises over 700 prevalent taxa (212). The roles of oral microbes in regulating health and diseases were first proposed by oral microbiologist W. D. Miller in the 1890s (131). According to Miller, oral microbes and their products might profoundly affect the development of diseases. In recent decades, the advances of high-throughput next-generation sequencing technologies, has widened tremendously our knowledge of this theory. Precisely, several studies have explored the possible roles of oral microbiome in the manifestation of diseases such as diabetes (213, 214), cancer (215, 216), inflammatory bowel disease (217, 218) and asthma (219, 220). Pathogenic bacteria enters into the blood circulation via the oral mucosal barrier can later result in chronic gut inflammation (132) through the induction of and reduction of Th17 cells and faecal IgA levels as well as an increase in the M1/M2 macrophage ratio (133). Other than its pathophysiological roles, oral microbiome is viewed as promising diagnostic biomarker for diseases due to its non-invasive sampling method (221) as well as its long term stability characteristics (222).

Food allergy is defined as an adverse immunologic response to ingested food antigens that associated with a range of disorders from IgE-mediated anaphylaxis to delayed cell-mediated reactions (223). Food allergy affects up to 10% of infants in some countries, some of the food allergies remain persistent for life time; furthermore, the prevalence of food allergy is increasing in industrialized regions (2). At present, there is no cure for food allergy, although immunotherapy option is available for treatment (7). The most common reactions to food allergy are gastrointestinal symptoms, which includes abdominal cramps, nausea, vomiting, and diarrhoea as well as cutaneous symptoms, which include hives, itching, and eczema (224). Although the etiology of food allergy is still not fully understood, previous studies have identified reduced diversity and altered composition of gut communities are clearly associated with food allergy (106, 107, 199).

Viewing that previous studies found an association between oral microbiome and the manifestation of diseases, it is reasonable to believe that the oral microbiome might play a role in affecting the pathogenesis of food allergy. Although oral dysbiosis has previously been associated with the alterations of immune responses and subsequent development of food allergy (134), the understanding of oral microbiome modulation and its impact on food allergy is still in its infancy stage and requires to be further addressed. In addition, the associations between oral microbiome and food allergy can be investigated further by network analysis (101), which offers an approach for identifying highly interconnected taxa within oral microbial communities rather than

individual taxa associated with food allergy by taking into account the complex interplay between oral microbial taxa and their hosts.

Herein, the purpose of this study is to apply 16S ribosomal RNA (rRNA) sequencing to comprehensively characterize the oral microbiome of food-allergic children in integration with network analysis by using weighted gene co-expression network analysis (WGCNA).

6.3 Methods

6.3.1 Study subject

This study was approved by the Human Research Ethics Committee (HREC), Perth Children's Hospital (RGS151 / HREC 2017060EP) and Curtin University (HRE2017-0712) and conducted in accordance with the National Health and Medical Research Council National Statement on Ethical Conduct in Human Research. Informed written consent was provided by the parents or guardians of the participants. This study included food-allergic children (n = 18), who were treated as outpatients at Perth Children's Hospital and non-food allergic children (n=16) from local community. Diagnoses of food allergy were determined by an immunologist on the day of recruitment based on the clinical outcomes of the patients. Food allergy was defined by immediate symptoms (1–2 h) after food allergen ingestion combined with either failed oral food challenge or with positive skin prick test wheal diameter $\geqslant$ 3 mm to any food allergen. In contrast, a negative skin prick test and/or a passed oral food

challenge or never have a reaction to any food allergens defined the subject as children without a food allergy.

6.3.2 Saliva sample collection and processing

Parents/ guardians of the participants were provided a saliva collection kit, which included a protocol of saliva collection, a sterile plastic container with yellow lid, a pair of disposable gloves and a sealed plastic bag with labels. The saliva sample would then be transported on ice by a researcher within 2 hours of collection to the laboratory -80°C freezers for storage.

DNA was then purified using a PureLink™ Microbiome DNA Purification Kit (Thermo Fisher Scientific, United States) in accordance with the manufacturer's instructions. Briefly, the sample was centrifuged at 14,000 x g for 10 minutes to pellet the microorganisms. The microbial pellet was resuspended in 800 µL of S1—Lysis Buffer before transferred to the bead tube. The suspension was then added with 100 µL of S2–Lysis Enhancer and incubated at 95°C for 10 min and later homogenized by the vortex mixer for 10 minutes at maximum speed. The sample was then centrifuged at 14,000 x g for 1 minute. The supernatant (500 µL) was transferred into a new tube and vortexed along with 900 µL of S4–Binding buffer. 700 µL of the sample mixture was transferred into a spin column-tube and centrifuged for 1 minute. After discarding the flow-through, the column was then washed with 500 µL of S5–Wash Buffer and centrifuged for 1 min. S6-Elution Buffer (100 µL) was added and incubated at room

temperature for 1 minute before centerifuge. The eluted DNA was stored at -80 ˚C until processing.

6.3.3 PCR amplication and sequencing

The PCR amplication and sequencing of thirty-four saliva samples were conducted by Beijing Novogene Bioinformatics Technology Co., Ltd. Briefly, PCR was conducted using Phusion® High-Fidelity PCR Master Mix and GC Buffer (New England Biolabs, Beijing, China) in accordance with manufacturer's instruction. PCR thermal cycling included an initial denaturation of 60s at 98°C, 35 cycles of 10s at 98°C, 30s at 50°C and 90s at 72°C, as well as a final extension of 5min at 72°C. The PCR sequencing of the V3-V4 region was performed on the Ion S5 XL platform (Thermo Fisher). A total of 2,497,798 sequences reads that passed the quality check (>Q20, error rate < 1%) were generated.

6.3.4 Quantitative Insights into Microbial Ecology (QIIME)

The raw sequences were demultiplexed and quality filtered using Quantitative Insights Into Microbial Ecology (QIIME) (194). The high quality reads were then assigned to operational taxonomic units (OTUs) using the open reference method in QIIME with the SILVA reference database (release 128) at a 97% similarity level (196). The Chao1 index, the observed OTUs index and the Shannon diversity index were used as measures of Alpha diversity while Beta diversity were measured using the weighted and unweighted UniFrac distance matrices. Beta diversity was then visualized with Principal Coordinate Analysis (PCoA). Differences of Alpha diversity between the

two groups were compared using Wilcoxon rank-sum test and inferences of Beta diversity were permuted using the Adonis permutational multivariate analysis (Adonis/PERMANOVA). A comparison of the relative abundance of OTUs between groups was computed using the Mann Whitney test. The sample size was not sufficient for multiple comparison adjustment; hence, $p< 0.05$ was considered statistically different.

6.3.5 Construction of microbial co-abundance network

In order to have a better understanding of the co-abundance network of the microbial taxa, Weighted Gene Correlation Network Analysis (WGCNA) package of R (140) was performed by using Hellinger transformation of the OTU count data (197) (with 97% identity threshold), as previously mentioned in chapter 5. Briefly, an adjacency matrix was constructed and a topological overlap matrix (TOM) was calculated based on the predefined soft-thresholding parameter. Highly co-occurred taxa were merged into different module clusters based on the default parameter (a minimum cluster size of 30 and merge cut height of 0.25) and these different module clusters were then assigned to different colours for visualization. Lastly, module trait association analysis was used to calculate the correlation coefficient between modules and food allergy as well as demographics traits such as age and gender. Modules with p-value <0.05 were considered as significant.

6.3.6 Identification of hub taxa

Next, an intramodular analysis was performed to determine the hub taxa by summing the connection strengths with other module taxa. Considering the small sample size of the present study, a more stringent cut-off for hub taxa (absolute value of the TaxaSignificance >0.5 and Module Membership >0.8) was applied. Taxa of the significant modules were then visualized using Cytoscape v3.8.0 (198).

6.3.7 KEGG Pathway Analysis

The generated OTUs table and OTUs taxonomy was mapped onto Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways using R package, Tax4Fun (225). Linear discriminant analysis (LDA) effect size (LEfSe) analysis (http://huttenhower.sph.harvard.edu/lefse/) was performed to detect biomarkers of the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways that differed significantly between non-food allergic children and food-allergic children. Default settings (alpha = 0.05, effect-size threshold of 2) were applied.

6.4 Result

6.4.1 Oral microbial alpha diversity

Saliva samples were obtained from 34 participants, which included 18 food-allergic children and 16 non-food allergic children. Seventy six percent of the subjects were boys, with the median age for non-food allergic children and food-allergic children of 6.1 years and 7.2 years, respectively. The groups did not differ from each other with

regard to age (p=0.137) and gender (p=0.317). The food allergies noted in the food-allergic children included nuts (n = 12), egg (n = 3) and mixed allergies (n = 3).

To determine the average species diversity in a habitat or specific area, alpha diversity was evaluated using Chao1, Shannon index and observed OTUs matrices. Chao1, Shannon index and observed OTUs showed that non-food allergic children and food-allergic children had similar average estimates for oral microbial community richness and evenness (Table 6-1).

Table 6-1 Comparison of oral microbial alpha diversity between food-allergic children and non-food allergic children. Values represent mean ± SD.

	Non-food allergic children	**Food-allergic children**	***p***
Chao1	544.1±50.1	546.9±50.8	0.848
Observed OTUs	473.7±51.7	471.0±55.3	0.896
Shannon diversity index	5.1±0.5	5.4±0.4	0.099

6.4.2 Oral microbial beta diversity

To determine the degree of inter-group dissimilarity, beta diversity was evaluated using unweighted and weighted UniFrac distance matrices. The weighted-UniFrac PCoA showed that oral microbiome of food allergic children was clearly separated from the oral microbiome of non-food allergic children (Figure 6-1). Similar to the PCoA plots results, the permutational multivariate analysis (Adonis) also revealed a

significant difference in oral microbial communities between food-allergic children and non-food allergic children ($p < 0.05$).

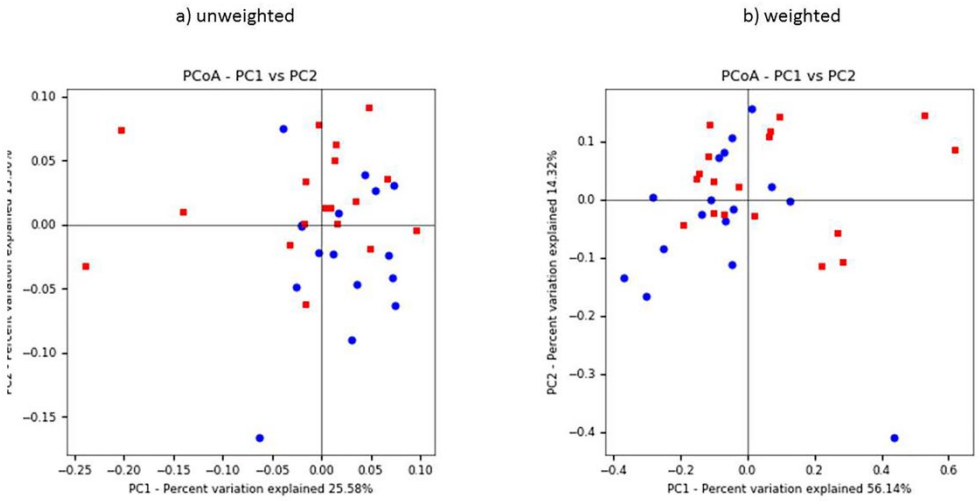


Figure 6-1 PCoA plots of individual oral microbiota in food-allergic children (red) and non-food allergic children (blue) derived from (a) unweighted and (b) weighted UniFrac distances. Each symbol represents a sample.

6.4.3 Oral microbial composition

OTU dataset for food-allergic children and non-food allergic children consisted of 9 phyla, 20 classes, 27 orders, 43 families and 80 genera. At the phyla level, the oral microbiota was dominated by Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria and Fusobacteria (Figure 6-2). Smaller contributions of Absconditabacteria (SR1), Saccharibacteria, Gracilibacteria and Cyanobacteria were also detected in the oral microbiota, which represented <2% of the total reads analyzed.

There was no significant difference in the phyla level between food-allergic children and non-food allergic children (Table 6-2).

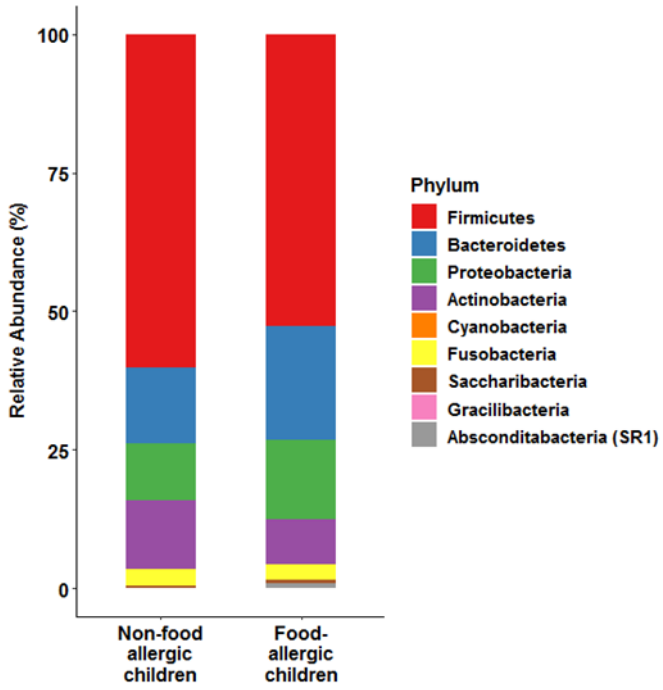


Figure 6-2 Relative abundance of oral microbial phyla

Table 6-2 The comparison of oral microbiota at the phyla level between food-allergic children and non-food allergic children.

Phylum	**Non-food allergic children**	**Food-allergic children**	***p***
Cyanobacteria	0.02	0.00	0.125
Bacteroidetes	13.69	20.47	0.157

Firmicutes	60.08	52.75	0.202
Proteobacteria	10.35	14.40	0.469
Fusobacteria	2.97	2.80	0.704
SR1 (Absconditabacteria)	0.10	0.92	0.756
Saccharibacteria	0.38	0.50	0.783
Actinobacteria	12.41	8.07	0.809
Gracilibacteria	0.01	0.08	0.971

Values expressed as relative abundance (%).

p: p-value.

Eighty genera were identified, and only 16 genera were accounted for more than 1% across all samples (Table 6-3). The relative abundance of *Prevotella 7* was marginally higher and the abundance of *Bergeyella* and *Granulicatella* was marginally lower in children with food allergy compared with children without food allergy. After adjusting for multiple comparisons, there was no significant difference in the genera level between food-allergic children and non-food allergic children.

Table 6-3 Relative abundance of predominant genera in oral microbiota between food-allergic children and non-food allergic children (≥ 1% across all samples)

Genus	**Non-food allergic children**	**Food-allergic children**	***P***	***FDR_p***
Bergeyella	1.2	0.09	0.032	0.153

Prevotella 7	4.17	9.62	0.053	0.225
Granulicatella	4.13	2.9	0.058	0.231
Actinomyces	1.99	2.75	0.129	0.412
Prevotella	0.94	1.64	0.147	0.436
Abiotrophia	1.15	0.83	0.202	0.538
Streptococcus	48.56	42.22	0.301	0.611
Haemophilus	3.65	4.56	0.334	0.611
Veillonella	0.67	1.27	0.352	0.611
Alloprevotella	2.16	2.32	0.427	0.700
Neisseria	4.39	6.05	0.512	0.728
Rothia	10.03	4.54	0.535	0.728
Porphyromonas	4.65	4.69	0.535	0.728
Gemella	4.64	4.03	0.730	0.823
Neisseriaceae_unclassified	1.16	1.66	0.783	0.858
Leptotrichia	2.26	1.76	0.945	0.969

Values expressed as relative abundance (%).

p: p-value.

FDR_p: adjusted p-value

6.4.4 Microbial co-abundance network

To better characterize saliva microbial taxa in food-allergic children, we applied WGCNA to identify clusters of microbial taxa whose differential representation was correlated with food allergy.

Through WGCNA, we were able to identify 8 modules of co-expressed taxa and the number of taxa within modules ranged from 30 to 214 (Table 6-4). Among all the genes, only 146 taxa (22%) were not included in any colour module, and these taxa were grouped into the grey module as per default.

Table 6-4 The number of taxa in the 8 modules

Module colours	Freq
Black	30
Blue	105
Brown	56
Green	38
Grey	146
Red	36
Turquoise	214
Yellow	49

6.4.5 Modules of saliva microbial taxa associated with food allergy

We further compared the module eigengenes between food-allergic children and non-food allergic children with using module trait association analysis to identify the food allergy-associated modules. Our results showed that a module (red) ($r = 0.38$ $p = 0.03$) to be positively correlated with food allergy and a module (turquoise) to be negatively correlated with food allergy ($r = -0.43$, $p = 0.01$) (Figure 6-3).

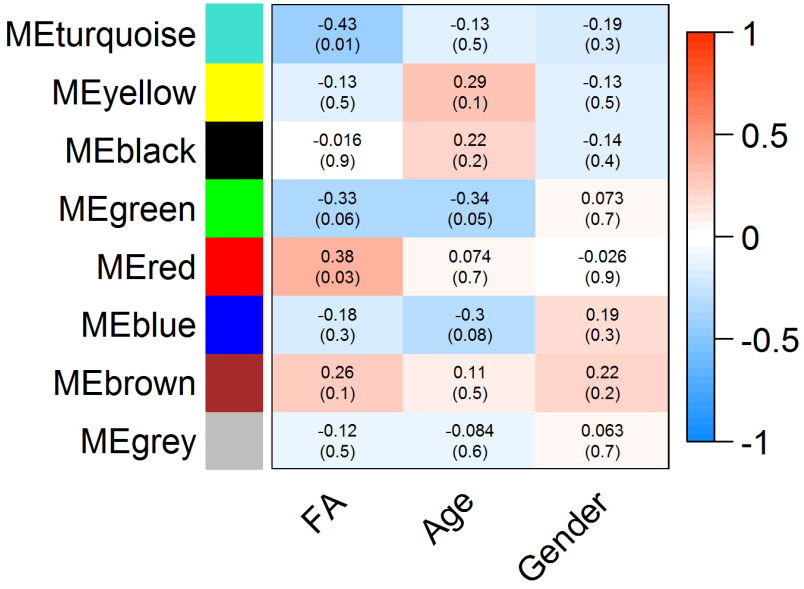


Figure 6-3 Module-trait associations. Each row corresponds to a module eigengene (ME) while each column corresponds to either phenotype (FA: food allergy) or demographic traits such as age and gender. Each cell contains the corresponding correlation coefficient (display at the top of the cell) and corresponding p-values for each module (display at the bottom of the cells within parentheses). Blue and red colours of the spectrum on the right denote low and high correlation, respectively.

Red module was revealed to be positively correlated with food allergy. In this module, 2 out of 36 taxa (> 1% relative abundance across all samples) were identified as dominant taxa, which included *Haemophilus* (Proteobacteria phylum) and *Prevotella*

7 (Bacteroidetes phylum). However, no hub taxa was identified to have the absolute value of the TaxaSignificance > 0.5 and Module Membership > 0.8 (Figure 6-4).

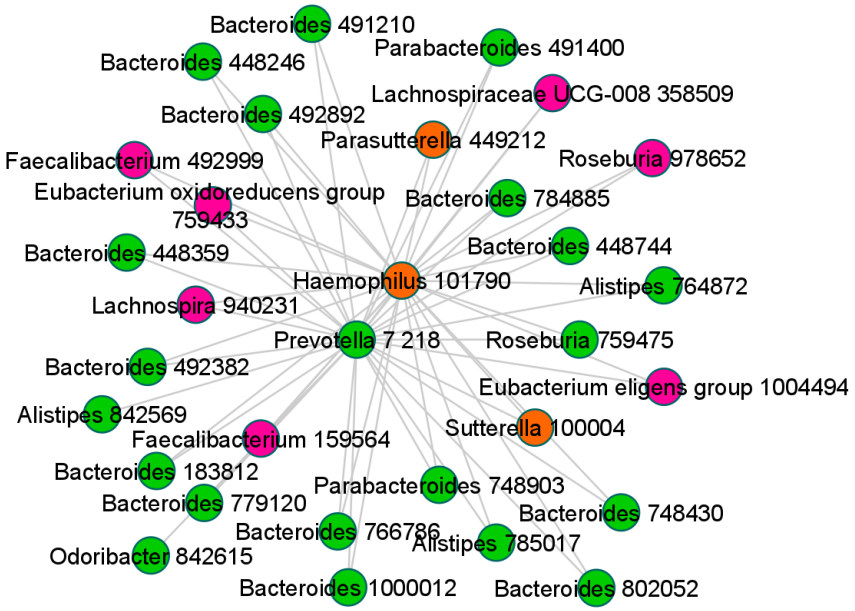


Figure 6-4 Network analysis identifies red module as a distinct module of co-associated taxa. The highly correlated taxa in the comparisons of food allergic children and non-food allergic children are indicated and colour coded according to the phylum. Green colour represents Bacteroidetes phylum, pink colour represents Firmicutes phylum while orange colour represents Proteobacteria phylum.

Turquoise module was revealed to be negatively correlated with food allergy. In this module, 10 out of 17 taxa were identified (> 1% relative abundance across all samples) as dominant taxa. The majority of the dominant taxa came from Firmicutes phylum,

which included the genera of *Abiotrophia*, *Gemella*, *Granulicatella* and *Streptococcus*. Other than this, the dominant taxa also included genera taxa from the phyla of Bacteroidetes (*Bergeyella*, *Porphyromonas*, and *Prevotella*), Proteobacteria (*Neisseria*), Actinobacteria (*Rothia*) as well as Fusobacteria (*Leptotrichia*). Particularly, *Streptococcus* was identified as be hub taxa of turquoise module, which had the absolute value of the TaxaSignificance > 0.5 and Module Membership > 0.8 (Figure 6-5).

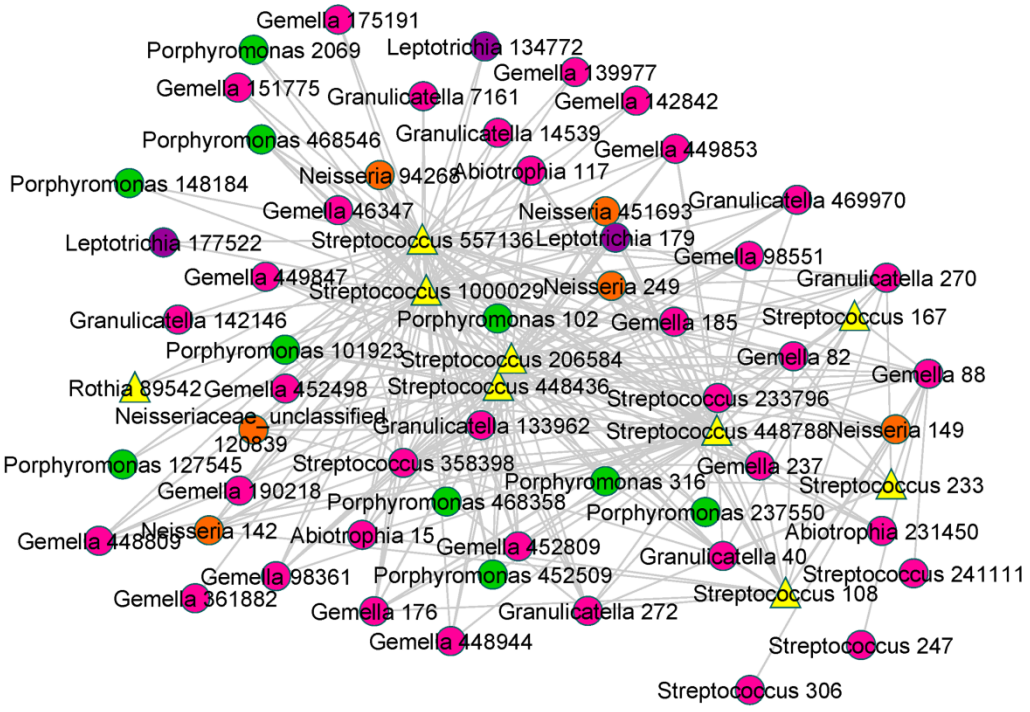


Figure 6-5 Network analysis identifies turquoise module as a distinct module of co-associated taxa. The highly correlated taxa in the comparisons of food allergic children and non-food allergic children are indicated and colour coded according to the phylum. Green colour represents Bacteroidetes phylum, pink colour

represents Firmicutes phylum, orange colour represents Proteobacteria phylum, blue colour represents Actinobacteria phylum while magenta colour represents Fusobacteria phylum. Hub taxa are indicated with yellow triangle shapes and other connector taxa are in round shapes.

6.4.6 Predicted functional pathway of oral microbial taxa associated with food allergy

To specifically identify functional pathway of oral microbial taxa associated food allergy, we performed LEfSe to compare KEGG functional pathway. No differentially KEGG categories were enriched in oral microbial taxa of food-allergic children when compared to non-food allergic children.

6.5 Discussion

While gut microbiome has previously been linked with the immune dysfunction and food allergy (106, 107, 109, 199), oral microbiome has also recently gained attention as a possible regulator of food allergy (134).

Our objective for this study was to perform 16S rRNA gene sequencing in integration with network analysis to compare the oral microbial compositions in food-allergic and non-food allergic children. To our knowledge, this is the first study comparing the oral microbiome in food-allergic children and non-food allergic children by using network analysis. In present study, we found the composition of oral microbiome in food-allergic children was different from that in non-food allergic children. Although we observed a marginally higher relative abundance of *Prevotella 7* and a marginally

lower relative abundance of *Bergeyella* and *Granulicatella* in children with food allergy compared with children without food allergy, the relative abundance of these taxa were not significantly different between children with and without food allergy. The confounded microbial composition analysis at the relative abundance was perhaps due to small sample size. In contrast, interrogation of the same data using network analysis, unmasked several taxa associated with food allergy including those microbial populations with low relative abundance but highly relevant to the onset of food allergy. Specifically, the co-abundance module of food-allergic children was dominated by *Prevotella 7* and *Haemophilus* while the co-abundance module of non-food allergic children was dominated by *Abiotrophia*, *Gemella*, *Granulicatella*, *Bergeyella, Porphyromonas*, *Prevotella*, *Neisseria*, *Rothia* and *Leptotrichia*. Furthermore, our data suggested that *Streptococcus* was the hub taxa of the co-abundance network module for non-food allergic children.

The oral microbiome has a high diversity of microbiota taxa, consisting of over 700 identified taxa at the species level (212). Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria were previously identified as the dominant taxa in the oral microbiome (217, 226). Similar to these studies, we also observed oral microbiome was dominated by Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria and Fusobacteria. Further to this, we also observed smaller contributions of Absconditabacteria (SR1), Saccharibacteria, Gracilibacteria and Cyanobacteria in the oral microbiome.

Through network analysis, we identified the co-abundance network module for non-food allergic children was characterized by a hub taxa, *Streptococcus*. *Streptococcus*, which belongs to Firmicutes phylum, is the dominant genus in human oral microbiome (134, 227). Consistent with our studies, a study conducted by Matsui et al also observed the counts of *Streptococcus* to be reduced in oral cavity of mice sensitized with ovalbumin compared with control mice and this reduction was found to be associated with increased levels of immunoglobulin A (134).

We also identified the co-abundance network module for non-food allergic children was more likely to be dominated by *Bergeyella*, *Granulicatella*, *Prevotella*, *Neisseria*, *Rothia*, *Leptotrichia*, *Abiotrophia* and *Gemella*. Our observations were largely consistent with the findings of Dzidic et al (219) in a longitudinal study, who noted that the oral cavity of children with allergic diseases were less colonized by genera *Bergeyella*, *Granulicatella*, *Prevotella*, *Rothia*, *Leptotrichia* and *Abiotrophia*, suggesting the protective roles of these taxa towards allergic diseases. However, this study also revealed the positive association of *Neisseria* and *Gemella* with allergy development, which was in contrast with our findings. Further studies are required to investigate if these inconsistencies were caused by the heterogeneity of study design.

Other than the above-mentioned taxa, we also found enrichment of *Porphyromonas* in the co-abundance network for non-food allergic children. *Porphyromonas gingivalis,* a species of *Porphyromonas* genus is usually found in the oral cavity of healthy children (228). A murine model with asthma demonstrated that *Porphyromonas*

gingivalis could reduce the airway expression of Interleukin 4, Interleukin 5, Interleukin 13, resulting in reduced airway eosinophilia and inflammation (229).

In contrast, the co-abundance network module for food-allergic children was more likely to be *Haemophilus* and *Prevotella 7* dominated. *Haemophilus*, which is the abundant genera in the oral cavity, is known for its pathogen role in causing inflammation. Enrichment of *Haemophilus* in the oral cavity region was previously revealed to be associated with increased activity of Eosinophilic Esophagitis (230). Likewise, relative abundance of *Haemophilus* in the sputum and airway has been associated with asthma exacerbation (231). The exact role of *Prevotella 7* in affecting the onset of allergic diseases remains unknown, but *Prevotella 7* was shown to have a positive correlation with bacterial antigen P6 (232).

Although our study found an association between dysbiotic oral microbiome network and food allergy, there were several limitations. First, our study can only be classified down to the genus level, but not species and strains. Secondly, causality cannot be inferred due to the cross-sectional of this study. Considering oral and gut are contiguous mucosal surfaces encompassing the whole GI tract, future studies with larger sample sizes could consider to apply a multi-omics approach by integrating gut and oral microbiome signature from same samples in order to explore further on the mechanisms behind this, and how oral microbiome changes impact food allergy development.

6.6 Conclusion

Much work remains to be done in order to better understand the association of oral microbiome to food allergy. Although the differences in oral microbiome that we identified in our study were not be able to directly linked to food allergy development, our findings suggested that the oral microbiome might have a potential association with food allergy.

7 CHARACTERIZATION OF HOUSE DUST MICROBIOME IN FOOD-ALLERGIC CHILDREN

This chapter described Aim6, the aim of which was investigate the house dust microbiome and pathways associated with food allergy. In this chapter, house dust microbiome profile was compared between households with food-allergic children and non-food allergic children. This chapter is presented in the manuscript format.

7.1 Abstract

Introduction: Exposure to diverse environmental microbes appears to affect the pathogenesis of allergic diseases. So far, only a few studies have considered the assessment of the indoor microbes in house dust and most of these studies focused on other allergic diseases instead of food allergy.

Method: At the present study, we analyzed house dust microbiome obtained from 23 participants (16 food-allergic children and 7 non-food allergic children) using 16S rRNA gene sequencing. WGCNA was used to construct a microbial co-abundance network and to identify microbial modules associated with food allergy. The microbial taxa were later mapped onto Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways using Tax4Fun.

Results: Our findings revealed food-allergic children have a distinct house dust microbiome compared to non-food allergic children. Our data suggested that *Streptococcus*, *Gemella*, *Haemophilus*, *Neisseriaceae_unclassified*, *Actinomyces*, *Rothia*, *Alloprevotella* and *Prevotella 7* were the hub taxa of the co-abundance network modules for house dust microbiome of food-allergic children. Further pathway analysis showed house dust microbiome of food allergic children were involved in the pathways of replication and repair as well as cofactors and vitamins metabolism while house dust microbiome of non-food allergic children were involved in the amino acid metabolism.

Conclusion: Obvious discrepancies were observed in the co-abundance network of house dust microbiome between food-allergic children and non-food allergic children. These discrepancies may be related to pathways of replication and repair as well as cofactors and vitamins metabolism.

7.2 Introduction

It is well known that there are variations in the prevalence of childhood allergic diseases in different geographic regions with higher prevalence of allergic diseases generally observed in Western, industrialized regions while lower prevalence of allergic diseases generally observed in Eastern, non-industrialized regions (2, 233, 234). Variations in the prevalence of allergic diseases in different geographic regions suggested a potential role of the environmental microbiome in modulating allergic responses.

Compared with outdoor environments, indoor environments contribute significantly to human exposure to environmental microbes, as people spend most of their time indoors. Indoor pollutant emissions are more likely to be inhaled than outdoor emissions, and one of the common generated indoor pollutants is house dust. The microbial communities of house dust in urban areas are less spatially variable than those found in more rural areas (235). House dust contains an average of 9,000 different species of microbes (235). To be specific, the average household has more than 2,000 different types of fungi and 7,000 different types of bacteria. Therefore,

horizontal transmission of house dust microbes, either through inhalation, ingestion or cutaneous, may be contributing commensal microbes to the onset of allergic diseases.

To understand the association between house dust microbiome exposure and allergy outcomes, researchers have initially compared the endotoxin concentrations in house dust of children with and without allergic diseases (73, 136). These studies revealed that exposure to high level of house dust endotoxin had a negative association with development of allergic diseases. Contradictory results also existed that exposure to high levels of house dust endotoxin had an increased rate of developing allergic diseases (76, 77). Nevertheless the discordant results, house dust endotoxin has an association with protection towards allergic diseases and the development of allergic diseases.

Recent studies using 16S rRNA gene sequencing have found several taxa from house dust microbiome associated with allergic diseases in children (72, 137, 138). For example, a case-control study of 104 children shown that reduced exposure to house dust microbiome, particularly Firmicutes and Bacteriodetes, in the first year of life is associated with an increased risk of developing atopy and atopic wheeze (137). Another study identified *Lactococcus* genus as a risk factor for asthma and twelve bacterial genera (mostly from the Actinomycetales order) as a protective factor towards asthma (138). Loo et al. identified an enrichment of *Anaplasmataceae*, *Bacteroidaceae*, and *Leptospiraceae* in house dust samples of allergic subjects (72). Although these studies have found an association between house dust microbiome and allergy, studies examining associations of house dust with food allergy are limited.

In this study, we would like to characterize the microbial composition in the house dust samples of food-allergic children and non-food allergic children by performing 16S rRNA gene sequencing in integration with network analysis of bacterial taxa in house dust.

7.3 Methods

7.3.1 Study subject

16 children with immunologist-diagnosed food allergy were recruited from the outpatient clinic at Perth Children's Hospital. Non-food allergic children (n=7) were recruited from the local community. All subjects were recruited under a protocol approved by the Human Research Ethics Committee (HREC), Perth Children's Hospital (RGS151 / HREC 2017060EP) and Curtin University (HRE2017-0712). Informed written consent was obtained from the parents or guardians upon enrolment.

7.3.2 House dust sample collection

House dust samples were collected from the participants' bedroom by a researcher. Prior to house dust sample collection, the parents of the participants were advised not to clean their children's bedroom. The researcher then used a sterile cotton swab to wipe across the smooth surface of the participant's bedroom, mainly on a cupboard using an even pressure and holding the swab flat against the surface. The cotton swab was then placed immediately back to its sterile tube, which was labelled with the participant's name. The house dust sample was then transported on ice by the researcher within 2 hours of collection to the laboratory -80°C freezers for storage. A

sterile scissor was used to cut the cotton from the swab stick and place the tip into the provided bead tube.

7.3.3 DNA extraction and sequencing

Total DNA was purified using PureLink™ Microbiome DNA Purification Kit (Thermo Fisher Scientific, United States) according to the manufacturer's procedure. The V3-V4 region of the 16S rRNA gene was amplified by PCR using barcoded primers and was sequenced using Ion S5 XL platform (Thermo Fisher). A total of 1,600,618 sequences reads that passed the quality check (>Q20, error rate < 1%) were generated.

7.3.4 Microbial co-abundance network construction and network visualization

Microbial co-abundance network was constructed with WGCNA package of R by using Hellinger transformation of the OTU count data (14) (197). The soft thresholding power was obtained based on the scale-free topology index (R2). Highly co-occurred bacterial taxa were then assigned into several module memberships. Next, the association of these module memberships were quantified with food allergy as well as demographics traits such as age and gender. Modules with p-value < 0.05 were regarded as significant food allergy-related modules. Considering the small sample size of the present study, a more stringent cut-off for hub taxa (absolute value of the TaxaSignificance >0.5 and Module Membership >0.8) was applied. These taxa were then visualized using Cytoscape v3.8.0 (198).

7.3.5 Quantitative Insights into Microbial Ecology (QIIME)

The 16S rRNA sequence analyses were performed using 7.3.4 Quantitative Insights Into Microbial Ecology (QIIME) (194). The operational taxonomic units (OTUs) were assigned with the SILVA reference database (release 128) based on 97 % similarity level(196). As previously described in chapter 5, relative abundance was calculated with the Chao1 index, observed species was evaluated with observed OTUs while relative abundance and evenness were measured with Shannon index. The comparison of beta diversity was calculated using the weighted and unweighted UniFrac distance matrices and later visualized in a two-dimensional structure using Principal Coordinate Analysis (PCoA). The Adonis permutational multivariate analysis (Adonis/PERMANOVA) was performed to compare beta diversity dissimilarity matrices while Mann Whitney test was performed to compare the relative abundance of OTUs between groups. The sample size was not sufficient for multiple comparison adjustments; hence, $p< 0.05$ was considered statistically different.

7.3.6 Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Analysis

7.1.12 Predictive functional analysis was performed using Tax4Fun with Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway abundance classification and biomarkers of KEGG pathways were analysed by Linear discriminant analysis (LDA) effect size (LEfSe) analysis (http://huttenhower.sph.harvard.edu/lefse/) with default settings (alpha = 0.05, effect-size threshold of 2).

7.4 Result

7.4.1 House dust microbial alpha diversity

House dust samples were obtained from 23 participants, including 16 food-allergic children and 7 non-food allergic children. Seventy percent of the subjects were boys, with the median age for non-food allergic children and food-allergic children of 3.7 years and 5.1 years, respectively. The groups did not differ from each other with regard to age (p=0.179) and gender (p=0.266). The food allergies noted in the food-allergic children included nuts (n = 12), egg (n = 3) and mixed allergies (n = 1).

Chao1, Shannon index and observed OTUs showed that there were no significant differences between the two groups by Mann Whitney U Test ($p > 0.05$) (Table 7-1).

Table 7-1 Comparison of house dust microbial alpha diversity between food-allergic children and non-food allergic children. Values represent mean ± SD.

	Non-food allergic children	**Food-allergic children**	***P***
Chao1	1014.4±127.7	1073.1±136.5	0.393
Observed OTUs	810.4±165.8	895.4±135.5	0.223
Shannon diversity index	6.5±1.4	7.0±0.7	0.316

7.4.2 House dust microbial beta diversity

The weighted-UniFrac PCoA showed that house dust microbiome of food allergic children was clearly separated from the house dust microbiome of non-food allergic children (Figure 7-1). Similar to the PCoA plots results, the permutational multivariate

analysis (Adonis) also revealed a significant difference in the house dust microbiome of food-allergic children and non-food allergic children ($p < 0.01$).

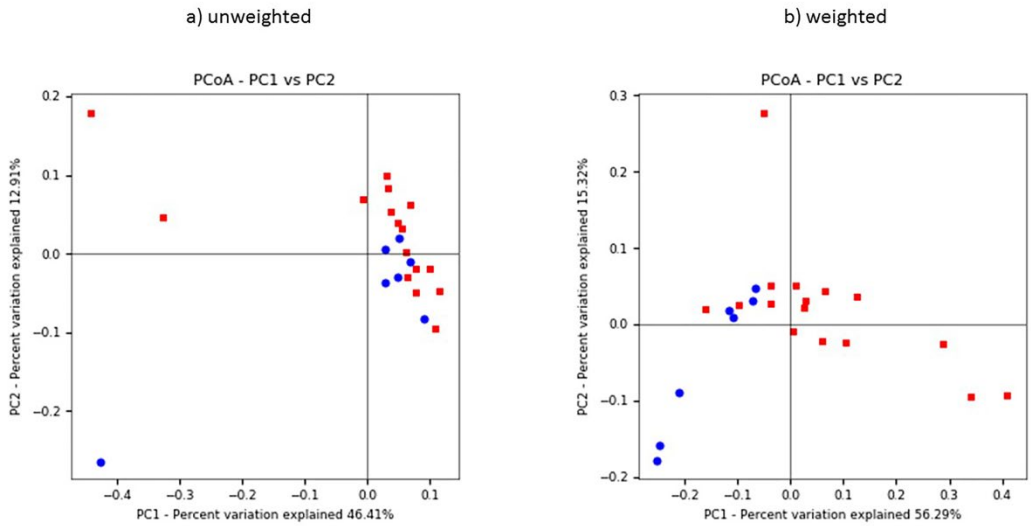


Figure 7-1 PCoA plots of individual house dust microbiome in food-allergic children and non-food allergic children derived from (a) unweighted and (b) weighted UniFrac distances. Each symbol represents a sample. PCoA: Principal Coordinate Analysis.

7.4.3 Microbial composition of house dust samples

OTU dataset for food-allergic children and non-food allergic children consisted of 1465 OTUs, which classified to 20 phyla, 44 classes, 86 orders, 181 families and 428 genera.

The most predominant phyla were Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria, which characterize the house dust microbiome. Other phyla were also

detected at relatively low abundances (<1%) including Fusobacteria, Saccharibacteria, Nitrospirae, Tenericutes. Absconditabacteria (SR1), Cyanobacteria, Acidobacteria, Planctomycetes, Spirochaetae, Synergistetes, Deinococcus-Thermus, Ambiguous taxa, Verrucomicrobia, Ignavibacteriae, Chloroflexi and Gemmatimonadetes (Figure 7-2). There was no significant difference in the phylum level between food-allergic children and non-food allergic children (Table 7-2).

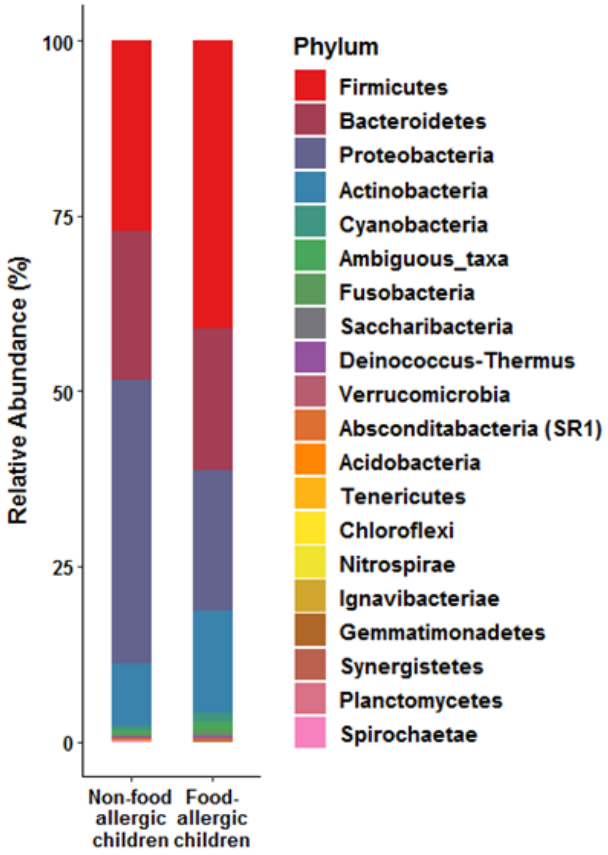


Figure 7-2 Relative abundance of house dust microbial phyla

Table 7-2 The comparison of house dust microbiome at the phylum level between food-allergic children and non-food allergic children

Phylum	**Non-food allergic children**	**Food-allergic children**	***P***	***FDR_p***
Fusobacteria	0.26	0.94	0.006	0.123
Actinobacteria	8.74	14.68	0.045	0.237
Saccharibacteria	0.12	0.25	0.045	0.237
Nitrospirae	0.01	0.02	0.051	0.237
Tenericutes	0.00	0.03	0.067	0.237
Firmicutes	27.17	41.16	0.071	0.237
Absconditabacteria (SR1)	0.042	0.11	0.109	0.311
Proteobacteria	40.48	19.89	0.124	0.311
Cyanobacteria	0.57	1.13	0.285	0.632
Acidobacteria	0.05	0.09	0.316	0.632
Planctomycetes	0.05	0.00	0.494	0.782
Spirochaetae	0.08	0.00	0.494	0.782
Synergistetes	0.00	0.01	0.508	0.782
Deinococcus-Thermus	0.11	0.19	0.593	0.847
Bacteroidetes	21.35	20.18	0.789	0.911
Verrucomicrobia	0.33	0.13	0.789	0.911
Ignavibacteriae	0.06	0.02	0.819	0.911

Chloroflexi	0.00	0.03	0.909	0.920
Gemmatimonadetes	0.01	0.01	0.92	0.920

Values expressed as relative abundance (%).

p: p-value.

FDR_p: adjusted p-value

Four hundred and twenty-eight genera were identified, and only 28 genera were accounted for more than 1% across all samples (Table 7-3). There was no significant difference in the genera level between food-allergic children and non-food allergic children after adjusting for multiple comparisons.

Table 7-3 Relative abundance (%) of predominant genera in house dust microbiome between food-allergic children and non-food allergic children (⩾ 1% across all samples)

Genus	**Non-food allergic children**	**Food-allergic children**	***p***	***FDR_p***
Streptococcus	7.50	24.27	0.001	0.146
Actinomyces	0.53	1.58	0.001	0.146
Rothia	0.82	2.30	0.005	0.146
Granulicatella	0.85	2.09	0.0065	0.146
Alloprevotella	0.49	1.33	0.008	0.162
Acidovorax	1.06	0.01	0.009	0.162

Gemella	0.76	2.22	0.009	0.162
Haemophilus	0.69	1.80	0.009	0.162
Prevotella 7	0.60	2.38	0.011	0.170
Neisseria	0.55	1.92	0.016	0.198
Bartonella	10.15	0.10	0.019	0.206
Faecalibacterium	3.90	1.79	0.023	0.206
Ruminococcus 2	1.12	0.31	0.023	0.206
Porphyromonas	0.62	1.64	0.033	0.253
Brevundimonas	2.20	0.19	0.071	0.372
Lachnospiraceae UCG-008	2.00	0.68	0.082	0.401
Pseudomonas	3.94	0.820	0.082	0.401
Bacteroides	13.95	7.36	0.095	0.414
Neisseriaceae_unclassified	0.41	1.71	0.095	0.414
Alistipes	1.26	0.76	0.124	0.463
Prevotella 9	1.08	3.38	0.181	0.532
Corynebacterium 1	1.42	3.85	0.256	0.605
Staphylococcus	0.78	1.96	0.285	0.632
Massilia	7.16	0.36	0.462	0.725
Comamonadaceae_unclassified	2.27	0.08	0.504	0.746
Paracoccus	1.00	1.36	0.548	0.779
Sphingomonas	2.29	1.57	0.593	0.816

Acinetobacter	3.26	2.88	0.738	0.868

Values expressed as relative abundance (%).

p: p-value.

FDR_p: adjusted p-value

7.4.4 Microbial co-abundance network

To better characterize house dust microbial taxa in food-allergic children, we applied WGCNA to identify clusters of microbial taxa whose differential representation was correlated with food allergy.

Through WGCNA, we were able to identify 10 modules of co-expressed taxa and the number of taxa within modules ranged from 43 to 424 (Table 7-4). Among all the taxa, only 96 taxa (6%) were not included in any colour module, and these taxa were grouped into the grey module as per default.

Table 7-4 The number of taxa in the 10 modules

Module colours	**Freq**
Black	78
Blue	280
Brown	207
Green	94
Grey	96
Red	43

Pink	61
Red	79
Turquoise	424
Yellow	103

7.4.5 Modules associated with food allergy

We further compared the module eigengenes between children with and without food allergy with using module trait association analysis to identify the food allergy-associated modules. Our results showed that two modules were significantly associated with food allergy (Figure 7-3), which were brown module (r = 0.60 p = 0.003) and green module (r = 0.49, p = 0.02).

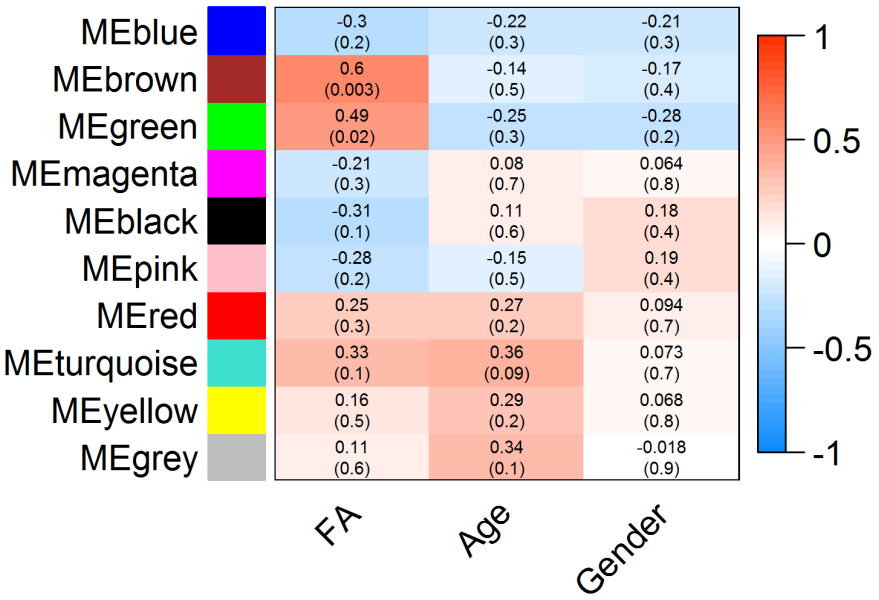


Figure 7-3 Module-trait associations. Each row corresponds to a module eigengene (ME) while each column corresponds to either phenotype (FA: food allergy) or demographic traits such as age and gender. Each cell contains the corresponding correlation coefficient (display at the top of the cell) and corresponding p-values for each module (display at the bottom of the cells within parentheses). Blue and red colours of the spectrum on the right denote low and high correlation, respectively.

Taxa from brown module were revealed to have the highest positive correlation with food allergy. In this module, 11 out of 42 taxa (> 1% relative abundance across all samples) were identified as dominant taxa, and all these taxa were correlated with each other. Particularly, *Streptococcus*, *Gemella*, *Haemophilus*, *Neisseriaceae_unclassified*, *Actinomyces* and *Rothia* were identified to be hub taxa of brown module with high TaxaSignificance and Module Membership (Figure 7-4).

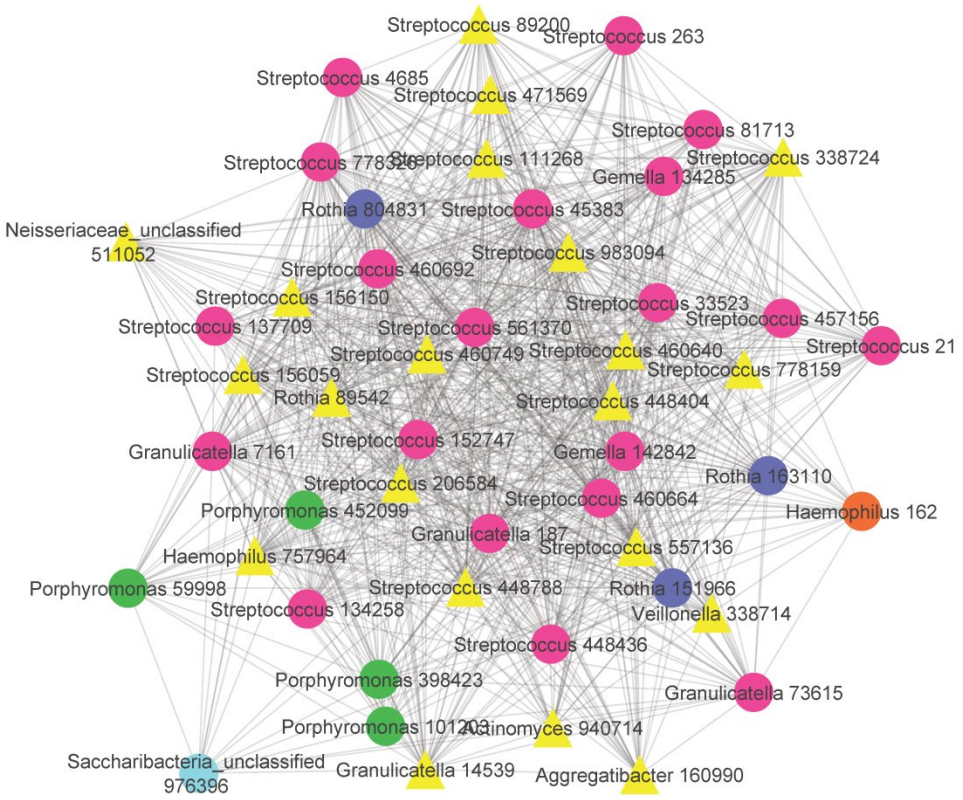


Figure 7-4 Network analysis identifies brown module as a distinct module of co-associated taxa. The highly correlated taxa in the comparisons of food allergic children and non-food allergic children are indicated and colour coded according to the phylum. Green colour represents Bacteroidetes phylum, pink colour represents Firmicutes phylum, orange colour represents Proteobacteria phylum, blue colour represents Actinobacteria phylum while baby blue colour represents Saccharibacteria phylum. Hub taxa are indicated with yellow triangle shapes and other connector taxa are in round shapes.

Taxa from green module were also revealed to have a positive correlation with food allergy. In this module, 7 taxa out of 28 taxa (> 1% relative abundance across all

samples) were identified as dominant taxa, and all these taxa were correlated with each other. Particularly, *Actinomyces*, *Alloprevotella*, *Prevotella 7* and *Streptococcus* were identified to be hub taxa with high TaxaSignificance and Module Membership (Figure 7-5).

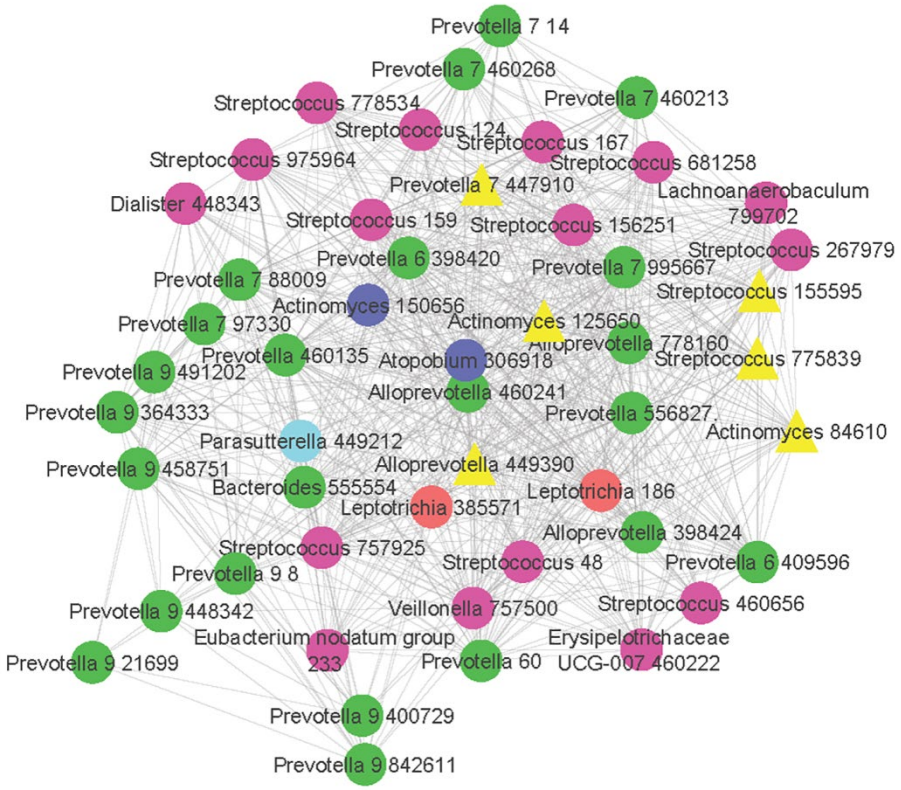


Figure 7-5 Network analysis identifies green module as a distinct module of co-associated taxa. The highly correlated taxa in the comparisons of food allergic children and non-food allergic children are indicated and colour coded according to the phylum. Green colour represents Bacteroidetes phylum, pink colour represents Firmicutes phylum, red colour represents Fusobacteria phylum, blue

colour represents Actinobacteria phylum while baby blue colour represents Saccharibacteria phylum. Hub taxa are indicated with yellow triangle shapes and other connector taxa are in round shapes.

7.4.6 Predicted functional pathway of house dust microbial taxa associated with food allergy

Linear discriminant analysis effect size (LEfSe) performed on the Tax4fun output showed several KEGG categories differentially present in each group (Figure 7-6). Using the threshold values (LDA > 2.0, $p < 0.05$), we found that replication and repair (nucleotide excision repair and base excision repair) and metabolism of cofactors and vitamins (folate biosynthesis, Nicotinate and nicotinamide metabolism as well as riboflavin metabolism) were enriched in food-allergic children. In contrast, we found amino acid metabolism (arginine and proline metabolism, histidine metabolism, as well as valine, leucine and isoleucine biosynthesis) was enriched in non-food allergic children.

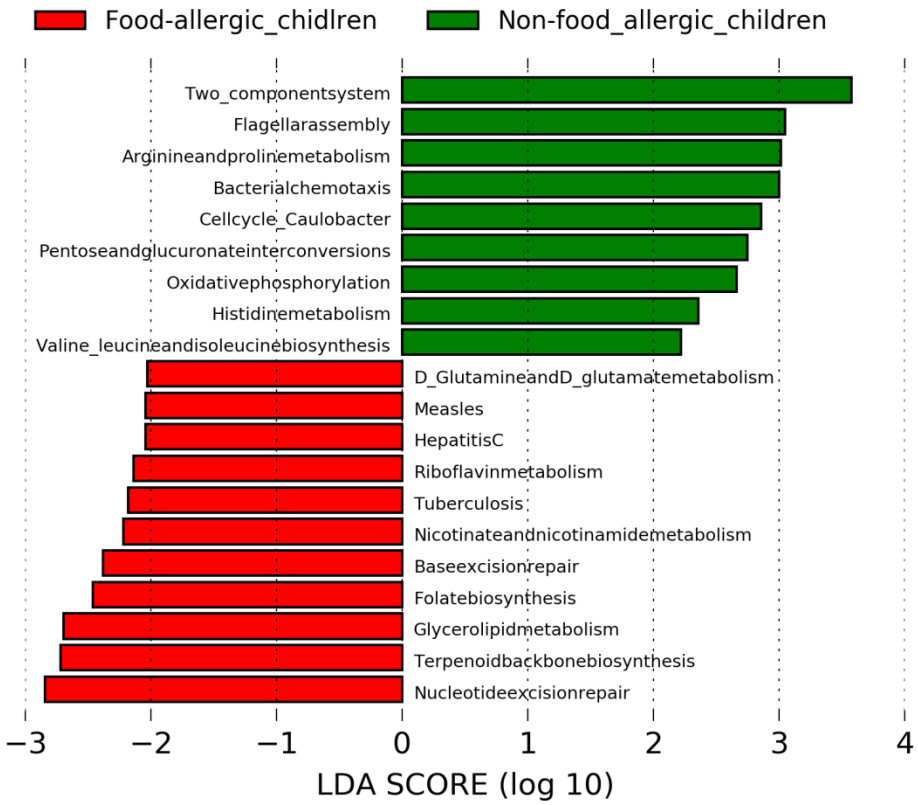


Figure 7-6 LEfSe analysis revealed distinct KEGG pathway differences in house dust microbiota between food-allergic children and non-food allergic children. KEGG pathway enriched in food-allergic children was indicated with red while the KEGG pathway enriched in non-food allergic children was indicated with green. Only the taxa that met a LDA significant threshold of >2 are displayed. LEfSe: Linear discriminant analysis effect size. LDA: Linear discriminant analysis. KEGG: Kyoto Encyclopedia of Genes and Genomes.

7.5 Discussion

Other than gut and saliva microbiome, exposure to diverse environmental microbes appears to modulate mucosal immunity (236, 237), which in turn affects the pathogenesis of allergic diseases (72, 137, 138, 238). So far, only a few studies have considered the assessment of the indoor microbes in house dust (72, 137, 138). Most of these studies focused on other allergic diseases, instead of food allergy. Our objective for this study was to perform 16S rRNA gene sequencing in integration with network analysis to compare the house dust microbial compositions in food-allergic and non-food allergic children. The present study demonstrated that food-allergic children showed a distinct house dust microbial composition and a significant shift in microbial function when compared to non-food allergic children.

The house dust microbiome has a high diversity of microbial taxa, consisting of approximately 1000 identified taxa at the species level (239). Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria were previously identified as the dominant taxa in the house dust microbiome (72, 106, 240, 241). Similar to these studies, we also observed house dust microbiome to be dominated by Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria. Further to this, we also observed smaller contributions of Fusobacteria, Saccharibacteria, Nitrospirae, Tenericutes. Absconditabacteria (SR1), Cyanobacteria, Acidobacteria, Planctomycetes, Spirochaetae, Synergistetes, Deinococcus-Thermus, Verrucomicrobia, Ignavibacteriae, Chloroflexi and Gemmatimonadetes in the house dust microbiome.

Through network analysis, we were able to identify two co-abundance network modules to be positively correlated with food allergy and these modules were characterized by several hub taxa, *Streptococcus*, *Gemella*, *Haemophilus*, *Neisseriaceae_unclassified*, *Actinomyces*, *Rothia*, *Alloprevotella* and *Prevotella 7*. Among these taxa, *Streptococcus* (242, 243), *Gemella* (219, 244), *Haemophilus* (245), *Neisseria* (246), *Alloprevotella* (219) and *Prevotella* (246) had been previously shown to be enriched in subjects with allergic diseases. Although *Actinomyces* (247) and *Rothia* (248-250) were not previously associated with allergy, they are known for their roles in exacerbating inflammation and causing infectious conditions. A mouse study demonstrated *Actinomyces naeslundii*, a species of *Actinomyces* genus was able to stimulate the secretion of pro-inflammatory cytokines such as interleukin 1β, interleukin 6 and tumour necrosis factor (251). Another study revealed *Rothia dentocariosa*, a species of Bacteroidetes, was able to activate toll-like receptors 2 and Nuclear factor kappa B, which induced the production of tumor necrosis factor alpha, leading to infection(252). Collectively, the above results showed an increase of pathogenic microbes in food-allergic children, which suggested that dysbiosis of the house dust microbiome was involved in the development of food allergy.

The KEGG pathway analysis indicated that these perturbed house dust microbiome in food-allergic children were strongly associated with dysregulation of cofactors and vitamins metabolic processes such as folate, nicotinate and nicotinamide. Viewing that gut microbiome is known for its role in modulating DNA methylation in host cells via production of epigenetically active metabolites such as folate (253), hence, exposure

to specific microbes in the indoor environment may affect gut microbiome to produce epigenetically active metabolites and later cause changes in DNA methylation patterns, leading to the pathogenesis of food allergy.

In contrast, the KEGG pathway analysis indicated that house dust microbiome in non-food allergic children were strongly associated with amino acid metabolism (arginine and proline metabolism, histidine metabolism, as well as valine, leucine and isoleucine biosynthesis). Considering that gut microbiome produces amino acids that modulate the intestinal immunity functions (254), hence, the exposure to specific microbes in the indoor environment may affect gut microbiome to produce amino acids, which may regulate the intestinal immunity functions, contributing to protection against food allergy.

Although we observed obvious discrepancies in the co-abundance network of house dust microbiome and predicted metagenome functions between food-allergic children and non-food allergic children, the relative abundance of these taxa was not significantly different between children with and without food allergy after multiple testing. This was likely due to a relatively small sample size, which affected the statistical power. In addition, we caution that our results cannot determine causality. Besides, we were aware that other environmental factors, such as cleaning practice, diet and pet contact might also be related to observations made in this study. Another limitation of our study is the single dust sample from each house as we assume this sample reflects the usual home condition. To the extent that a single sample is not a good reflection of the usual exposure, this limitation would tend to bias our results

toward the null rather than result in false-positive associations. Further studies should consider to use vacuum devices with filters to collect house dust samples with several locations in the house along with the standardization of sampled location and the collected data should be analysed in conjunction with environmental factors to further elucidate the association between food allergy and the house dust microbiome.

Despite these limitations, the findings of this study are valuable for understanding the association of house dust microbiome and food allergy due to two reasons: First, the present study is the first study to thoroughly characterize the house dust microbiome of food-allergic children by applying network analysis. This approach allowed a relatively comprehensive characterization of house dust microbial communities associated with food allergy including those microbial populations with low relative abundance but highly relevant to food allergy. Second, rather than assessing endotoxin levels as an indirect measurement of house dust microbiome, we utilized 16S rRNA gene sequencing approach to identify specific house dust microbial composition.

7.6 Conclusion

Our findings indicate that the microbiome in food-allergic children is different from that in non-food allergic children. We also found enrichment of *Streptococcus*, *Gemella*, *Haemophilus*, *Neisseriaceae_unclassified*, *Actinomyces*, *Rothia*, *Alloprevotella* and *Prevotella 7* in the house dust microbiome of food-allergic children. The house dust microbiome of these food-allergic children was mainly involved in

cofactors and vitamins metabolism. In contrast, house dust microbiome of non-food allergic children was mainly involved in amino acid metabolism.

8 GENERAL DISCUSSION AND CONCLUSION

8.1 Overall findings

The prevalence of food allergies and food-related anaphylaxis admissions have increased dramatically over the last few decades and this bears a globally significant health burden (1). The underlying mechanisms that contribute to food allergy are thought to be heterogeneous (2). Several risk factors, particularly related to genes and the human microbiome, have been previously reported to play a role in the pathogenesis of food allergy. At present there are no clear food allergy related diagnostic biomarkers and the pathophysiology of food allergy remains poorly understood. Notably there are no studies that report a comprehensive evaluation of food allergy susceptibility with the host transcriptome and microbial composition of multiple sites at once. The present study aims to fill this knowledge gap by exploring both the host transcriptome profile and microbiome profiles of food-allergic children and non-food allergic children, and seeks to systematically elucidate the association of gene expression and microbiomes with food allergy. We also investigated the house dust microbiome in the bedroom of these children with and without food allergy.

The present study finds significant differences in the profiles of both the host transcriptome and the microbiomes in children with and without food allergy, thereby extending opportunities to identify prognostic biomarkers as well as therapeutic interventions. In particular, we demonstrate for the first time that the *IFIH1*, *DRAM1* and *ZNF512B* genes are nut allergy susceptibility genes. Our study also demonstrates that the cellular immune response in children with nut allergy is characterised by a significantly lower CD4+ T-cell /Treg response and a higher neutrophil response

compare to children without nut allergy. This finding significantly improves the understanding of the detailed molecular mechanism of food allergy. Moreover, we also observe a significant difference in the beta diversity of both the saliva microbiome and the house dust microbiome when comparing food-allergic children and non-food allergic children. We identify an enrichment of pro-inflammatory microbial taxa in the gut and house dust microbiome and a depletion of anti-inflammatory microbial taxa in the saliva of food-allergic children when compared to non-food allergic children. Collectively, our study suggests that the pathogenesis of food allergy is connected to an upregulation of the food allergy susceptibility genes, to dysregulated immune cell responses, including upregulated neutrophil responses and downregulated CD4+ T-cells /Treg responses, and to alterations of the gut and oral microbiome configuration, including a perturbed taxonomic composition, along with the environmental house dust microbiome.

The host transcriptome has received much attention in order to understand the pathogenesis of allergic diseases (87-90). Detailed functional studies are needed to determine the functional roles of the novel genes, *IFIH1*, *DRAM1* and *ZNF512B*, which are identified in present study. So far pathway analysis shows that these genes are enriched with type 1 interferon signalling. Type 1 interferons can activate interferon-alpha receptors, as well as Janus kinase/signal transducers and activators of the transcription pathway, leading to the secretion of inflammatory cytokines (178). Attenuation of type 1 interferon responses has been previously observed in subjects who achieved oral tolerance (90).

In addition to an enrichment of type 1 interferon-related pathways in nut allergy, we also observed a downregulation of the CD4+ T-cells /Treg response in nut allergy. CD4+ T-cells /Treg responses have been previously shown to play a critical role in immune response and oral tolerance, and downregulation of CD4+ T-cells /Treg responses are commonly associated with the pathogenesis of allergic diseases, including food allergy (88, 91, 92). CD4+Foxp3+ Treg cells can inhibit the activation of dendritic cells, mast cells, basophils, and eosinophils, suppress the production of allergen-specific IgE, inhibit effector functions and the migration patterns of Th1, Th2, and Th17 cells, as well as promote the secretion of IgG4. Hence, a reduced CD4+Foxp3+ Treg cell responses can result in the breakdown of oral tolerance.

We also observed an upregulation of neutrophil responses in nut allergy. Consistent with our findings, previous studies identified that neutrophils play an important role in inducing anaphylactic reactions (87, 89). The exposure to allergens can trigger the activation of neutrophils which in turn produce interleukin 1, interleukin 6, interleukin 8 and interleukin 12, tumour necrosis factor-α, and transforming growth factor-β. The activation of neutrophils can also express the high-affinity receptor for both immunoglobulin E and immunoglobulin G, leading to the amplification of the allergic reaction (255). Our and previous findings collectively support that targeting type 1 interferons, CD4+ T-cells /Treg responses and neutrophil activity may help to advance therapy of food allergy and to identify biomarkers associated with food allergy.

Other than host gene regulation, the microbiomes of multiple sites in the human body are also involved in the development of allergic diseases, including food allergy (72,

106-109, 134). To investigate the potential association between these microbiomes and food allergy, we compared the microbiome composition between food-allergic children and non-food allergic children in the gut and oral cavity, and characterized the functional capacity of these microbiomes using network analysis. The gut microbiome of the food-allergic children is characterized by a hub taxon, namely *Ruminococcaceae UCG-002*, and the other dominant taxa are mainly from Firmicutes phylum, which have been previously associated with allergic diseases (106, 107, 205). These gut microbial taxa are involved in functions related to methane metabolism and glycerolipid metabolism. A high-fat diet has been shown to stimulate the production of methane and glycerolipid (207), which in turn induces gut dysbiosis and gut permeability, stimulates mast cell accumulation, and promotes total IgE responses, contributing to the development of food allergy (204). In contrast, the gut microbiome of non-food allergic children is enriched in the metabolism of cofactors and vitamins, particularly in ubiquinone and other terpenoid-quinone biosynthesis. Coenzyme Q10, which is a kind of ubiquinone, has been previously suggested to have an inverse association with allergic diseases (210, 256). Coenzyme Q10 has been suggested to have protective effects towards allergic diseases through decreasing the accumulation of eosinophils (210) as well as reducing IgE levels (257) and inflammation (258).

The oral microbiome improves the host immunity and contributes to modulating inflammation (131) and allergic reactions (134). *Streptococcus*, one of the most predominant genera of the oral cavity, is identified as the hub taxon for the oral cavity of the non-food allergic children in this study. Consistent with our findings, a study

conducted by Matsui et al also observed that the counts of *Streptococcus* increased in the oral cavity of control mice compared with mice sensitized with ovalbumin (134). Mouse studies have further demonstrated that *Streptococcus* aids to suppress allergic inflammation (259-261). *Streptococcus* was shown to redirect the Type 2 T helper (Th2) allergic response to a Type 1 T helper (Th1) regulatory response (262), to increase the proportion of regulatory T cells (Treg) (259), to increase the expression of anti-inflammatory cytokines such as Interleukin 10, to decrease the expression of pro-inflammatory cytokines such as Interleukin 5 and Interleukin 13 as well as to decrease the total circulating Immunoglobulin E (259), which in turn attenuated allergic immune responses. These observations show that certain oral microbial taxa take part in regulating the allergic inflammation.

There are several possible explanations for the correlation of the gut and oral microbiome with food allergy outcomes. The human gut microbiome is unstable in the first few years of life during the initial colonization and development of the gut, and gut microbial colonization have previously been associated with immune development (263-265), allergic disease (266), and the response to food allergens (267). Our study identifies an alteration of the oral microbiome in food-allergic children and a previous study demonstrates that oral microbes can modify the gut microbiome to induce inflammatory responses (132). These results suggest that oral microbial dysbiosis can interact with the gut microbiome and contribute to the susceptibility to food allergy. Our study could not investigate this crosstalk between the oral and gut microbiome in further detail due to the limited sample size of children participating in both studies.

The microbial abundance of house dust influences the host immune system and has a complex interaction with the development of allergic diseases (72, 137, 138). The present study shows the enrichment of *Streptococcus*, *Gemella*, *Haemophilus*, *Neisseriaceae_unclassified*, *Actinomyces*, *Rothia*, *Alloprevotella* and *Prevotella 7* in the house dust microbiome of food-allergic children. These taxa are involved in functions related to the inflammation and dysregulation of cofactors as well as vitamin metabolic processes such as folate, nicotinate and nicotinamide. It has been suggested that a high concentration of unmetabolized folic acid and an abnormal nicotinate and nicotinamide metabolism in the host body can increase oxidative stress (268). Such excessive expression of oxidative stress may enhance the activity of Nuclear factor kappa B, which in turn increases the production of inflammatory cytokines, such as tumor necrosis factor alpha and interleukin 6, as well as acute phase reactants (269), leading to inflammation.

In contrast, the house dust microbiome of non-food allergic children is strongly associated with amino acid metabolism. Amino acid metabolism is known for its protective role towards food allergy. Mice model demonstrated that an amino acid based diet can help to alleviate allergic symptoms through an increase of FoxP3+ cell counts and reduction of serum IgG2a and IgG1 levels (270). Thus, we speculate that the environmental exposure to these house dust microbial taxa may modulate the host immune system via the dysregulation of cofactors, vitamin metabolic processes and amino acid metabolism, and as a consequence potentially cause the pathogenesis of food allergy. How exactly the domestic exposure to these house dust microbial taxa

affects the regulation of the host immune response and the development of food allergy remains to be determined.

8.2 Key findings

- Nut allergy was associated with the upregulation of neutrophil responses, and downregulation of CD4 T cell/Treg responses.
- *IFIH1*, *DRAM1* and *ZNF512B* were identified as hub genes for nut allergy.
- The InnateDB pathway analysis revealed that the upregulated genes were associated with type 1 interferons.
- *Ruminococcaceae UCG-002* was identified as the hub taxon of the gut microbiome co-abundance network module for food-allergic children and this network module was involved in the methane metabolism and glycerolipid metabolism.
- Functional prediction showed an enrichment of ubiquinone and other terpenoid-quinone biosynthesis in the gut microbiome of non-food allergic children.
- Food-allergic children had a distinct oral microbiome compared to non-food allergic children.
- *Streptococcus* was identified as the hub taxon of the oral microbiome co-abundance network module for non-food allergic children.
- *Streptococcus*, *Gemella*, *Haemophilus*, *Neisseriaceae_unclassified*, *Actinomyces*, *Rothia*, *Alloprevotella* and *Prevotella 7* were identified as the

dominant taxa of the house dust microbiome in bedrooms of food-allergic children and these microbiome taxa were mainly involved in cofactors and vitamins metabolism.

- Functional prediction showed an enrichment of amino acid metabolism in house dust microbiome of non-food allergic children.

8.3 Strengths of the study

One of the strengths of this thesis study was the robust phenotyping of the study population using clinically proven food allergy. The diagnosis of food allergy was based on the clinical examination by an immunologist on the day of recruitment. Another strength of this thesis study was the unbiased and comprehensive approach for the identification of the key genes and the microbial taxa for food allergy. RNA sequencing detected low-expressed genes and improved the quantification accuracy at the gene level of the phenotype while 16S rRNA gene sequencing allowed for a robust identification and profiling of the microbial communities. In addition, the application of network analysis identified the global connectivity structure of the gene expression and microbial taxa, and distinguished system-level features of the underlying biology for food allergy.

8.4 Limitations of the study

We discuss several limitations of this study and directions for future studies.

First, this study identified several key genes and microbiome taxa that are associated with food allergy, yet the gene expression and the relative abundance of the taxa were

not significantly different between children with and without food allergy after adjusting for multiple comparisons. This was likely due to the relatively small sample size which affects the statistical power. In addition, this is a cross-sectional study that adopt convenience sampling method, hence causal inference in regards the role of gene expression and microbiome taxa in the pathogenesis of food allergy cannot be made. Future study should consider applying a longitudinal study approach to investigate the association of multiple environmental factors, such as mode of delivery, presence of a sibling, maternal history of atopy, eczema, timing of allergenic food introduction, consumption of yoghurts and probiotic supplements with the pathogenesis of food allergy as well as the effects of these risk factors on the gene expression and microbiome composition.

Second, the primary aim of this thesis study was to utilize transcriptomic and microbiome signatures and to integrate those with network analysis to identify key genes and microbial taxa associated with nut allergy. Hence, this thesis did not address the functional roles of the identified key genes and microbial taxa and we discussed their possible roles based on published literature. Therefore, future mechanistic studies are needed to understand the role of the identified key genes and microbial taxa in more detail. The key genes and microbiome taxa identified in this thesis could inform the selection of targets for future mechanistic studies, which might involve in germ-free mice models to better illustrate the function of those targeted genes and microbiome taxa.

Third, using flow cytometry-based assays to target multiple cell populations was not feasible because the volume of blood collection from children was restricted. Instead we have applied an unbiased deconvolution approach to infer the proportions of 12 types of human cells in the whole blood transcriptome. In addition, our study cannot determine whether changes in cellular proportions precede or follow transcriptomic changes. Further experiments are needed to identify the cellular origin of the gene expression signals associated with nut allergy.

Forth, the studies described in chapters 5, 6 and 7 of this thesis have utilised 16S rRNA gene sequencing to comprehensively characterize the gut, saliva and house dust microbiome profile of children with and without food allergy. 16S rRNA gene sequencing allows for a cost effective, easy and robust identification and profiling of the microbial communities, yet can only identify and profile microbial communities down to the genus level, and not specific species and strains (271, 272). Moreover, 16S rRNA gene sequencing is unable to identify the microbial taxa directly and can only predict their functional profile. In contrast, shotgun metagenomics is able to identify and profile all genomic DNA (from bacteria to viruses) at a species or even strain level. In addition, shotgun metagenomics is also able to provide a functional characterization of microbial taxa (271, 272). Therefore, shortgun metagenomics should be utilized in future studies to further elucidate the association between food allergy and the microbiome at a species and strain level as well as identify the potential role of these microbial taxa.

Lastly, our samples were collected from different study subsets and we were unable to study the whole blood and microbiome samples from the same participants. Hence, we are unable to directly correlate the results of the genes and the microbiome. Future studies can apply a multi-omics approach by integrating transcriptomic and microbiome signatures for a better understanding of the immune mechanism of food allergy. To better characterize mechanisms of host–microbiome dysregulation during food allergy, future studies could consider to apply an integrated longitudinal molecular profiles of microbial and host activity. To the extent possible, multiple molecular profiles should be obtained from the same sets of samples, including transcriptomes, gut microbiome, oral microbiome and house dust microbiome profile, allowing concurrent changes to be observed in multiple types of host and microbial molecular over time.

8.5 Concluding remarks

In conclusion, integration of transcriptional profiling and network analysis identified several novel genes and associated immune cells that were different between children with and without nut allergy. This thesis study also identified a different microbiome profile in the gut and oral cavity of food-allergic children when compared to non-food allergic children. In addition, this thesis study also found a distinct house dust microbiome profile in the children's bedroom between food-allergic and non-food allergic children. Taken together, the results of this thesis suggest that the host transcriptome as well as gut, oral and house dust microbiomes are correlated with food

allergy, and that the transcriptome and microbiome exhibit a co-existence relationship with a complex correlation pattern likely resulting in the pathogenesis of food allergy. The overall findings of this thesis emphasize that the transcriptome and microbiome profiles have to be investigated as a linked entity, i.e. as a network, to gain a better and systematic understanding about the aetiology of food allergy. These data can assist in the intervention, management, treatment and possible prevention of food allergy.

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10 APPENDICES

APPENDIX A FOR CHAPTER 1

Chapter 1 has been reformatted from Lee, K.H., Song, Y., Wu, W. et al. The gut microbiota, environmental factors, and links to the development of food allergy. Clin Mol Allergy 18, 5 (2020). https://doi.org/10.1186/s12948-020-00120-x, published under a Creative Commons BY license (http://creativecommons.org/licenses/by/4.0/)

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REVIEW Open Access

The gut microbiota, environmental factors, and links to the development of food allergy

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Abstract

Food allergy appears to have its roots in an insufficient exposure to a diverse range of environmental microbiota during early life. Microbial exposure ensures the colonization of the gastrointestinal tract with commensal microbes, which is necessary for the induction of a balanced and tolerogenic immune function. High-throughput sequencing technology has facilitated in-depth studies of the gut microbiota as well as bacterial-derived metabolites. Although the role of the microbiota in allergies is now widely studied, its importance for food allergy was only recently noted. Studies in human cohorts have shown that there is an association of dysbiosis and pathogenesis of food allergy, while studies from animal models have demonstrated the capacity of specific species in the gut microbiota to alter immune response, which may lead to the desensitization of food allergy. This article reviews the role of the gut microbiota in food allergy, and discusses the influence of environmental factors as well as prevention and management strategies relating to such regulatory mechanism.

Keywords: Environmental factors, Food allergy, Immune system, Microbiota, Probiotics

Introduction

For decades, many cultivated microorganisms have been discovered by the microbiological culture technique, but these represent only a minority of the microbial species of the gut. The use of next generation high-throughput sequencing techniques has widened tremendously our knowledge of the human microbiota composition and its relationship to disease. The human body is estimated to consist of 10–100 trillion microbes, and more than 1000 bacterial species [1]. The highest number of microbes are found in the human gut [2]. The number of microbes found in the human gut are 10 times the number of cells making up the human body [3], although this ratio has been disputed [4]. Studies in the past few years have identified a critical role of the gut microbiota in shaping the immune system [5–8]. The gut microbiota is involved in the development of the organs of the immune system, and determines the tendencies of host immune responses. Research on the association between immune diseases and gut microbiota has reported that alterations in commensal bacteria can induce changes to the immune system [2, 9] which affect regulation of host metabolism, immune system maturation, and development of oral tolerance [10]. Thus, attempts to mitigate allergic diseases through adjusting the diversity and individuality of the gut microbiota have increased.

This review article outlines recent insights into the role of the gut microbiota in food allergy, the influence of environmental factors as well as prevention and management strategies involving the gut microbiota.

Dysbiosis and food allergy

Dysbiosis refers to a change in the microbiota composition and function such that it disrupts gut homeostasis and contributes to diseases [11]. There are increasing evidence from human studies suggesting that dysbiosis is associated with pathogenesis of food allergy (Table 1) [12–23]. Although these studies are not able to identify specific bacterial taxa that are

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Table 1 Main gut microbiota differences between patients with and without food allergy

Study	Types of food allergy	Association with food allergy	References
Bunyavanich et al.	Cow's milk	↓Clostridia, Firmicutes	[12]
Savage et al.	Cow's milk, egg, wheat, soy, nuts	↓*Citrobacter, Oscillospira, Lactococcus, Dorea*	[14]
Azad et al.	Cow's milk, egg, peanut	↑*Enterobacteriaceae* ↓*Bacteroidaceae*	[15]
Hua et al.	Peanut	↓Clostridiales ↑Bacteroidales	[16]
Inoue et al.	Egg, wheat, soybean, sesame, cow's milk, peanut, shrimp, crab	↓*Dorea, Akkermansia* ↑*Veillonella*	[17]
Ling et al.	Cow's milk, egg, wheat, nut, peanuts, fish, shrimp, soy beans	↓Bacteroidetes, Proteobacteria, Actinobacteria ↑Firmicutes	[18]
Chen et al.	Egg white, cow's milk, wheat, peanut, soy bean	↓Bacteroidetes ↑Firmicutes	[19]
Fazlollahi et al.	Egg	↑*Lachnospiraceae, Streptococcaceae, Leuconostocaceae*	[20]
Dong et al.	Cow's milk	↑Lactobacillaceae ↓*Bifidobacteriaceae, Ruminococcaceae*	[115]
Diaz et al.	Cow's milk	↓*Coriobacteriaceae*	[21]
Berni Carnani et al.	Cow's milk	↑*Bacteroides, Alistipes*	[22]
Kourosh et al.	Tree nuts, fish, milk, egg, sesame, soy	↑*Oscillibacter valericigenes, Lachnoclostridium bolteae, Faecalibacterium* sp.	[23]

consistently associated with food allergy due to heterogeneity in study design, such as different sampling time points, different techniques used to characterize the gut microbiota, and different allergic phenotypes, these studies show microbiota diversity and composition are significantly associated with the onset of food allergy.

Until now, it remains unclear how dysbiosis exactly affects the immune system in the development of food allergy but studies suggest that the gut microbiota influences the immune system by affecting host metabolism [2, 24–26] and the alteration of adaptive immunity [27, 28].

Metabolic and immune effects

Major attention has been directed to the possible role of short-chain fatty acids, such as butyrate, propionate, and acetate in affecting the immune system (Table 2) [29–32], since short-chain fatty acids are the main product of the digestive action of the gut microbiota [29, 30]. Production of short-chain fatty acids, particularly butyrate, is able to enhance the Vitamin A metabolism, in turns inducing the activity of ALDH in CD103+MLN DCs, and increasing the percentages of T regulatory (Tregs) cells and increasing IgA production [33]. Meanwhile, short-chain fatty acids are able to inhibit histone deacetylases activity, resulting in regulation of *aldh1a1* expression, which contributes to immune tolerance. Other than this, short-chain fatty acids can bind metabolite-sensing G-protein coupled receptors, GPR43 or GPR109A [34],

Table 2 Production of the short-chain fatty acids by the gut microbiota

Short-chain fatty acids	Pathway	Microbiota	References
Acetate	Acetyl-CoA	Most enteric bacteria	[31]
	Wood–Ljungdahl pathway	*Blautia hydrogenotrophica*	
Propionate	Succinate pathway	Negativicutes, Bacteroidetes	[32]
	Acrylate pathway	*Megasphaera elsdenii, Clostridium propionicum*	
	Propanodiol pathway	*Salmonella enterica, Roseburia inulinivorans, Ruminococcus obeum*	
Butyrate	Butyrate kinase and phosphotransbutyrylase	*Coprococcus* species	[31]
	Butyryl-coenzyme A (CoA):acetate CoA-transferase	*Faecalibacterium prausnitzii, Roseburia* spp., *Eubacterium rectale, Eubacterium hallii Anaerostipes* spp.	

in turn promoting the tolerogenic CD103+ DC function and protecting against food allergies [35]. Moreover, short-chain fatty acids can reduce the production of pro-inflammatory cytokines including IL-1β, IL-6, IL-17 [36], and meanwhile increase the production of anti-inflammatory mediators including IL-10 [36, 37]. Thus, short-chain fatty acids are viewed as a key factor in promoting immunological tolerance towards harmless antigen and preventing inflammation.

Adaptive immunity

Adaptive immune responses are divided into two types: humoral immunity, regulated by B cells [38, 39], and cell-mediated immunity, regulated by T cells. The role of Tregs, subset of CD4+ T cells in oral tolerance development to food allergen, have been confirmed in animal models [40, 41] as well as human studies [42, 43] in which the induction of allergen-specific Treg cells is highly associated with a favourable allergy outcome. Microbiota, especially Clostridia species, in this case, were shown to be able to induce the production of Tregs [44, 45], which helps to inhibit allergic inflammation and promote oral tolerance [12, 46–48].

Specific Taxa related to food allergy across multiple studies

Bacteroidetes and Firmicutes comprise 90% of the microbiota population [49] and the colonization of these two microbiota phyla is associated with the pathogenesis of food allergy [50], as summarized in Table 3.

Bacteroidetes

An association between Bacteroidetes phyla in the gut and food allergy was found in a few epidemiologic studies [15, 18, 19]. Using gene sequencing methods, these studies identified that children with various types of food allergy had a lower relative abundance of Bacteroidetes compared with healthy children. The exact role of Bacteroidetes is unclear. However, a murine study indicated that *Bacteroides fragilis*, a species of Bacteroidetes, was able to produce polysaccharide A (PSA), which increased the suppressive capacity of the Treg cells and increased the production of IL-10 from Foxp3+ T cells [51]. The accumulated IL-10 mediates tolerance at mucosal surfaces and prevents intestinal inflammation. Further clinical studies in humans are needed to examine the regulatory role of Bacteroidetes in the development of food allergy.

Firmicutes

Firmicutes is the largest microbiota phylum containing more than 200 genera [52]. Most of the Firmicutes detected in the gut belong to the *Clostridium* clusters *XIVa* and *IV*. Both groups comprise members of the genera *Clostridium*, *Eubacterium* and *Ruminococcus*. Firmicutes are the most important butyrate producer in the gut and help to reduce inflammation through the inhibition of histone deacetylase [30]. A number of studies using 16S rRNA gene sequencing have identified a lower relative abundance of bacterial class *Clostridia* (bacterial phylum Firmicutes) in children with food allergy compared to healthy children [12, 13, 53, 54]. It is an accepted scientific knowledge that *Clostridia* promotes the accumulation of Treg cells [44, 45, 47, 55]. However, a study conducted by Stefka st al [55] demonstrated that Clostridia elevated IL-22 expression by RAR-related orphan receptor gamma (RORγt)+ ILCs and T cells, which in turn increasing an early, innate, barrier protective response. At the same time, Clostridia also elevated the production of IL-17, which in turn reducing intestinal epithelial permeability. Contradictory results also exist that patients with food allergy showed an increase in levels of *Clostridia* [18, 19, 56]. The genera of Clostridia that have been identified so far to have an association with food allergy are *Clostridium* [13, 18, 19, 53] and *Ruminococcus* [13, 15, 19, 54]. Diesner et al. reported that mice tolerant to food allergy have higher abundances of Ruminococcaceae [54] compared with intolerant counterparts, supporting the potential protective effects of *Ruminococcus* on food allergy. Nevertheless some discordant results the Clostridia class is that more clearly involved in mechanism of food tolerance induction and in food allergy protection.

Early-life colonization and environmental factors

Some studies argue that the gut microbiota is critical in early life for the development of the immune system as well as the development of allergic diseases [15, 18,

Table 3 Gut microbiota taxa and alterations associated with food allergy

Phylum	Class	Genus	Species	Action in the Gut	References
Bacteroidetes	*Bacteroidetes*	*Bacteroidetes*	*Bacteroides fragilis*	Increase the suppressive capacity of Tregs and induce the production of IL-10 from Foxp3+ T cells	[51]
Firmicutes	*Clostridia*	*Clostridium* *Lactobacillus* *Ruminococcus*		Promote the accumulation of Tregs	[116]

19, 53, 57]. The gut microbiota is unstable in the first 2–3 years of life during the initial colonization and development of the gut. Disruptions of microbial colonization during this period have been shown to increase the disease susceptibility during life. After the age of three the gut microbiota progresses towards an adult-like configuration and it essentially remains stable unless perturbed [1, 5, 53].

The dramatic rise of food allergy in modern society has led to the postulation of the hygiene hypothesis [58]. The hygiene hypothesis proposes that a lack of early childhood exposure to infectious agents suppresses the development of the immune system which leads to the rise of atopic diseases. Recent work has revisited the hygiene hypothesis model to include mode of delivery, antibiotic intake, diet [59] and synthetic chemicals as factors in altering gut microbiota (Fig. 1).

Mode of delivery

Perturbation of gut microbiota may influence susceptibility to food allergy [60]. It is evidenced that infants born vaginally are exposed to both maternal fecal and vaginal microbiota while infants born by caesarean section are exposed to maternal skin and environmental microbes only [50]. For instance, infants born by caesarean section are more likely to have lower abundance of Bacteroides and higher abundance of Firmicutes. Both Bacteroides and Firmicutes were revealed to associate with development of food allergy [50]. A population based birth cohort study revealed that children born by caesarean section had a threefold higher chance of developing food allergy [61]. However, the role of the delivery mode in influencing the food allergy outcome remains unclear as the delivery mode only exhibits an effect on the immune system for the first 90 days [62].

Antibiotic intake

Mothers undergoing a caesarean section are more likely to receive antibiotics [62], which can perturb the composition of the gut microbiota and modify the risk for allergic outcomes [63].

A number of studies have attempted to elucidate how antibiotics modify the composition of the gut microbiota and lead to the pathogenies of food allergy [64–66]. These studies revealed that antibiotic administration altered and reduced the microbiota diversity. A study suggested that antibiotic administration may cause reconstitution of the flora, thereby resulting in failure of the signal transmission via Toll-like receptor 4 (TLR4) [64]. The inability to signal via TLR4 resulted in markedly increased peanut-specific IgE and Th2 cytokine responses, contributing to allergic responses. Nevertheless, the association of antibiotic intake in early life and the development of food allergy is debated [67]. In a Chicago Family Cohort Food Allergy study, 1359 families were recruited and structure questionnaires were administered. This study did not find any association between antibiotic intake in the first year of life and the diagnosis of food allergy [67].

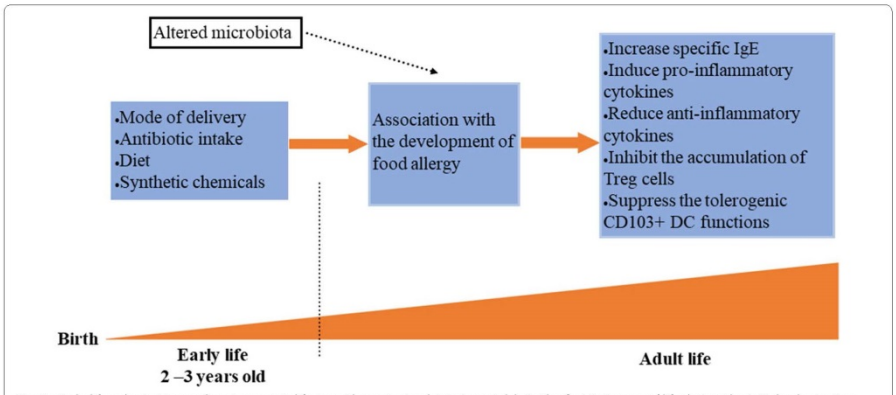

Fig. 1 Early-life colonization and environmental factors. The gut microbiota is unstable in the first 2–3 years of life during the initial colonization and development of the gut. During this period, the gut microbiota is vulnerable to some disruptions such as mode of delivery, antibiotic intake, diet and synthetic chemicals, which may increase specific IgE, induce pro-inflammatory cytokines, reduce anti-inflammatory cytokines, inhibit the accumulation of Treg cells, and suppress the tolerogenic CD103+ DC (dendritic cell) functions, contributing to the pathogenesis of food allergy. After the age of 3 the gut microbiota progresses towards an adult-like configuration and this remains the same throughout the life

Diet

Diet plays an important role in the establishment of the gut microbiota and affects allergic symptoms [68, 69]. The dietary intake of infants starts with milk, either breast milk or formula [70]. Breast milk contains human milk oligosaccharides which stimulate the growth of *Bifidobacterium bifidum* [71] and genus *Lactobacillus* [72], which are the main probiotic organisms in the gut, forming an acidic environment with enriched short-chain fatty acids [73]. Consequently, breastfed infants are colonized with *Bifidobacteria* and *Lactobacilli* compared with formula fed infants [74]. Additionally, the breastfeeding duration also has an influence on the development of food allergy as infants with a very brief breastfeeding duration have a higher risk of developing cow's milk allergy [75].

The milk intake is gradually replaced by a solid food diet that is similar to that of the adult and composed of a wide-variety of dietary macronutrients such as proteins, fats, carbohydrates and fibres [76]. These macronutrients are fundamental in determining microbiota composition and its effect on health outcomes.

After the introduction of complementary diet, the gut of infants exhibit more *Lactobacillus*, *Ruminococcus*, *Bacteroides*, *Peptostreptococcus* and *Clostridium*, which are tryptophan-catabolizing species [77]. Tryptophan is an amino acid commonly presented in high protein food, which is required for protein biosynthesis. Tryptophan metabolism plays a fundamental role in regulating the immune response as well as T cell proliferation. Other than this, tryptophan metabolism also induces IL-10 receptor-1 (IL-10R1) expression, which is essential in determining whether cells respond to IL-10, a potent anti-inflammatory cytokine that inhibits the release of pro-inflammatory cytokines [78].

Other than protein, fibre can also affect microbiota composition. The microbiota composition of the human gut associated with a high fibre diet is different from that seen with a high fat diet [79]. The gut microbiota with a high fat diet consisting of animal-based foods has higher abundance of *Alistipes*, *Bilophila* and *Bacteroides* and lower abundance of *Roseburia*, *Eubacterium* and *Ruminococcus* at genus level when compared to the gut microbiota with a high fibre diet [79]. The microbial conversions of dietary fibre to monosaccharides involve a number of metabolic pathways catalysed by the enzymatic activities of the gut microbiota and short-chain fatty acids are the major end-products of these microbial conversions [29, 30]. Mice studies were conducted in order to investigate the role of dietary fibre in suppressing immune response to food antigens [35, 80]. In these studies, mice were sensitized with food antigen and fed with either high fibre diet or no fibre diet. After few weeks of exclusion diet, mice receiving high fibre diet, showed significantly reduced symptoms of food allergy. Therefore, the "Western" diet with its high fat but low fibre content is proposed to be one of the reasons for the high prevalence of food allergy in Western countries [35, 81].

Synthetic chemicals

Synthetic chemicals play a role in perturbing the composition of gut microbiota as microbial metabolism of chemicals by gut microbiota may cause microbial dysbiosis [82], which in turn triggers allergy reaction [83, 84]. For example, food additives, one of the synthetic chemicals, are commonly found and used in our food as antioxidants, colourings, and flavourings, sweeteners and preservatives [83]. Mouse studies have indicated that such chemicals are associated with increased risks for food allergy [83, 84]. Food additives inhibited the accumulation of Treg cells, which is necessary for acquisition of oral tolerance [83]. The food additives also promoted the induction of allergic CD11b+ DCs, reduced the accumulation of tolerogenic CD103+ DCs as well as inhibited the induction of CD4+CD25hiT cells. Further to this, this study also suggested that intake of multiple food additives could increase the risk of developing food allergy.

Other synthetic chemicals, such as air pollutants, can be responsible for increasing the appearance of food allergy. Higher ambient levels of air pollutants, especially nitrogen dioxide have been consistently demonstrated to be associated with the increasing risk of allergies, including food allergies [85–87]. The mechanism of how air pollutant can cause food allergy remains unknown but studies suggested that inhaled air pollutants were able to directly or indirectly modify the composition of gut microbiota [88], which may cause an increase in the gut permeability and inflammation. Furthermore, inhaled air pollutants can lead to a substantial inflammatory response via reactive oxygen species (ROS) production and nuclear factor kappa B (NF-κB) activation in GI tract.

Therapeutic strategies

Human [12, 15, 18, 19, 53] and murine [54] models have demonstrated that study subjects with food allergy have distinct gut signatures and a different gut microbiota composition. Moreover, there is growing evidence that alteration of the gut microbiota may explain the development of food allergy [40, 41]. Microbial colonization has been shown to promote the induction of Treg cells, which is necessary to modulate the immune system and maintain tolerance to self-antigens [40, 41]. Microbiota may also influence the epigenetic modification of genes. It has been demonstrated that various forms of epigenetic

changes, such as DNA methylation and histone modification, regulate the immune system. Also microbial derived metabolites, such as short-chain fatty acids, have been shown to reduce pro-inflammatory cytokines and induce anti-inflammatory mediators as well as inhibit histone deacetylases [36].

Research indicates that environmental factors affect the microbiota composition making it an ideal target of research to find new interventions to desensitize food allergy.

Probiotic supplementation

The use of probiotic supplementation seems an attractive option for the prevention and treatment of allergic diseases. Probiotics are defined as "live microorganisms which, when administered in adequate amounts as part of food, confer a health benefit on the host" [89, 90]. Probiotics can act as promoters of an adequate balance in the gut microbiota to prevent the development of allergies. The beneficial effects of probiotics involve restoring intestinal permeability to normal, improving the intestine's immunological barrier function (both physical and mucous layer), promoting IgA production and inhibiting the release of proinflammatory cytokines through regulating gut microbiota composition [91, 92].

A well-characterised bacterial probiotic in desensitizing food allergy, especially cow's milk allergy, is *Lactobacillus rhamnosus* GG [93]. Dietary intervention with *Lactobacillus rhamnosus* GG was found to reduce allergic responses towards cow's milk in murine [94, 95] and human studies [93, 96, 97]. *Lactobacillus rhamnosus* GG was able to increase the production of different cytokines with proinflammatory (*TNF-α* and *IL-6*) or regulatory (*IL-10*) functions [98]. In addition, *Lactobacillus rhamnosus* GG also induced the accumulation of colonic Treg cells in the intestine [99]. In addition, *Lactobacillus rhamnosus* GG was able to increase *FoxP3* demethylation rate, increase IL-4 and IL-5 DNA methylation rate, reduce IL-10 and IFN-γ DNA methylation rate, increase the expression of miR-155, -146a, -128 and -193a, to promote the acquisition of cow's milk tolerance 12 months after treated with extensively hydrolyzed casein formula containing the probiotic *Lactobacillus rhamnosus* GG [97].

One study found that *Lactobacillus rhamnosus* GG showed a weak effect in desensitizing peanut allergy [100]. Most of the participants who received this probiotic and peanut oral immunotherapy passed the oral food challenge after receiving the treatment for 18 months and still passed the oral food challenge after 2 to 5 weeks after that. Moreover, participants who received probiotic and peanut oral immunotherapy showed a decrease in peanut sIgE levels and skin prick test wheal size, as well as an increase in peanut sIgG4 levels, at the end of treatment. Three months after treatment ended, these participants still had low sIgE levels and a small skin prick test wheal size. However, there was no convincing evidence that probiotic was effective in reducing allergic reactions to food as this study only compared between participants who received probiotic and peanut oral immunotherapy and participants who received placebo without including oral immunotherapy group only and probiotic group only. Also the prolonged tolerance towards peanut remains unclear as the participants in this study were not followed up beyond 3 months.

A mouse model of shellfish allergy demonstrated that oral administration of the probiotic strain *Bifidobacterium longum* reduced the specific IgE and stimulated dendritic cell maturation and CD103+ tolerogenic DCs accumulation in gut-associated lymphoid tissue. This, in turn, increased Tregs differentiation and suppressed Th2 responses [95, 101]. A murine study of cow's milk allergy showed that oral administration of *Bifidobacterium longum* subsp. *Infantis* LA308 strain induced the expression of IL-10 and skewed the immune response to Th1. These results are encouraging to find a candidate probiotic strain to perform a clinical trial.

Two other murine studies have reported the capacity of *Clostridium butyricum* to reduce adverse reactions to egg and cow's milk [46, 102]. The mechanism of how *Clostridium butyricum* is able to inhibit allergic inflammation remains unknown. Shi [102] suggested that the therapeutic effect of *Clostridium butyricum* may be generated by IL-10, as the IL-10-producing antigen specific Breg was found in mice with egg allergy which were administered with specific immunotherapy and *Clostridium butyricum*. On the other hand Zhang [46] suggested that *Clostridium butyricum* increased sIgA, CD4+CD25+Foxp3 Treg cell as well as reversed the imbalance of Th1/Th2 and Th17/Treg. These interesting results require further validation from human clinical trials.

There are three limitations on probiotic dietary intervention. First, the effect of probiotic is strain specific [103] and may vary depending on the individual's lifestyle and baseline gut microbiota profile [104]. Secondly, abrupt termination of probiotic dietary intervention may further increase the gut dysbiosis [105]. Lastly, there are only a few studies about probiotic dietary intervention and further studies are needed to validate the findings in human system.

Prebiotic supplementation

Prebiotic is defined as "nonviable food component that confers a health benefit on the host through modulation of the gut microbiota" [106]. An animal study reported

that cocoa, a source of antioxidant polyphenols, may be used to desensitize oral allergy [107]. In this study, rats administered with a 10% cocoa diet achieved oral tolerance and had a lower relative abundance of bacterial phylum Firmicutes and Proteobacteria and a higher relative abundance of Tenericutes and *Cyanobacteria* spp. compared to rats who received a standard diet, either orally sensitized or non-sensitized rats. It was suggested that the cocoa diet was able to increase the proportion of TCRγδ+ and CD103+CD8+ cells and decrease the proportion of CD62L+CD4+ and CD62L+CD8+ cells in mesenteric lymph nodes [108], regulate Treg cells function and reduce IgA production [107] through modulation of gut microbiota. This suggests that a cocoa diet has potential in regulating allergic immune responses in the human body and is of interest to investigate further.

Synbiotic supplementation

Synbiotics refers to a mixture of probiotics and prebiotics, designed to improve the survival of the beneficial microbiota as well as stimulate the growth of beneficial microbiota in the gastrointestinal tract [106].

A pioneer study tested a prebiotic blend of fructo-oligosaccharides with the probiotic strain Bifidobacterium breve M-16V. Infants with suspected non-IGE cow's milk allergy were administered with a hypoallergenic, nutritionally complete amino-acid based formula either with or without the synbiotics [109]. The relative abundance of *Bifidobacteria* was increased in infants with cow's milk allergy who were administered synbiotics. Moreover, the microbiota profile of these allergic infants became similar to that of healthy breastfed infants. More studies are required to confirm this therapeutic effect of synbiotics supplementation in desensitizing food allergy. It is a promising therapeutic strategy for improving the gut ecosystem and reducing food allergy responses.

Fecal microbiota transplantation

Fecal microbiota transplantation is a possible therapeutic for food allergy. Transplantation of fecal bacteria from a healthy donor to a disease recipient can re-establish gut microbiota diversity leading to the resolution of symptoms [110, 111]. As dysbiosis affects the development of food allergy [12], restoration of immune homeostasis and reconstruction of the impaired gut microbiota barrier by fecal microbiota transplantation may be able to promote the development of oral tolerance [110]. Recently, a human study has revealed that fecal microbiota transplantation is able to induce remission of infantile allergic colitis through restoration of gut microbiota diversity [112]. Although the available data in this field remain limited and the relevant scientific work has only just begun, this recent success in reducing infantile allergy colitis symptoms suggests that fecal microbiota transplantation can be a feasible strategy to arrest food allergy responses.

Synthetic stool substitute

Considering the strain specific effect of probiotic [103] and limited patient acceptance of fecal microbiota transplantation [113], a synthetic stool substitute was proposed in a pilot study [113]. In this study, a stool substitute with 33 strains was developed based on the fecal microbiota diversity of a healthy donor, also known as RePOOPulate. *Clostridium difficile* infection symptoms of patients were eradicated after 2–3 days of RePOOPulate treatment and this symptom free state lasted for 6 months. This synthetic stool substitute is so far only tested on two patients with *Clostridium difficile* infection, yet its potential benefit of reverting normal bowel pattern and restoring immune homeostasis may help to reduce allergic reactions towards food.

Microbiome-based therapy

Microbiome-based therapy can be viewed as a potential way in treating food allergy. In a mouse study, germ free mice were colonized with human feces from infants with cow's milk allergy and age and gender matched healthy infants [114]. Healthy-colonized mice had a higher abundance of *Anaerostipes caccae* when compared with cow's milk allergy colonized mice. Mice were then administered with *Anaerostipes caccae* in order to further investigate the role of *Anaerostipes caccae* in regulation of gene expression. *Anaerostipes caccae* were able to reduce the expression of Th2 dependent, antibody (serum BLG-specific IgE and IgG1), *Acot12* expression as well as cytokine responses IL-13 and IL-4, which promoted oral tolerance towards cow's milk allergy. This opens up a new perspective of food allergy therapy on human.

Conclusion

Studies have shown that the gut microbiota composition is vulnerable to disruptions in early life and that associated changes in host-microbiota homeostasis can cause food allergy. These studies support a regulatory role of the gut microbiota in the manifestation of food allergy, particularly in early life, but many questions remain and the underlying mechanisms are yet to be defined. The majority of our knowledge stems from animal studies and more human studies are required to validate the precise role of the gut microbiota in the development of food allergy. Evidence is emerging to suggest that therapeutic strategies in modifying gut microbiota composition are useful in the prevention, management and treatment of food allergy. Such strategies are apparent future research directions for developing prophylactic and therapeutic approaches against food allergy.

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KHL contributed to the preparation of the manuscript and the figure. YS, WW, KY and GZ conceived the review and revised the manuscript. All authors read and approved the final manuscript.

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APPENDIX B FOR CHAPTER 5

Chapter 5 has been reformatted from Lee, K.H., Guo, J., Song, Y., Ariff, A., O'Sullivan, M., Hales, B., Mullins, B.J., Zhang, G. Dysfunctional gut microbiome networks in childhood IgE-mediated food allergy. International Journal of Molecular Sciences, published under a Creative Commons BY license (http://creativecommons.org/licenses/by/4.0/).

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Dysfunctional Gut Microbiome Networks in Childhood IgE-Mediated Food Allergy

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Abstract: The development of food allergy has been reported to be related with the changes in the gut microbiome, however the specific microbe associated with the pathogenesis of food allergy remains elusive. This study aimed to comprehensively characterize the gut microbiome and identify individual or group gut microbes relating to food-allergy using 16S rRNA gene sequencing with network analysis. Faecal samples were collected from children with IgE-mediated food allergies ($n = 33$) and without food allergy ($n = 27$). Gut microbiome was profiled by 16S rRNA gene sequencing. OTUs obtained from 16S rRNA gene sequencing were then used to construct a co-abundance network using Weighted Gene Co-expression Network Analysis (WGCNA) and mapped onto Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. We identified a co-abundance network module to be positively correlated with IgE-mediated food allergy and this module was characterized by a hub taxon, namely *Ruminococcaceae UCG-002* (phylum Firmicutes). Functional pathway analysis of all the gut microbiome showed enrichment of methane metabolism and glycerolipid metabolism in the gut microbiome of food-allergic children and enrichment of ubiquinone and other terpenoid-quinone biosynthesis in the gut microbiome of non-food allergic children. We concluded that *Ruminococcaceae UCG-002* may play determinant roles in gut microbial community structure and function leading to the development of IgE-mediated food allergy.

Keywords: 16S rRNA gene sequencing; food allergy; microbiome; WGCNA; Ruminococcaceae

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1. Introduction

Emerging evidence has pointed towards the critical role of microbial communities in human health and disease, including regulation of the mucosal barrier function [1–4], metabolism [5–7] and host immune responses [3,4,8]. This is particularly evident in the gastrointestinal (GI) tract, where the diversity and richness of microorganisms are highest [9]. Changes in the gut microbiome commonly referred to as dysbiosis, may disrupt gut homeostasis and increase intestinal permeability, thereby causing immune system disorders such as autoimmune diseases and allergic disorders including food allergy [10–12].

Previous studies have started to unveil an association between the gut microbiome and the development of food allergy. A large observational cohort study in the United States showed that food-allergic children had a higher abundance of Bacteroidetes and a lower abundance of Firmicutes than children with resolved food allergy [13], while some studies showed the opposite results [14,15].

Considering the complexity of structure, function and compositional variability, the gut microbiome can be modelled and expressed as networks to infer the dynamic nature of the host–microbe interactions [16]. One approach to construct co-abundance network modules is to apply weighted gene co-expression network analysis (WGCNA) to quantify the co-abundance of operational taxonomic units (OTUs) across multiple samples. Developed by Horvath and colleagues, WGCNA was initially used to construct gene networks based on their similar biological functions and identify the hub gene that may associated with phenotypic traits [17]. We used WGCNA in this study to analyse the association between gut microbiome and disease phenotype by forming the complex microbial communities into different co-abundance network modules in order to identify hub taxa, the centralities of these co-abundance modules. Through this, we expect that WGCNA will identify potential target microbes, which may play a key role in regulating/influencing the microbe–microbe interactions, leading to the onset of food allergy.

2. Results

2.1. Gut Microbial Alpha Diversity

A total of 60 samples were included in our final analysis (33 food-allergic children and 27 non-food allergic children). Thirty-nine percent of the subjects were boys, with the median age for non-food allergic children and food-allergic children of 5.9 years and 5.0 years, respectively. The groups did not significantly differ from each other with regard to age ($p = 0.200$) and gender ($p = 0.525$). The food allergies noted in the food-allergic children included nuts ($n = 23$), egg ($n = 4$) and mixed allergies ($n = 6$).

To determine the average species diversity in a habitat or specific area, alpha diversity was evaluated using Chao1, Shannon index and observed OTUs matrices. Chao1 showed that non-food allergic children had lower species richness compared to food-allergic children, while Shannon index and observed OTUs showed that non-food allergic children and food-allergic children had similar gut microbial community richness and evenness (Table 1).

Table 1. Comparison of gut microbial alpha diversity between food-allergic children and non-food allergic children. Values represent mean ± SD.

Alpha Diversity	Non-Food Allergic Children	Food-Allergic Children	p
Chao1	565.7 ± 91.7	622.3 ± 87.4	**0.02**
Observed OTUs	458.9 ± 86.0	502.8 ± 83.9	0.058
Shannon diversity index	5.3 ± 0.7	5.5 ± 0.7	0.395

Bold value indicates a statistically significant difference with a p-value less than 0.05.

2.2. Gut Microbial Beta Diversity

To determine the degree of inter-group dissimilarity, beta diversity was evaluated using unweighted and weighted UniFrac distance matrices. Beta diversity did not show a significant difference between food-allergic children and non-food allergic children (Supplementary Figure S1).

2.3. Gut Microbial Composition

OTU dataset for food-allergic children and non-food allergic children consisted of 7 phyla, 14 classes, 16 orders, 28 families and 105 genera. At the phyla level, the gut microbiota was dominated by Firmicutes and Bacteroidetes, with lower abundance of Proteobacteria, Verrucomicrobia, Actinobacteria, Tenericutes and Cyanobacteria (Supple-

mentary Figure S2) in children with and without food allergy. There was no significant difference in the phylum level between food-allergic children and non-food allergic children (Supplementary Table S1, Online Supplemental Notes).

One hundred and five genera were identified, and only 18 genera were accounted for more than 1% across all samples (Supplementary Table S2, Online Supplemental Notes). There was no significant difference in the genera level between food-allergic children and non-food allergic children.

2.4. Microbial Co-Abundance Network Modules

To better characterize gut microbial taxa in food-allergic children, we applied WGCNA to identify clusters of microbial taxa whose differential representation was correlated with food allergy. Each cluster was represented as a colour module.

Through WGCNA, we were able to identify 14 modules of co-abundant taxa and the number of taxa within modules ranged from 32 to 167 (Table 2). Among all the taxa, only 167 taxa (17%) were not included in any colour module, and these taxa were grouped into the grey module as per default.

Table 2. The number of taxa in the 14 modules.

Module Colours	Frequency
Black	54
Blue	88
Brown	88
Green	67
green-yellow	34
Grey	167
Magenta	48
Pink	51
Purple	47
Red	66
Salmon	32
Tan	33
Turquoise	114
Yellow	82

2.5. Hub Taxa Associated with Food Allergy

The module eigengenes between children with and without food allergy were further compared with using module trait association analysis to identify the food allergy-associated modules.

Our results showed that a co-abundance network module (turquoise) was positively correlated with food allergy ($r = 0.27$ $p = 0.04$) (Figure 1). Particularly, *Ruminococcaceae UCG-002* was identified as the hub taxon (TaxaSignificance > 0.2 and Module Membership > 0.8) (Figure 2) for this module. In addition, 10 dominant taxa (>1% relative abundance across all samples) were also identified in the module. The majority of the dominant taxa came from Firmicutes phylum, including the genera of *Ruminococcaceae UCG-002*, *Eubacterium oxidoreducens group*, *Eubacterium coprostanoligenes group* and *Lachnospiraceae* (*NK4A136* and *UCG-008*). Other than this, the dominant taxa also included genera taxa from the phyla of Bacteroidetes (*Bacteroides*, *Alistipes*, *Parabacteroides* and *Prevotella* 2) as well as Proteobacteria (*Rhodospirillaceae*).

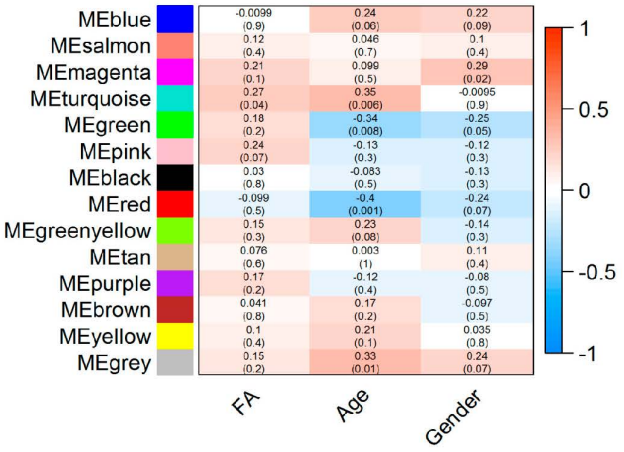

Figure 1. Module-trait associations. Each row corresponds to a module eigengene (ME) while each column corresponds to either phenotype (FA: Food allergy) or demographic traits such as age and gender. Each cell contains the corresponding correlation coefficient (display at the top of the cell) and corresponding p-values for each module (display at the bottom of the cells within parentheses). Blue and red colours of the spectrum on the right denote low and high correlation, respectively.

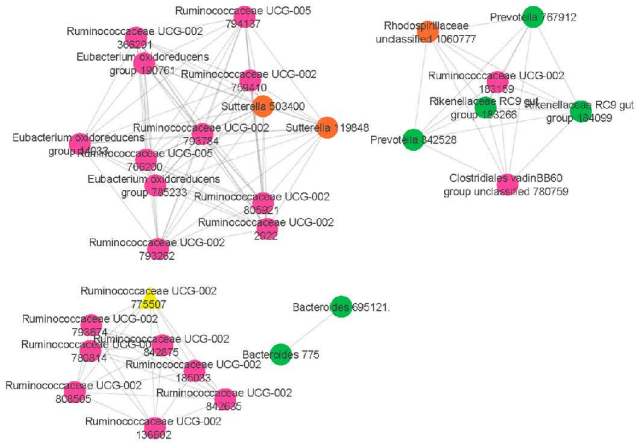

Figure 2. Network analysis identifies a distinct module of co-associated taxa. The highly correlated taxa in the comparisons of food allergic children and non-food allergic children are indicated and colour coded according to the phylum. Green colour represents Bacteroidetes phylum, pink colour represents Firmicutes phylum while orange colour represents Proteobacteria phylum. Hub taxon (yellow triangle) exhibits greatest intramodular connectivity, whereas connector taxa (circles) exhibit a higher frequency of intramodular connectivity.

2.6. Predicted Functional Pathway of Gut Microbial Taxa Associated with Food Allergy

In order to have a better understanding of the functional pathway of gut microbial taxa that are associated with food allergy, linear discriminant analysis effect size (LEfSe) was performed by using the Tax4fun output. Using the threshold values (LDA > 2.0, $p < 0.05$), LEfSe revealed distinct KEGG pathway differences between gut microbiota of food-allergic children and non-food allergic children (Figure 3). Specifically, methane metabolism and glycerolipid metabolism were found to be enriched in food-allergic children. In contrast, ubiquinone and other terpenoid-quinone biosynthesis, as well as *Vibrio cholerae* pathogenic cycle were found to be enriched in non-food allergic children.

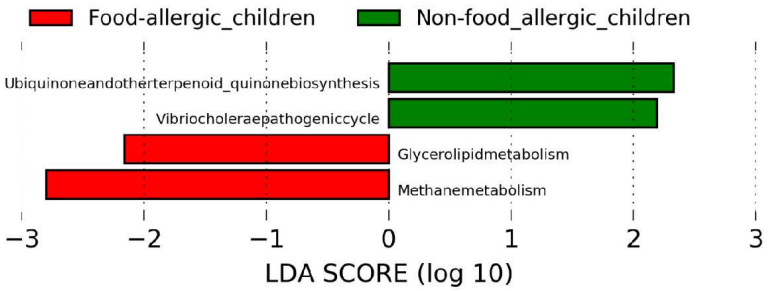

Figure 3. Linear discriminant analysis effect size (LEfSe) analysis revealed distinct KEGG pathway differences in gut microbiota between food-allergic children and non-food allergic children. KEGG pathway enriched in food-allergic children was indicated with red while the KEGG pathway enriched in non-food allergic children was indicated with green. Only the taxa that met a LDA significant threshold of >2 are displayed. LEfSe: Linear discriminant analysis effect size. LDA: Linear discriminant analysis. KEGG: Kyoto Encyclopedia of Genes and Genomes.

3. Discussion

There is increasing evidence that alterations in the gut microbiome are related to the development of food allergy [13–15,18], although the specific microbe associated with the pathogenesis of food allergy remains elusive. Our objective for this study was to perform 16S rRNA gene sequencing in integration with network analysis to characterize the gut microbiome and identify individual gut microbes or network modules of them that differ between food-allergic children and non-food allergic children. To our knowledge, this is the first study to characterize the gut microbiome of food-allergic children by applying network analysis.

Through network analysis, we identified a co-abundance network module (turquoise) to be positively correlated with food allergy and this module was characterized by a hub taxon, *Ruminococcaceae UCG-002* (Firmicutes phylum). It is suggested that a high relative abundance of *Ruminococcaceae* is associated with both food allergies [15], and a high fat diet in murine models [19–21], a factor which is known for its association with the development of food allergy. Taken together, these findings suggest that the high relative abundance of *Ruminococcaceae*, induced by a high fat diet, may produce acetic and propionic acid that possibly promote the synthesis of lipogenesis and cholesterol [22], which in turn dysregulated intestinal effector mast cell responses, as well as increased intestinal permeability and gut dysbiosis [23], leading to exacerbations of allergic responses.

We also identified a number of dominant taxa in this co-abundance network module that were highly related with food allergy, with the majority of them coming from phylum Firmicutes. Firmicutes has been suggested to play a role in modulating the immune system and subsequent development of allergic diseases [14,24]. A case-control study was conducted to investigate the association of gut microbiome and food allergy by comparing the gut microbiota composition between 34 infants with food allergy and 45 healthy

controls [14]. The data revealed that the relative abundance of Firmicutes in food-allergic subjects was higher than that of the control subjects. Another study conducted by Chen et al. [24] also showed that Firmicutes was enriched in food-sensitized children.

The enrichment of pathways related to methane metabolism and glycerolipid metabolism (a subcategory of lipid metabolism) in the gut microbiome of food-allergic children was observed. However, KEGG pathways related to metabolism of cofactors and vitamins (ubiquinone and other terpenoid-quinone biosynthesis) was significantly enriched in the gut microbiome of non-food allergic children. Methane is the anaerobic fermentation product of endogenous and exogenous carbohydrates through intestinal microbiota [25]. The increase production of methane caused by high fat diet [26] may cause gastrointestinal disorders [25,27]. Our finding of enriched glycerolipid metabolism in food-allergic children was consistent with recognized roles of dietary lipid in regulating inflammation and food allergy [23,28]. A high-fat diet has been previously shown to change gut microbiota composition, leading to inflammation and food-allergic reactions. In contrast, the key role of ubiquinone in protecting against food allergy has been gaining attention lately. The deficiency of coenzyme Q10, which is a kind of ubiquinone, may develop and worsen the progress of food allergy in children [29].

Our finding of increased gut microbiota diversity in food-allergic children when compared with non-food allergic children appears contrary to several other food allergy studies, in which gut microbiota diversity was higher in healthy controls than food-allergic subjects. However, a study conducted by Fazlollahi et al. [15] has also shown that gut microbiota diversity could be higher in children with egg allergy compared to controls. Some other studies reported no association between gut microbiota diversity and food allergy [14,30]. This has indicated a subtle relationship between gut microbiota diversity and food allergy. Hence, the role of microbiome in food allergy was suggested to be considered along with the interplay between different taxa and their metabolic effects rather than only examining a single dimension, bacterial diversity.

Taken together, we speculate that increased abundance of *Ruminococcaceae* along with other dominant microbial taxa, may remodel the normal gut microbial ecosystem into a state of dysbiosis through the pathways of methane metabolism and glycerolipid metabolism, which in turn elicit a host IgE-mediated allergic response. Our findings highlight the usefulness of network analysis in disentangling the hub taxon, *Ruminococcaceae* that play determinant roles in gut microbial community structure and functions leading to IgE-mediated food allergy. The differences in the co-abundance patterns of gut microbiome between children with and without food allergy can help us understand the complex interrelationships between gut microbiome and pathogenesis of food allergies. This information potentially aids targeted dietary or probiotic strategies for clinical practice to improve food allergy outcomes. Although our study revealed there was an association between gut microbiome network and development of food allergy, there were several limitations in the study. Firstly, the sample size was small. However, the application of network analysis in our study has deciphered key microbial populations that may be associated with food allergy, including those with low relative abundance but highly relevant to the onset of food allergy through characterizing the interactions of microbes at the community scale. Secondly, 16S rRNA gene sequencing is only sensitive to the genus level, but not species and strains. Thirdly, as this was a cross-sectional study, our results could not indicate a causal relationship between the gut microbiome and food allergy. Finally, as our study aimed to construct a microbial network through 16S rRNA gene sequencing and weighted correlation network analysis, the actual roles of these taxa predicted to be related to food allergy have not yet been evaluated. Therefore, further studies utilizing metagenomic analysis or real-time PCR in larger cohorts are required to confirm our results.

4. Materials and Methods

4.1. Study Subjects

From January 2018 to March 2019, children with immunologist-diagnosed food allergy, were recruited from Immunology Outpatient Clinic, Perth Children's Hospital. Children from 1 year old to 7 years of age with immunologist-diagnosed food allergy were eligible for participation. Non-food allergic children, with age and gender matched were recruited from the local community.

All parents of the subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the National Health and Medical Research Council National Statement on Ethical Conduct in Human Research, and the protocol was approved by the Human Research Ethics Committee (HREC), Perth Children's Hospital (RGS151/HREC 2017060EP) and Curtin University (HRE2017-0712).

4.2. Faecal Sample Collection and Processing

Parents/guardians of the participants were provided a faecal collection kit, which included a protocol of faecal collection, a screw cap faecal container (Sarstedt, Germany), an underpad sheet, a pair of disposable gloves, a white paper bag and a sealed plastic bag with labels. Once collected, the faecal sample would then be transported on ice by a researcher within 2 hours of collection to the laboratory −80 °C freezers for storage.

DNA was then extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Germany) in accordance with the manufacturer's instructions. The PCR amplication and sequencing of sixty stool samples were performed by Novogene Bioinformatics Technology Co., Ltd. (Beijing, China). Briefly, PCR was carried out using Phusion® High-Fidelity PCR Master Mix and GC Buffer (New England Biolabs, Beijing, China) in accordance with the manufacturer's instruction. PCR thermal cycling was set as follows: initial denaturation at 98 °C for 1min, followed by 35 cycles at 98 °C for 10 s, 50 °C for 30 s and 72 °C for 90 s, and a final extension at 72 °C for 5 min. The samples were then subjected to electrophoresis on a 2% agarose gel for detection. Samples with a bright main strip between 400 and 450 bp were chosen for further analysis. The PCR products were purified using the Gene JET Gel Extraction kit (Thermo Scientific), and the sequencing libraries were constructed using Ion Plus Fragment Library Kit (Thermo Fisher Scientific, USA) in accordance with the manufacturer's instruction. The library quality was monitored using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, St. Louis, MO, USA) and a Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA). Lastly, the library, which targeted the V3–V4 region of the 16S rRNA gene was sequenced on the Ion S5 XL platform (Thermo Fisher). A total of 4,858,507 sequences reads that passed the quality check (>Q20, error rate < 1%) were generated.

4.3. Quantitative Insights into Microbial Ecology (QIIME)

The raw sequences were then demultiplexed and quality filtered using QIIME [31] by removing those raw sequences with read-quality score less than 19, setting length fall below 3bp and consecutive quality base below 75%. The filtered sequences were then screened for chimeras using the usearch61 algorithm [32] and putative chimeric sequences were removed from the dataset. Sequences were clustered into operational taxonomic units (OTUs) at a 97% similarity level against the SILVA reference database (release 128) [33]. The OTUs with low relative abundance (less than 0.005%) were removed. All further analyses were performed at a rarefied depth of 22,178 sequences per sample to correct for differences in the read depth across samples. Alpha diversity and beta diversity of microbial communities were analysed using QIIME. Alpha diversity was estimated using two different indices: (1) Chao1, which takes into accounts only the abundance; (2) observed OTUs, which takes into accounts only the observed species; (3) the Shannon index, which takes into accounts the abundance and evenness of OTUs. Beta diversity was measured using the weighted and unweighted UniFrac distance matrices. Principal Coordinate Analysis (PCoA) was obtained to visualise unweighted and weighted Unifrac

distances in a two-dimensional structure. The Adonis permutational multivariate analysis (Adonis/PERMANOVA) was performed to compare beta diversity dissimilarity matrices. A comparison of the relative abundance of OTUs between groups was computed using the Mann–Whitney test. A probability value of $p < 0.05$ was considered statistically significant.

4.4. Construction of Microbial Co-Abundance Network

In order to have a better understanding of the co-abundance network of the microbial taxa, Weighted Gene Correlation Network Analysis (WGCNA) package of R [17] was then performed to conduct network analysis by using OTU count data (with 97% identity threshold), which has undergone Hellinger transformation, by transforming OTU count data from absolute to relative abundance that gives low weights to variables with low counts and many zeros [34].

Taking into account that the use of correlation analysis in analysing the microbiome data can lead to a spurious association, WGCNA applied few steps to reduce the number of false positive connections introduced by spurious associations [17]. A soft thresholding power β was determined based on scale-free topology index (R2) of 0.85. The most appropriate soft thresholding power was then used to construct a weighted adjacency matrix to which the co-abundance similarity has been raised. By raising the absolute value of the correlation to a soft thresholding power ($\beta \geq 1$), this step emphasized a strong correlation coefficient. Then, to further minimize the effects of noise and spurious associations, the adjacency matrix was transformed into a topological overlap matrix and the corresponding dissimilarity was calculated. This topological overlap matrix was particularly useful when the original adjacency matrix was sparse or susceptible to noise by replacing the isolated connections with weighted neighbourhood overlaps, thus, reducing the effects of spurious associations leading to a more robust network. The modules were subsequently identified using a dynamic tree cut algorithm with a minimum cluster size of 30 and merge cut height of 0.25 and later assigned the clusters of highly co-occurred taxa to different colours for visualization.

After that, module trait association analysis was used to calculate the correlation coefficient between modules and food allergy as well as demographics traits such as age and gender. Modules with p values < 0.05 were regarded significant food allergy-related modules.

4.5. Hub Taxa Selection and Visualization

Next, an intramodular analysis was performed to determine the hub taxa by summing the connection strengths with other module taxa. Moreover, the hub taxa have to meet the absolute value of the TaxaSignificance > 0.2 and Module Membership > 0.8. Taxa of the significant modules were then visualized using Cytoscape v3.8.0 [35].

4.6. Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Analysis

All OTUs table and OTUs taxonomy were mapped onto Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways using R package, Tax4Fun. Linear discriminant analysis (LDA) effect size (LEfSe) analysis (http://huttenhower.sph.harvard.edu/lefse/ (accessed on 18 February 2021)) was performed to detect biomarkers of the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways that differed significantly between non-food allergic children and food-allergic children. Default settings (alpha = 0.05, effect-size threshold of 2) were applied.

5. Conclusions

Our study provides a better understanding of the gut microbiome with respect to the presence of *Ruminococcaceae UCG-002* interacting with other dominant taxa including *Eubacterium oxidoreducens group*, *Eubacterium coprostanoligenes group*, *Lachnospiraceae* (*NK4A136* and *UCG-008*), *Bacteroides*, *Alistipes*, *Parabacteroides*, *Prevotella* 2 as well as *Rhodospirillaceae* in the pathogenesis of IgE-mediated food allergy and these microbial taxa were mainly

involved in methane metabolism and glycerolipid metabolism. Integrative view of gut microbial ecology based on the microbial module in our study may help to understand the microbial interactions associated with IgE-mediated food allergy.

Supplementary Materials: The following are available online at https://www.mdpi.com/1422-0067/22/4/2079/s1, Supplementary Figure S1: PCoA plots of individual gut microbiota in food-allergic children (red) and non-food allergic children (blue) derived from (a) unweighted and (b) weighted UniFrac distances. Each symbol represents a sample. PCoA: Principal Coordinate Analysis, Supplementary Figure S2: Relative abundance of gut microbial phyla, Supplementary Table S1: The comparison of gut microbiota at the phyla level between food-allergic children and non-food allergic children, Supplementary Table S2: Relative abundance of predominant genera in gut microbiota between food-allergic children and non-food allergic children ($\geq$1% across all samples), Online Supplemental Notes.

Author Contributions: K.H.L. and G.Z. designed the study. K.H.L. carried out the experiments and data analysis with assistance from Y.S., J.G., A.A., and G.Z. M.O. and B.H. helped supervise the project. K.H.L. wrote the manuscript with consultation with all authors. B.J.M. and G.Z. coordinates all aspects of the study. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the National Health and Medical Research Council National Statement on Ethical Conduct in Human Research, and the protocol was approved by the Human Research Ethics Committee (HREC), Perth Children's Hospital (RGS151/HREC 2017060EP) and Curtin University (HRE2017-0712).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: All raw sequencing reads are available at NCBI BioProject database (PRJNA699997) (https://www.ncbi.nlm.nih.gov/bioproject/, accessed on 18 February 2021).

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Abbreviations

LDA	Linear discriminant analysis
LEfSe	Linear discriminant analysis effect size
OTUs	Operational Taxonomic Units
ME	Module Eigengene
PCoA	Principal Coordinate Analysis
QIIME	Quantitative Insights Into Microbial Ecology
WGCNA	Weighted Gene Correlation Network Analysis

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APPENDIX C

This appendix contains one first-authorship paper published during candidature which is not included by the thesis. This appendix only includes the accepted manuscript version of the article, but not the published version. The published version is available on Lee, K.H., Song, Y., O'Sullivan, M. et al. The Implications of DNA Methylation on Food Allergy. Int Arch Allergy Immunol 173 (2017). https://doi.org/10.1159/000479513, published under a CC BY-NC-ND licence.

1 **The Implications of DNA Methylation on Food allergy**

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Keywords

Food allergy · DNA methylation · Gene expression · Gene-environment interactions

Abstract

Food allergy is a major clinical and public health concern worldwide. To date, risk factors are well defined, however, the mechanisms by which they affect immune development remain largely unknown, and unfortunately, the effective treatment or prevention for food allergy is still under development. Recent studies show that the genes that are critical for the development of food allergy are regulated through DNA methylation. Environmental factors can affect host DNA methylation status and subsequently predispose people to food allergy. DNA methylation is therefore an important mediator of gene-environment interactions in food allergy and key to understanding the mechanisms underlying the allergic development. Indeed, modification and identification of the methylation levels of specific genetic loci have gained increasing attention for therapeutic and diagnostic application in combating food allergy. In this review, we summarize and discuss the recent developments of DNA methylation in food allergy, including pathogenesis, therapy and diagnosis. This review will also summarize and discuss the environmental factors that affect DNA methylation levels in food allergy.

Introduction

The prevalence of food allergies has been increasing rapidly worldwide, particularly in children [1]. For example, in the US, around 8% of children are reported to have food allergy [2]. In Europe, the lifetime prevalence and point prevalence for self-reported food allergy are around 17% and 6%, respectively [3]. An Australian cross-sectional population study found that 10% of children aged 1 to 5 years developed an allergy to foods [4]. Food allergies are regarded a new wave of allergy epidemic because of their increasing prevalence, their potentially fatal implications and their vast medical and economic impacts [5]. The most common food causing allergies include peanut, milk and hen's egg [6]. Peanut allergy is potentially a lifelong disease, unlike cow's milk and hen's egg allergy, and may cause life-threatening reactions. To date risk factors are well defined, however, the mechanisms by which they affect immune development remains largely unknown for food allergy, and unfortunately the effective treatment or prevention for food allergy is still under development [6]. Among all of the current treatments, immunotherapy has shown great promise by desensitizing patients with food allergies [7]. However, larger, well-designed studies are needed to fully evaluate the effects of food immunotherapy.

Nowadays there is an increasing recognition that epigenetic disruptions play an important role in the pathogenesis of diseases. Epigenetic disruptions regulate genomic adaption to environmental factors, and can thus initiate disease development [8]. Among all of the epigenetic disruptions, DNA methylation is the most commonly known. DNA methylation occurs by the addition of a methyl (CH3) from S-adenosyl methionine to the DNA strand itself, often to the 5-carbon of a cytosine ring that resides at a CpG site [9-11]. By doing this, DNA methylation modifies the function of the DNA, leading to changes in gene expression. Moreover, DNA methylation can render the chromatin, or protein structure surrounding the DNA molecule, into an ''inactive'' state, leading to gene silencing [12].

In this review, we report on the recent developments in epigenetic studies of food allergy, with a specific focus on DNA methylation.

DNA Methylation in Pathogenesis of Food allergy

To date, the majority of epigenetic studies have focused on asthma [13-16]. Nonetheless disruptions in DNA methylation levels may also play a critical role in the development of allergic diseases [17]. DNA methylation levels can affect the development of allergic reaction to different types of foods (Table 1).

Interleukins

The interleukins are a group of cytokines, which are biologically active protein mediators [18], which play a crucial role in immune responses and are related to food allergy.

Song et al conducted an animal study in order to examine the relationship of allergy susceptibility and Th2-biased epigenetic alterations [19]. Offspring of mothers with peanut allergy (O-PAM mice) and offspring of naive mothers (O-NM mice) were both sensitized with peanut. Blood sera were collected weekly during sensitization and 1 day before challenge by means of submandibular venepuncture to monitor the antibody response to peanut sensitization. It was found that the DNA methylation of *IL-4* in offspring of mothers with peanut allergy was lower than in offspring of naive mothers. The study also found that *IL-4* methylation levels showed a negative correlation with IgE production. This demethylation of *IL-4* was suggested to impair Th2 responses and IgE class switching, resulting in the development of food allergy.

In another study investigating the underlying mechanisms for the development of food allergy, Berni Canani and colleagues assessed the DNA methylation of CpGs in the promoter regions of genes from peripheral blood mononuclear cells as well as the serum levels of IL-4, IL-5, IL-10 and IFN-γ in forty children [20]. These children were divided into three groups: children with active IgE-mediated cow's milk allergy (group 1), children who acquired tolerance to cow's milk proteins (group 2) and healthy children (group 3). A venous blood sample (4 ml) was obtained from these children for epigenetic analysis. This study showed that the DNA methylation of *IL-4* and *IL-5* in patients with cow's milk allergy was lower, and the DNA methylation of *IL-10* and *IFN-γ* was higher, compared to the healthy controls. Moreover, this study also found a significant and negative correlation between methylation levels of the cytokine promoter regions and the respective serum concentrations. The data revealed that demethylation of *IL-4* and *IL-5* and increased methylation of *IL-10* and *IFN-γ* played an important role in the pathogenesis of food allergy.

Other than the above interleukins, IL-17A is also found to be associated with the development of food allergy and is suggested to be one of the key mediators in regulating inflammatory processes [21]. The concentrations of IL-17A were quantified and compared between 30 patients with food allergy and 10 healthy subjects. The patients with food allergy had a higher level of IL-17A than that in the healthy controls. The mechanism by which IL-17A affects the pathogenesis of food allergy remains unknown. However, it was suggested that the *IL-17A* gene was regulated by promoter methylation, which affects pro- and anti-inflammatory responses of the immune system leading to allergy development [22]. Further studies are required to confirm the relationship between methylation of *IL-17A* and development of food allergy.

***HLA-DR* and *-DQ gene* region**

Major histocompatibility complex (MHC) class II system mediates the cell-mediated adaptive immune response [23-25]. There are three pairs of MHC class II α- and β-chain genes, called the human leukocyte antigen (HLA) system, which includes *HLA-DR*, *HLA-DP*, and *HLA-DQ*. *HLA-DR* is the most prominent gene and more than 500 different HLA-DR protein sequences have been identified to date. *HLA-DQ* is closely linked to *HLA-DR*. The diversity of *HLA-DR/DQ* alleles signifies a versatile immune system of the host that can respond to a variety of pathogens, both at the level of the individual and at the level of the population [26]. DNA methylation affects *HLA-DQ* by silencing the *HLA-DQ* region CCCTC transcription factor (CTCF)-binding insulators that separate the MHC-II sub-regions [27].

The first genome-wide association study of well-defined food allergy was conducted in Chicago [28]. This study recruited 2,759 participants who were sensitized to peanut. Of those 1,315 were children and 1,444 were parents. This study revealed that ethnic background was associated with DNA methylation of *HLA-DQB1* and *HLA-DRB1* genes, and a linkage was found between DNA methylation and single-nucleotide polymorphism (SNP). It was revealed that 2 SNPs, namely rs7192 and rs9275596, influenced the DNA methylation pattern. DNA methylation sites that were specific for peanut allergy were identified in the *HLA-DR* and *HLA-DQ* gene region at 6p21.32, tagged by SNP rs7192 and rs9275596, in 2,197 participants of European ancestry. Proteins coding by *HLA-DQB1* and *HLA-DRB1* serve to present processed peptide antigens to $CD4^+$ T-lymphocytes, which in turn can stimulate the initiation of an antigen-specific immune response.

An epigenome-wide association study also confirmed the importance of *HLA-DQB1* in the development of allergic disease. 12 children with IgE-mediated food allergy (hen's egg, cow's milk and peanut) and 12 healthy children were recruited. Genome-wide DNA methylation profiles (~450 000 CpGs) from $CD4^+$ T-cells were measured using Illumina Infinium Human Methylation 450 Bead Array platform. The study showed that the DNA methylation status in specific regions of *HLA* might vary in different food allergies. Such change determines which antigen-derived peptides are presented to the T cells and potentially results in the dysregulation of food tolerance [29].

FOXP3

The regulatory T-cell (Treg) is crucial in maintaining immune tolerance to allergens. The development and function of Treg depends on the transcription factor forkhead box *(FOXP3)*. The expression of *FOXP3* is regulated by the DNA methylation status of a methylation-sensitive enhancer (TSDR, Treg cell-specific demethylation region) [30], which is an alternative mechanism of disruption of the immune balance, causing host intolerance to exogenous substances.

A case control study investigated the function of Treg cells by investigating whether Treg cells were associated with immune tolerance in oral immunotherapy for peanut allergy [30]. 23 cases with peanut allergy were compared with 20 age-matched allergic control subjects. The participants were required to undertake oral immunotherapy to peanut protein over the course of 24 months. Subsequently the therapy was withdrawn for 3 months and followed by an oral food challenge at 27 months. The data revealed that the *FOXP3* DNA methylation level of patients with peanut sensitisation was higher than that of the control participants. Moreover, a further 24 month follow-up study showed that the *FOXP3* DNA methylation level in patients who remained without allergic reactions was decreased, compared with patients who re-sensitized to peanut [30]. Therefore, increased methylation of *FOXP3* is likely to play a crucial role in the development of peanut allergy.

An Italian cohort study also confirmed the importance of *FOXP3* in the development of food allergy and that its expression was regulated by DNA methylation [31]. Forty children aged 3 to 18 months were divided into four groups: 10 children with active IgE-mediated cow's milk allergy, 10 children who outgrew cow's milk allergy after dietary treatment with an

155 extensively hydrolyzed casein formula containing the probiotic Lactobacillus rhamnosus GG,
156 10 children who outgrew cow's milk allergy after treatment with other formulas, and 10
157 healthy controls. The *FOXP3* TSDR demethylation and expression were measured in
158 mononuclear cells that were purified from peripheral blood. This study showed that *FOXP3*
159 TSDR demethylation was significantly lower in children with active IgE-mediated cow's
160 milk allergy, compared to either children who outgrew cow's milk allergy or the healthy
161 children. This study further revealed that extensively hydrolyzed casein formula containing
162 the probiotic Lactobacillus rhamnosus GG increased *FOXP3* TSDR demethylation, resulting
163 in a tolerance reaction. Thus, the *FOXP3* methylation level appears to be important in cow's
164 milk allergy and this can be influenced by the formula selection.

165 ***Filaggrin***

166 The presence of mutations in *filaggrin* is the most frequently noted genetic factor for damage
167 in the anatomical and functional barrier of the epidermis [32]. Mutations in the filaggrin-
168 coding gene are linked to the occurrence of a constitutional reaction to protein antigens in
169 food. Since the *filaggrin* mutation is often identified with atopic dermatitis, which usually
170 coexists with food allergy, the independent effect of *filaggrin* on food allergy remains to be
171 determined.

172 A recent study investigated the association of *filaggrin* and food allergy [33]. 1456 children
173 were recruited and a skin prick test was used to identify food allergen sensitization. The
174 results showed no direct effect of *filaggrin* mutations on food allergy at any age. However,
175 there was a significantly indirect effect of *filaggrin* mutations on the risk of food allergy
176 through eczema later in childhood, between the ages of 10 and 18. The barrier defect due to
177 *filaggrin* deficiency makes the study subjects susceptible to the development of cutaneous
178 sensitization through antigen-presenting cells and systemic atopic response [33]. Peanut
179 sensitization can occur through the topical application of peanut oil to the skin and cutaneous
180 exposure through the presence of peanut allergen in the environment, and this process might
181 be enhanced by a deficient barrier caused by *filaggrin* mutations or eczema [33].
182 Nevertheless, the importance of methylation at the CpG sites in the regulation of *filaggrin*
183 expression in buccal cells is debated [34]. The study performed by Tan et al did not indicate a
184 relationship between the methylation of the *filaggrin* gene promoter and allergy diseases [34].

185 **Other genes**

186 Other than the above mentioned genes, differences in *DHX58*, *ZNF281*, *EIF42A* and *HTRA2*
187 gene DNA methylation are suggested to be associated with development of cow's milk
188 allergy as observed in the Dutch EuroPrevall birth cohort study [35]. In this study, bisulfite
189 converted DNA from the blood samples of 20 children with cow's milk allergy, 23 healthy
190 control, and 10 boys who outgrew food allergy. DNA was analysed using the 450K infinium
191 DNA-methylation array [35]. This study revealed that increased methylation of *ZNF281* and
192 *HTR2A* and demethylation of *EIF4E2* and *DHX58* caused allergic reaction. The increased
193 methylation of *ZNF281* and *HTR2A* was suggested to be related to gastrointestinal symptoms
194 while the demethylation of *EIF4E* and *DHX58* could lead to allergic inflammation.

195 Additionally, an epigenome-wide association study of children from the US revealed that the
196 demethylation of *NDFIP2, EVL, TRAPPC9* genes were associated with development of
197 cow's milk allergy [36]. This study was conducted in two stages, the discovery stage and the
198 replication stage. In the discovery stage, whole blood samples were collected from 106
199 Caucasian children with cow's milk allergy and 76 nonallergic and nonatopic Caucasian
200 children. DNA methylation was measured at 485, 512 genomic loci using the Illumina
201 Human Methylation 450 arrays. In the replication stage, the top significant DNA methylation
202 loci were validated using childhood whole blood samples from 25 Caucasian children from
203 the Chicago Food Allergy Study and cord blood samples from 140 Afro-American children
204 from the Boston Birth Cohort. This study found that the DNA methylation of *NDFIP2*, *EVL*,
205 *TRAPPC9* genes was lower in children with cow's milk allergy compared to healthy children.
206 These 3 candidate genes were suggested to be relevant for the immune system. The exact role
207 of these annotated genes in cow's milk allergy remains to be determined.

208 Altered DNA methylation is observed in a number of different genes, implying that multiple
209 cell pathways are required for allergic disease development.

210 **DNA Methylation and Allergic Tolerance**

211 The significance of DNA methylation for cellular regulation potentially provides new
212 strategy of allergy intervention and treatment. If DNA methylation can be artificially

213 modified, it can change the corresponding gene and protein expression to prevent allergic
214 reaction or reverse allergic sensitisation.

215 Tolerance can be induced through alterations in DNA methylation status of dendritic and
216 $CD4^+$ cells [30,37-40]. The function of dendritic cells is to either induce protective immunity
217 or maintain the state of tolerance to self-antigens and allergens. These dendritic cells induce
218 different lineages of T cells, such as Th1, Th2, and Treg cells, which suppress the
219 development of allergy and asthma, thus providing anti-inflammatory responses and
220 protective immunity. DNA methylation regulates the differentiation of human monocyte-
221 derived dendritic cells, affecting the cells to become either activated or tolerogenic [38]. In
222 $CD4^+$ cells, Treg cells maintain the demethylation status of TSDR and retain the ability to
223 reactivate *FOXP3* expression and its suppressive function upon activation. TSDR changes
224 when patients undergo treatment, but its cause or effect remains unknown for food allergy
225 [38]. In a clinical trial, 20 out of 23 participants developed tolerance to 4 g of peanut protein
226 after 24 months of oral immunotherapy. This was accompanied by an increase in antigen-
227 induced Treg and a greater suppressive function due to elevated levels of *FOXP3*
228 methylation. After withdrawal from the treatment for 6 months, four participants regained
229 peanut sensitivity along with increased methylation of *FOXP3* CpG sites in antigen-induced
230 Treg [30]. The data were consistent and showed that immunotherapy altered the *FOXP3*
231 methylation level to achieve the therapeutic effect.

232 A mice study revealed that epicutaneous immunotherapy could reduce the risk of further
233 sensitization to peanut [41]. The mice were first sensitized with milk, followed by
234 epicutaneous treatment for 8 weeks and lastly they were sensitized to peanut. The study
235 found an increase in the methylation level of the *GATA-3* promoter, which is located in the
236 promoter region of *IL-5* and *IL-13*. Furthermore, the study also showed that the methylation
237 status was sustained over two months. Therefore, this study suggested that epicutaneous
238 immunology modified the *GATA-3* methylation level to inhibit further allergic sensitisation to
239 other allergens. Another study noted that epicutaneous immunotherapy appeared to be more
240 effective compared with oral immunotherapy and a sustainable treatment for food allergy
241 sensitization [42]. After 4 weeks of epicutaneous immunotherapy and oral immunotherapy,
242 mice developed tolerance to peanut, accompanied by the occurrence of *FOXP3*
243 demethylation in $CD4^+$ T cells. However, following the end of treatment, only the *FOXP3*
244 demethylation level in mice treated with epicutaneous immunotherapy remained persistent

[42]. The persistent low *FOXP3* demethylation level was suggested to be essential for outgrowing the peanut sensitivities.

A study showed that the efficacy of antigen-specific immunotherapy could be increased through DNA methylation [41]. In a food allergy study with mice, the mice were divided into two groups: half of the mice were treated with Allergen Specific Immunotherapy while the other half of the mice were treated with both Allergen Specific Immunotherapy and recombinant IL-8 [43]. The findings indicated that IL-8 significantly enhanced the therapeutic effect of Allergen Specific Immunotherapy and suppressed allergy inflammation via the promoter demethylation. Yang developed a food allergy mouse model in order to examine the relationships of IL-13, tolerogenic dendritic cells and the development of food allergy. The mice were divided into two groups: a group of mice were treated with modified ag-specific immunotherapy (MSIT) alone while the other group of mice were treated with MSIT and anti IL-13 antibody [44]. The mice were orally challenged and sacrificed the next day. The study showed that there was a significant difference in inflammation due to the allergy in mice treated with MSIT and anti IL-13 antibody compared to mice treated with MSIT alone. IL-13 increased the methylation of Thrombospondi-1 (TSP1) gene DNA in B cells, which increased development of tolerogenic dendritic cell (ToIDC) in mice with food allergy and inhibited allergic inflammation in the intestine. These studies suggest that interleukins can improve the efficacy of allergen specific immunotherapy by suppressing the allergic inflammation.

Diet is also a promising method for accelerating tolerance acquisition in children with cow's milk allergy [40]. In a clinical trial, two hundred sixty children were divided into five groups based upon the formula used for treatment: group 1 (extensively hydrolyzed casein formula), group 2 (extensively hydrolyzed casein formula and Lactobacillus rhamnosus), group 3 (hydrolyzed rice formula), group 4 (soy formula), and group 5 (amino acid based formula) [40]. After 12 months of exclusion diet with the different formulas, the groups receiving extensively hydrolyzed casein formula (43.6%) or extensively hydrolyzed casein formula and Lactobacillus rhamnosus (78.9%) had a higher rate of achieving oral tolerance compared to groups receiving hydrolyzed rice formula (32.6%), soy formula (23.6%), and amino acid based formula (18.2%). The underlying mechanism was related to an increase in *FOXP3* TSDR demethylationas well [40]. Thus, CpG methylation within *FOXP3* appears to be a key therapeutic target for different types of food allergies.

Considering that the observed changes in methylation occurred in multiple genes, other therapeutic targets should also be considered and evaluated in order to achieve long-term and synergistic effects.

Clinical Utility

The analysis of DNA methylation-based biomarkers is a rapidly developing field. Since the DNA methylation profile can be inherited from one generation to next, it may have potential to predict offspring's susceptibility to disease by examining the parent's genome [45,46].

DNA methylation may be suitable as a diagnostic signature for food allergy in three ways. Firstly, DNA methylation levels can be used to discriminate phenotypes of food allergy as well as other allergic diseases [47,48]. In an Australian infant cohort study, it was discovered that the DNA methylation signature of 96 CpG sites could distinguish between food allergic, food-sensitized and non-food allergic individuals, and predict clinical outcomes for food allergy [47]. The authors performed genome-wide methylation profiling on blood mononuclear cells from infants aged 11 to 15 months who underwent concurrent skin-prick testing and oral food challenges. Reproducibility was assessed with an additional 12 case and control samples respectively, using methylation data from a publicly available data set. This study found that DNA methylation in *MAP* kinase signalling genes was more accurate in predicting food challenge outcome compared to allergen-specific IgE and skin prick and had a 79.2 percent accuracy rate.

Secondly, certain DNA methylation signatures can be used to differentiate people with food tolerance from those with food intolerance. The gold standard diagnostic test in food allergy is double blind oral food challenge [49]. However, oral food challenge requires the food-sensitised children to produce IgE-mediated response to antigens before food sensitization is confirmed. This causes threatening anaphylaxis to the children and makes the parents stressful. As discussed earlier, *FOXP3* methylation can be used to discriminate tolerance and intolerance to peanut [30]. In this way, *FOXP3* methylation is a better way to diagnose food sensitization compared to oral food challenge, as this does not cause any discomfort to the patients with food allergy. Apart from *FOXP3* methylation, DNA methylation level of selected Th1 and Th2 cytokine genes can be used as potential biomarkers in predicting tolerance acquisition. In a clinical trial, it was observed that healthy subjects had different

DNA methylation profiles in selected Th1 and Th2 cytokine genes such as *IL-4*, *IL-5*, *IL-10* and *IFN-γ* if compared to active cow's milk allergy patients [20]. In this way, DNA methylation analysis was clearly able to separate active cow's milk allergy patients from healthy controls.

Finally, with the aid of genome-wide technologies such as DNA methylation microarrays, high-throughput screening of patient samples becomes feasible and can be developed into prognostic markers, as some DNA methylation sites correlate with disease development and clinical outcomes [48].

There is a growing consensus that development of food allergy is likely to be caused by epigenetic disruptions and the information of food allergy can be transmitted from one generation to the next generation (transgenerational effects). Maternal heredity is suggested to be a stronger determinant of allergy risk than paternal heredity, and allergic women are more likely to have infants with food allergy [50]. This was demonstrated through a birth cohort study, in which food allergy (positive food SPT and history of IgE-mediated symptoms) occurs in 13% of 1-yr-old infants of atopic (SPT+) women compared to only 4% of infants of non-atopic (SPT−) women [5]. This study suggests that allergic women have modified immune interactions with their fetus during pregnancy.

The development of food allergy is likely to be caused by epigenetic disruptions. Considering that DNA methylation is one of these epigenetic disruptions, it might be linked with the initiation of food allergy. Further studies are needed to confirm the role of DNA methylation as a causal mediator of food allergy.

Environmental Factors Affect DNA Methylation in Food allergy

The rise in maternal allergy may also be affected by environmental changes. Around 13% of allergic women recruited after 2005 had a child with IgE-mediated food allergy at 1 year of age, compared with 9% infants from a cohort of allergic women recruited in 1999 [5].

Maternal diet during pregnancy might alter DNA methylation and gene expression in the offspring and affect allergy outcomes in the offspring. A mouse study showed that maternal peanut allergy plus non-toxic cholera toxin subunit B consumption induced peanut tolerance

in mice [51]. Mothers with peanut allergies were divided into two groups: one group was administered with low doses of peanut and non-toxic cholera toxin subunit B during pregnancy and lactation, while the other group was administered with water as controls. The offspring of these mothers was challenged with peanut after 8 weeks of peanut sensitization. Following the challenge, there were no anaphylactic symptoms for the offspring of the treated mothers while the offspring of mothers who were administered with water did show anaphylactic symptoms. The newborn mice of the treated mothers showed a significant decrease in plasma histamine, peanut-IgE, IL-4 and IL-17 and significant increase in IL-10 production compared to the newborn mice of the mothers that were administered with water. In addition, they showed a significant increase in $CD4^{+}CD25^{+}$Foxp3 T regulatory cells methylation. This suggested the increased peanut tolerance was due to non-toxic cholera toxin subunit B consumption which modified the expression of plasma histamine, peanut-IgE, IL-4, IL-17 and IL-10 through DNA methylation.

Vitamin D deficiency in pregnancy may be another risk factor for developing food sensitization through the suppression of Treg cells [52]. Two groups of female mice were fed different diets, a vitamin D-deprived diet and a balanced diet respectively. The female mice of each group were mated and continued the same diet during gestation and lactation. The offspring from these two groups were injected with ovalbumin and aluminium hydroxide to establish an animal model of food allergy at the age of 6 weeks. It was shown that the offspring of mothers that fed on the vitamin D-deprived diet had a higher risk of developing food allergies and a lower expression of Treg cells compared to the offspring of mothers fed on the balanced diet. Since a stable expression of Treg depends on the *FOXP3* DNA methylation, low vitamin D might reduce the Treg population through a change in methylation of *FOXP3* and result in increased susceptibility to development of food allergy. In contrast, a LINA (Lifestyle and environmental factors and their influence on Newborns Allergy risk) cohort study showed that high vitamin D levels in pregnancy and at birth increased the risk for developing food sensitization [53]. In the study, 629 mother–child pairs were examined. Blood samples were collected from the mother at the 34th week of pregnancy, at delivery and annually after birth. Treg in cord blood was detected by methylation-specific PCR using a Treg-specific demethylated region in the *FOXP3* gene. A negative correlation between vitamin D and Treg in cord blood was identified, and low numbers of Treg were associated with a higher likelihood of developing food allergy. The high amount of vitamin D was believed to suppress the Treg expression through methylation

368 of *FOXP3*, which led to a higher risk of developing food allergies. Evidently, the association
369 of vitamin D with food allergy is controversial, and needs further investigations.

370 The composition of the microbiota may also contribute to the development of food
371 sensitization [54-57]. Among all of the microbiota, Bifidobacterium, Bacteroides and
372 Staphylococcus have a protective impact towards milk allergy in mice [55,56]. A mice study
373 revealed that *Ruminococcaceae* (belonging to the system of Clostridiales) might be
374 associated with IL-22. The study found higher levels of IL-22 and higher abundances of
375 Ruminococcaceae in tolerant mice compared to allergic mice. *Ruminococcaceae* was
376 suggested to be able to increase induced IL-22, which had a protective impact on food allergy
377 in mice [58]. Microbiota composition can affect the DNA methylation in host cells [59]. The
378 gut microbiota produces large amounts of epigenetically active metabolites such as folate and
379 butyrate, which may cause changes in DNA methylation patterns of host cells resulting in
380 allergy development. Folate is essential for DNA methylation and acts as a methyl donor for
381 the regeneration of the intracellular methyl substrate S-adenosyl methionine. A recent Finnish
382 study indicated that folate intake and folic acid supplement use during pregnancy were
383 associated with an increased risk of a cow's milk allergy in the offspring [60]. Folate acid
384 altered the DNA methylation pattern which in turn increased the susceptibility to cow's milk
385 allergy [50]. Butyrate is a short-chain fatty acid (SCFA) and it can be produced by
386 Faecalibacterium prausnitzii which belongs to the cluster of Firmicute bacteria [61]. In a
387 clinical trial, after 6 months of dietary intervention with extensively hydrolyzed casein
388 formula supplemented with Lactobacillus rhamnosus GG, it was observed that children who
389 have tolerated cow's milk allergy presented a significant increase in faecal butyrate levels
390 compared to children with cow's milk allergy [62]. Interestingly, another study also found
391 that children with cow's milk allergy became tolerant after receiving a dietary treatment with
392 extensively hydrolyzed casein formula containing the probiotic Lactobacillus rhamnosus GG.
393 It was found that the tolerance development was caused by increased *FOXP3* TSDR
394 demethylation [31]. Therefore, these two studies suggest a potential link between butyrate,
395 *FOXP3* TSDR demethylation and development of allergic disease. In other words, butyrate
396 that is produced by microbiota can help to achieve tolerance by reducing *FOXP3* TSDR
397 methylation.

398 In Australia, there is a remarkable increase of food allergy for new immigrants from Asian
399 countries, and in particular the second generation of immigrants are three times more likely to

develop nut allergy compared to the local children [63]. The genetic predisposition in these children implies that epigenetic changes occur in their parents when they are exposed to their new environment and that these epigenetic modifications are inherited by the next generation. In our recent study, we showed that Chinese immigrants living in Australia for a longer period have increased global genome methylation levels [64]. Several environmental and lifestyle factors are suggested to cause the increased risk of allergies in Westernized countries, including exposures to new allergens or environmental microbiome, Western diet and lifestyles, ultraviolet radiation exposure and housing conditions [63]. Thus, multiple environmental factors may contribute to the development of food allergy through DNA methylation mechanisms.

Conclusion

DNA methylation modulates the development of the innate and adaptive immune systems, and plays an essential role in the development of allergic diseases. This research field is still at its infancy, particularly in relation to food allergy. Although further studies are clearly needed to determine how specific disease processes are mediated by DNA methylation, the currently available literatures indicate the importance of DNA methylation in the development and manifestations of food allergy. It provides a new paradigm of allergy intervention, treatment and diagnosis. The DNA methylation related technologies are developing rapidly e.g. measuring increasing numbers of CpG sites as well as single-cell methylome sequencing. We can anticipate that these emerging technologies will greatly help us to understand the biological and immunologic role of DNA methylation in food allergy, and will identify novel targets for clinical use.

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Disclosure Statement

The authors declare that they have no conflict of interest.

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630 Table 1. Genes associated with immune system regulated by DNA methylation in food
631 allergy

Genes	Cell Type	Function	Type of Allergy	Potential role	References
IL-4	$CD4^+$ (Th2)	Enhance the differentiation of naive $CD4^+$ T cells into IL-4-producing Th2 cells	cow's milk	Demethylation of *IL-4* impairs Th2 responses and IgE class switching	[19,20]
IL-5	$CD4^+$ (Th2)	Regulate eosinophilic inflammation	cow's milk	Demethylation of *IL-5* increases the risk of developing food allergy	[20]
IL-10	$CD4^+$ (Th2)	Suppress allergic inflammation	cow's milk	Increased methylation of *IL-10* induces oral tolerance	[20]
IFN-γ	$CD4^+$ (Th1/2)	Suppress Th2 responses of allergic diseases	hen's egg, cow's milk, peanut	Increased methylation of *IFN-γ* is associated with food allergy	[20]
IL-17A	$CD4^+$	Increase allergic inflammation	food allergy	Methylation of *IL-17A* is associated with food allergy	[21]
HLA-DR and -DQ gene	$CD4^+$ T Cells	Present antigen-derived peptides, mostly of exogenous origin, to CD4+ helper T cells.	peanut	Recognize allergenic proteins, including peanut proteins, which then lead to specific IgE responses	[26-29]
FOXP3	T cells	Maintain immune tolerance to allergens	peanut, cow's milk	*FOXP3* DNA methylation level is associated with food allergy	[30,31]
DHX58	$CD8^+$ and $CD4^+$ T cells, Natural killer cells, B cells, Monocytes, Granulocytes	Antigen recognition in the innate immune response to various RNA viruses and some DNA viruses	cow's milk	Demethylation of *DHX58* is associated with allergic inflammation	[35]
ZNF281	$CD8^+$ and $CD4^+$ T cells, Natural killer cells, B cells, Monocytes, Granulocytes	Repress GAST which stimulate the stomach mucosa, produce digestive enzymes, ensure smooth muscle contraction and	cow's milk	Increased methylation of *ZNF281* is associated with the gastro-intestinal reactions	[35]